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ON RESPIRATORY EPITHELIAL CELLS

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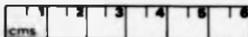
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NEUTRALIZATION OF INFLUENZA VIRUS
ON RESPIRATORY EPITHELIAL CELLS.

Mark Charles Outlaw

B.Sc. (Hons) Biochemistry (University of York)

A thesis submitted for the degree of
Doctor of Philosophy

Department of Biological Sciences
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May, 1969

SUMMARY

The host cell, probably plays an important role in neutralization but previous work on influenza virus neutralization used dedifferentiated cultured cells derived from tissues which are not the natural target of the virus. The aim of this investigation was to study the mechanisms of neutralization of influenza virus by different antibody isotypes using fully differentiated, ciliated epithelial cells of tracheal organ cultures. Tracheal organ cultures derived from the mouse were used together with mouse pathogenic strains (A/FPV/Rostock/34 (adpFPV/R) and A/PR/8) and mouse antibodies, so that all the participating components came from the same species. Parallel experiments were performed using cultured BHK cells and erythrocytes to enable comparison between different cell types. As previous work had used only a single saturating amount of antibody I decided to use a range of antibody concentrations with a constant amount of virus. Three potential mechanisms of neutralization were investigated: aggregation of virus, inhibition of virus attachment to cells and inhibition of virus internalization by cells.

Each isotype (IgG, IgM and IgA) aggregated influenza virus (by EM determination) when used in sub-saturating amounts. Increasing the amount of antibody reduced aggregation and at higher antibody concentrations, the virus was monodisperse. Sub-saturating amounts of IgG monoclonal antibody partially inhibited the attachment of adpFPV/R to tracheal organ culture cells, BHK cells and erythrocytes. Together, aggregation and inhibition of virus attachment by IgG accounted for more than a 90% loss of infectivity, although this was usually less than the amount of neutralization observed. It is argued that these represent potentially important mechanisms of neutralization. Increasing the IgG concentration increased neutralization and increased attachment of virus to tracheal organ culture and BHK cells but not to erythrocytes. Therefore when there is a high ratio of IgG to virus, neither aggregation or inhibition of virus attachment accounted for any loss of infectivity. Under these conditions, IgG did not prevent internalization of virus that had attached to tracheal organ culture or BHK cells and it was concluded that inhibition of an intracellular stage of the virus infectious pathway was the major mechanism of neutralization.

Polyclonal IgM partially inhibited attachment of virus to organ culture cells, BHK cells and erythrocytes. Increasing the amount of IgM did not increase virus attachment to any of the cell types. Internalization of IgM-neutralized virus that had attached to tracheal organ culture and BHK cells was blocked, confirming earlier observations when a single saturating concentration of IgM was used.

Neutralization of A/PR/8 by sub-saturating amounts of monoclonal polymeric IgA also inhibited virus attachment to BHK cells and erythrocytes. There was no rise in attachment when the amounts of IgA were increased and internalization of attached virus was inhibited. Sub-saturating amounts of IgA also greatly reduced virus attachment to tracheal organ culture cells. However increasing the relative IgA concentration resulted in increased attachment. The highest concentration of IgA used enhanced virus attachment by more than five-fold compared to infectious virus but this was accompanied by no increase in infectivity. Enhanced attachment was neuraminidase-resistant and was possibly mediated via an IgA-specific Fc receptor.

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ABBREVIATIONS

All abbreviations are defined at first mention in the text. The following list defines key abbreviations used in this thesis.

adpFPV/R	mouse adapted influenza variant of A/FPV/R (H7N1)
A/FPV	influenza A/Fowl Plague Virus/Rostock/34 (H7N1)
A/PR/8	influenza A/Puerto Rico/8/34 (H1N1)
A/WSN	influenza A/Wilson Smith Neurotropic/40 (H1N1)
BHK	baby hamster kidney
BSA	bovine serum albumin
CEF	chick embryo fibroblast
CPM	counts per minute
cRBC	chicken erythrocyte
Fab'	antigen binding fragment
(Fab') ₂	bivalent antigen binding fragment
Fc	crystalizable fragment
GAM	goat anti-mouse
HA	haemagglutinin
HAU	haemagglutination units
HI	haemagglutination inhibition
HIU	haemagglutination inhibition units
Ig	immunoglobulin
Log HIU:HAU	logarithmic ratio of HIU to HAU (also Log H/H)
Min.	minute
Mr	relative molecular mass
MW	molecular weight
NA	neuraminidase

NCS	newborn calf serum
NI	neuraminidase inhibition
NP	nucleocapsid protein
O.C.	organ culture
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming units
p.i.	post-infection
RNP	ribonucleoprotein
SC	secretory component
SDS	sodium dodecyl sulphate
sIgA	secretory IgA
TBS	Tris buffered saline
v/v	volume:volume
w/v	weight:volume

DECLARATION

I hereby declare that this thesis has been composed myself and has not been accepted in any previous application for a degree. The work presented was done by myself, with the exception of those instances where the contributions of others has been acknowledged. All sources of information have been specifically acknowledged by reference.

Mark C. Outlaw

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Finally I wish to acknowledge Elaine Bland, without whom none of this could have been completed.

INTRODUCTION.

1. A General description of type A influenza viruses.

Influenza viruses belong to the Orthomyxoviridae and are divided into types A, B or C depending on the antigenicity of the nucleocapsid (NP) structural virus protein. Type A influenza viruses are the most important from the stand-point of morbidity and mortality in man and are the subject of this thesis.

Using an electron microscope, influenza viruses appear as highly pleomorphic, spiked particles (Figure 1.1a). The size and shape of the particles varies considerably depending on the virus strain and the method of isolation used. Spherical particles (80-120nm diameter) are commonly found in virus preparations that have been repeatedly passaged in chorio-allantoic membrane of fertile eggs whereas freshly isolated virus preparations have a more filamentous morphology (Horne *et al.*, 1960).

The detailed structure of the influenza virus particle (reviewed by Lamb, 1983) is shown in a stylized form in Figure 1.1b. The envelope is acquired by the virus during the process of maturation as the progeny virus particles bud through the host cell membrane (Compans & Dismock, 1969). The lipid composition therefore closely reflects that of the host cell membrane from which the virus was derived (Klenk & Choppin, 1968). A higher proportion of phosphatidic acid has been found in the virion compared to the cells (Kates *et al.*, 1961), suggesting that the virus incorporates newly synthesized lipids as well as those lipids already present in the host cell at the time of infection.



Fig.1.1a. An electron micrograph of an influenza virus particle visualized by negative staining (x335,000). Courtesy of Dr.S.J.Armstrong (University of Warwick).

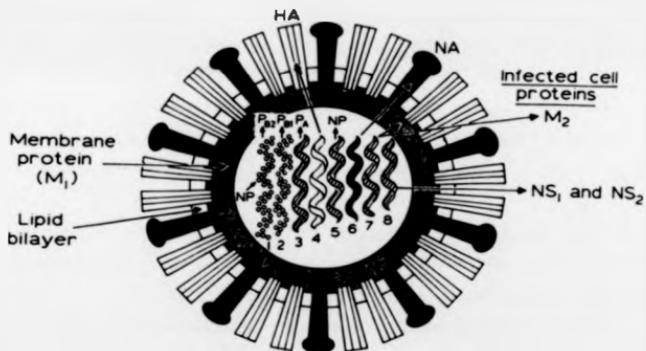


Fig.1.1b. A schematic diagram of the structure of the influenza virion. The coding assignments of the 8 RNA segments are also illustrated. The diagram is not drawn to scale (Lamb, 1983).

Into the envelope are inserted two types of virus-coded transmembrane glycoprotein spikes; haemagglutinin (HA) and neuraminidase (NA) (Laver & Valentine, 1969). It is against these two surface glycoproteins that the host's humoral immune response is mounted during infection. There are approximately 700-1000 spikes per virion with a ratio of roughly five haemagglutinin spikes to every neuraminidase spike (Webster *et al.*, 1982). The haemagglutinin spikes are evenly distributed over the virus surface and extend approximately 13.5nm from the envelope. They are trimers and have a cylindrical, rod shape with a triangular cross section. The haemagglutinin is the virus attachment protein (VAP), and also mediates virus penetration and fusion steps during the infectious process. The neuraminidase spikes are tetramers and are clustered in discrete patches on the virus surface (Murti & Webster, 1986). The major functions of the neuraminidase includes the prevention of self-aggregation (Palese *et al.*, 1974), improving virus mobility by freeing virus bound to respiratory tract mucous and freeing progeny virus from the host cell surface (Palese & Schulan, 1974). The detailed structures of both the HA and NA have been resolved using X-ray crystallography and are described in greater detail below.

Influenza virus 'cores' (diameter of roughly 65nm) are prepared by removing the envelope and surface glycoproteins (Schulze, 1972). The most abundant protein in the virion is the matrix protein (M1) (Compans *et al.*, 1970) and this forms a 3-4nm thick shell (Requister & Nermut, 1976). M1 is a type specific (Schild *et al.*, 1982) but sub-type variable influenza antigen (LeCompte & Oxford, 1981). It is a hydrophobic protein (Lenard *et al.*, 1974) which gives it a great avidity for lipid bilayers (Davis & Bucher, 1981; Karadaghi *et al.*, 1984). A specific fragment (5kD) of M1 embeds in the lipid bilayer (Gregoriades, 1981) and may allow interactions to take

place between the virus surface glycoprotein spikes and the virus core (Basak et al., 1984). Joassin et al. (1987) demonstrated M protein epitopes on the outer face of the viral envelope using monoclonal antibodies, indicating that it is a transmembrane protein. Positive cooperation between the NA and M1 does occur and enhances NA incorporation into lipid bilayers (Davis & Bucher, 1981). It is thought that the spike/core interactions result in a clustering of both HA and NA on the infected cell surface and the asymmetry of the interaction causes a local curvature of the membrane, facilitating the budding of progeny virus (Karadaghi et al., 1984). This is disputed by Patterson et al. (1988) who found that there was little M1 on the cytoplasmic surface of infected cell membranes. They thought that virus cores formed in the cytoplasm before migrating to the cell surface. The progeny budded rapidly once the cores and the glycoproteins came together. Others have also found M1 evenly distributed between the cytoplasm and nucleus of infected cells (Saith et al., 1987). The M1 protein inhibits the transcriptase activity of the virus, indicating that it is also closely associated with the ribonucleoprotein (RNP) complexes which lie within the virus core (Zvonarjev & Ghendon, 1980; Mikhejeva & Ghendon, 1983).

A second matrix protein (M2) is also coded for by the virus in a reading frame that overlaps with that for M1. This small (Mr15,000) protein was not thought to be present in the virion (Laab & Chopin, 1981; Zebede et al., 1985; Hay et al., 1985), although it is present in large quantities inserted into infected cell membranes (Laab et al., 1985; Saith et al., 1987). It has an integral membrane anchor domain with at least 18 residues external and a role in organising virus assembly has been proposed (Laab et al., 1985). Recently M2 has been found in small quantities in the virion structure (Zebede & Laab, 1988).

The ribonucleoprotein (RNP) complexes (90% protein; 10% RNA) lie inside the 'core' shell and are composed of four different proteins (NP, PA, PB1 and PB2) and eight single-stranded RNA segments of negative polarity. The RNP complexes are rod shaped structures with terminal loops and helical mid-sections (Pons et al., 1969). They can be separated into five size classes, each class reflecting the different size of RNA segment contained (Rees & Dismock, 1981).

The nucleocapsid protein (NP) is the most abundant protein in the RNP (Compans et al., 1970). Each NP contacts 20 base pairs of RNA and interacts with itself, PA, PB1, PB2, possibly M1 but not HA (Rees & Dismock, 1981; Patterson et al., 1988). It is a group specific antigen (Shekel & Schild, 1971) with at least five distinct antigenic determinants in three non-overlapping sites (van Wyke et al., 1980). These sites do undergo some antigenic variation, but one is common to all strains. Monoclonal antibodies against two of the sites inhibit transcription of viral RNA *in vitro*, which implies that NP is an integral part of the transcriptase complex (van Wyke et al., 1981). Others disagree with this suggestion. Scholtissek et al. (1971) found RNA transcription in the absence of NP.

PB1, PB2 and PA together make up the virus transcriptase enzyme (Palese et al., 1977; Braas et al., 1983; Detjen et al., 1987). PB1 and PB2 are involved in mRNA synthesis, with PB1 initiating transcription (Ulanen et al., 1981 & 1983) and PB2 binding the cap of host mRNA (Perrin et al., 1982; Ulanen et al., 1981; Blass et al., 1982; Braas-Markson et al., 1985)). The

role of PA is less clear but may be involved in mRNA priming and RNA transcription (Nichol et al., 1981). All RNA synthesis occurs in the nucleus (Shapiro et al., 1987).

Although the host's humoral response is primarily directed against the virus surface spikes, the internal structural proteins of the influenza virus 'core' are important antigens for cell-mediated immunity. Cytotoxic T lymphocytes (Tc or CTL) recognise NP and M1 determinants in association with the target cell major histocompatibility complex (MHC) class I antigens (MHC class I restricted) (Townsend et al., 1984; Gotch et al., 1987). The major influenza virus antigen is NP which has at least three distinct Tc epitopes. These are different from those recognised by antibodies because anti-NP does not inhibit Tc recognition. It is thought that the Tc epitopes are presented in a processed form in a manner similar to that found for MHC class II restricted presentation to T helper (Th) cells and that the MHC class I receptor can influence which epitope is presented (Townsend et al., 1985).

The influenza genome comprises eight single-stranded RNA segments (MW 0.27-1.00 $\times 10^6$) with negative polarity (Deesberger & Palese, 1978). The RNA segments code for the eight structural proteins described above plus two (possibly three) other non-structural proteins (Reviewed by Palese, 1977; McCauley & Mahy, 1983). The segmented nature of the influenza virus genome allows recombination events to occur during mixed infections leading to the phenomenon of 'antigenic shift' described below.

The virus-coded non-structural proteins, NS1 and NS2, are made in large amounts in the infected cells (Compans *et al.*, 1980) and are found to accumulate in the polysomes and nucleolus (Dismock, 1980; Shaw & Compans, 1978; Davey *et al.*, 1985). The functions of these proteins have not been established but NS1 may be involved in host-cell protein shut-off and NS2 in vRNA synthesis (Wolstenholme *et al.*, 1980). A third possible non-structural protein (NS3) has yet to be found and it exists only as an open-reading frame within the virus genome.

1.1. Antigenic 'drift' and antigenic 'shift' of type A influenza viruses.

The segmented nature of the influenza virus genome allows the virus to undergo recombination events through the exchange of segments during a mixed infection. When new HA or NA segments are acquired from an antigenically unrelated type A virus this is termed antigenic 'shift'. Variation in the HA and NA also arises by the progressive acquisition of point mutations, termed antigenic 'drift'. Together these mechanisms of genetic alteration combine to create one of the most striking features of influenza epidemiology, that is emergence of new, distinct virus strains which can differ in antigenicity, virulence, host range and transmissibility. Although RNA segment exchange and mutations occur within all the influenza virus genome segments, it is those affecting the two surface glycoproteins that ^{have attracted} most interest. These are the antigens against which the host's humoral immune responses are directed and the variations that occur give rise to new influenza virus strains which make the control of influenza by vaccination difficult (reviewed by Webster *et al.*, 1983).

Type A influenza viruses can be divided into subtypes on the basis of serological cross-reactivities to HA and NA. To date thirteen HA (designated H1-H13) subtypes and nine NA (designated N1-N9) subtypes are known (WHO memorandum, 1980). All the subtypes are found in birds whereas a more limited repertoire is found in higher animals, such as man, horses and swine. In man only three HA subtypes (H1, H2 and H3) and two NA subtypes (N1 and N2) have been found this century. A nomenclature system is used to identify influenza virus strains (WHO memorandum, 1980) which designates the host of origin (except for human viruses), the geographical origin, the strain number and the year of isolation. The antigenic description of the virus is given in parenthesis e.g. A/Hong Kong/1/68 (H3N2) is a type A influenza virus of human origin isolated in Hong Kong in 1968, the strain number is 1 and it possesses the H3 haemagglutinin and N2 neuraminidase.

1.2. Antigenic 'drift' within the haemagglutinin and neuraminidase genes.

Antigenic drift arises from the gradual accumulation of point mutations within the HA or NA gene. A virus strain then arises which is of the same subtype but that can be serologically distinguished from its predecessor e.g. the A/Bangkok/79 (H3N2) strain is related to, but differs from A/Hong Kong/68 (H3N2) strain. Antigenic 'drift' has been well documented for both HA and NA subtypes, and phylogenetic relationships calculated (Webster *et al.*, 1982; Martinez *et al.*, 1983; Colman *et al.*, 1983; Wiley *et al.*, 1981; Young *et al.*, 1979; Cox *et al.*, 1988; Laver *et al.*, 1980; Wang *et al.*, 1986; Robertson, 1987).

In vitro, antigenic 'drift' can be forced using monoclonal and polyclonal antibodies (Laver & Webster, 1968; Wiley et al., 1981; Webster et al., 1982). Mutations arise, usually point mutations, which cause the resulting virus strain to escape either the neutralizing or neuraminidase inhibiting effects of the eliciting antibody but not other antibodies to different antigenic sites. A single amino acid substitution is sufficient to evade the adverse effects of a monoclonal antibody and occur with a frequency of roughly 10^{-5} (Webster & Laver, 1980; Colman et al., 1983; Webster et al., 1982). On the HA molecule these mutations cluster into distinct antigenic sites (Gerhard & Webster, 1978; Laver et al., 1979; Wilson et al., 1981) whereas on the NA molecule the mutations cover almost all the exposed surface of the spike head (Colman et al., 1983). In neither case do the mutations arise in the receptor binding or catalytic pockets of the respective molecules. It is thought that the mutations occur at the site of antibody binding and observations using electron microscopy support this view (Wrigley et al., 1983).

Selection of variants by immunological pressure allows the mutants to escape neutralization (Laver & Webster, 1968). In vivo substitutions need to arise within each of antigenic sites on the HA molecule for a natural mutant strain to arise because the hosts serum is polyclonal (Wiley et al., 1981). Therefore the frequency of new strains occurring should be very low and in support of this, no escape mutants have been successfully produced in vitro using polyclonal sera. Netti et al. (1981) dispute the fact that mutations need to occur in each of the antigenic sites to give a viable variant in nature. Variants of A/Texas/77 (H5N2) produced in vitro were tested against the sera of patients positive to the parental strain. They found 40% of the patients sera failed to react against such variants, even

though only a single antigenic site was altered. It is also argued that not all the sites are equally immunogenic and that one site may be immunodominant (Webster & Laver, 1980). There are also non-immune selection pressures in vivo where the host cell exerts an effect. Viruses isolated from eggs have an antigenically different HA from viruses isolated in MDCK cells (Schild et al., 1983, 1984; Katz et al., 1987; Rott et al., 1984; Robertson et al., 1987; Oxford et al., 1987). The haemagglutinin genes for H3 viruses isolated from wild ducks are conserved compared to those isolated over the same time period from man (Kida et al., 1987). The mutations that had occurred were randomly distributed throughout the molecule and not confined to antigenic sites.

1.3. Antigenic 'shift' of the haemagglutinin and neuraminidase genes.

Antigenic shift arises from a reassortment event between RNA segments in dual infections and leads to a strain in which the HA or NA is serologically unrelated to the strain immediately preceding it. The antigenic 'shifts' recorded this century in man are H1N1 (1918-1957), the H2N2 (1957-1968) and H3N2 (1968-present day).

Antigenic 'shift' can be demonstrated in vitro by carrying-out mixed infections and selecting for recombinants using either monoclonal or mono-specific polyclonal antibodies. The origin of new influenza subtypes in nature is far less certain. Considerable evidence indicates that the emergence of new subtypes may arise by recombination between human and animal viruses in nature, with a tendency to acquire the HA of the more virulent parent virus (Kilbourne, 1974; Webster et al., 1973 & 1971). Non-human hosts have glycoproteins similar to the human H1, H2 and H3

haemagglutinin subtypes (Laver & Webster, 1973; 1979; Austin & Webster, 1986), and the N1 and N2 neuraminidase subtypes (Webster & Pereira, 1968; Schild & Newman, 1969). The human H3 subtype possibly comes from an animal source, either duck or equine (Scholtissek *et al.*, 1978; Laver *et al.*, 1973).

An alternative explanation for the emergence of new subtypes in nature is that a subtype is recycled after lying dormant in some unknown way and re-emerges at a later date e.g. H1N1 reappeared in 1977, twenty seven years after the last occurrence but has none of the expected evolutionary changes of 27 years replication (Nekajima *et al.*, 1978; Scholtissek *et al.*, 1979; Young *et al.*, 1979; Bean *et al.*, 1980). No explanation as to how this occurs has been found.

1.4. Structure of the haemagglutinin glycoprotein.

The structure and functions of the influenza virus haemagglutinin has been extensively researched, it is possibly one of the best understood proteins (Reviewed by Wiley & Skehel, 1987).

The haemagglutinin is translated as a single polypeptide chain and inserted into the endoplasmic reticulum membrane where the N-terminal signal sequence is removed. As the HA progresses through into the Golgi apparatus it is glycosylated and cleaved into HA1/HA2 before it reaches the plasma membrane (Reviewed by Gething *et al.*, 1986).

The structure of bromelain digested haemagglutinin of A/Hong Kong/68 (H3N2) has been determined by X-ray crystallography (Wilson *et al.*, 1981) and is reproduced in Figure 1.2. It is thought likely that the haemagglutinins of other subtypes have a similar structure because many of the important structural amino-acid residues (especially proline and glycine) are conserved, as are the disulphide bonds (of which there are five in the H3 subtype). The haemagglutinin spike is a trimer (Mr 224,640) of HA1/HA2 dimers linked by a single disulphide bond. The spike extends 13.5nm from the membrane and has a triangular cross-section. There are two structurally distinct regions within each HA1/HA2 dimer: (i) a stem region which extends 7.6nm away from the virus envelope. This region is composed of both HA1 and HA2 polypeptide chains and is centred on two anti-parallel alpha-helices; (ii) a globular head region which sits on top of the stem. The head is composed entirely of HA1 polypeptide folded into eight anti-parallel beta-structures. It is the head region that contains the antigenic sites and the receptor binding site.

Each HA1/HA2 dimer of H3 has seven glycosylation sites, all of which are utilised, and carbohydrate makes up 19% of the HA molecular weight (about Mr 13,000). The carbohydrate is mainly located on the lower half of the haemagglutinin and covers 17-20% of the spike surface. The glycosylation sites on the HA2 polypeptide are highly conserved and are possibly involved in trimerisation (Klenk & Schwarz, 1987). The glycosylation sites and carbohydrate moieties on HA1 are more variable (Klenk & Schwarz, 1987), but are important as they can mask antigenic sites (Skehel *et al.*, 1984).

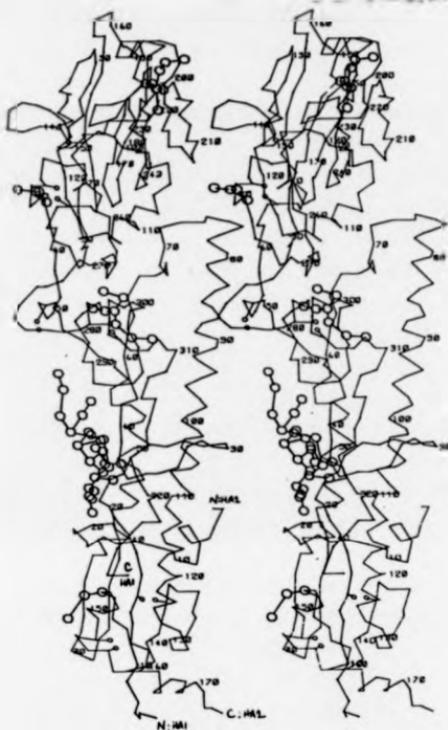


Fig.1.2. A stereo drawing of the alpha-carbon tracing of the H3 haemagglutinin monomer. The receptor binding site is at the distal (top) end of the molecule and the positions of the carbohydrate moieties (O) are also shown. (Wilson et al., 1981).

The trimeric spike is stabilised by non-covalent interactions between the HA1/HA2 dimer units. Most interaction occurs between the stem regions which twist together to form a triple-stranded helix. This stabilises the trimer by hiding a 3.6nm hydrophobic domain on the top half of the stem structure. The membrane anchor domains may also be important in trimer stabilisation (Doms & Helenius, 1986). There are fewer interactions between the globular head regions, the contact between the heads is not extensive but a carbohydrate moiety attached to amino acid residue 165 of HA1 does span the interface and may provide some stabilization.

The cell receptor for influenza virus is sialic acid bearing glycoproteins or glycolipids. The influenza viruses isolated from man have a specificity for $\alpha(2,6)$ linked sialic acid whilst strains isolated from equine or avian hosts show a specificity for $\alpha(2,3)$ linked sialic acid (Pritchett *et al.*, 1987). These workers were able to block the binding of virus to erythrocytes using sialosides of known linkage. The human A/Memphis/102/72 strain was only slightly inhibited by $\alpha(2,3)$ linked sialosides but $\alpha(2,6)$ linked sialosides (especially branched forms) blocked binding very efficiently. The influenza receptor binding pocket is highly conserved depression at the distal end of the H3 haemagglutinin (Wilson *et al.*, 1981). Weis *et al.* (1988) examined by X-ray crystallography the the same H3 haemagglutinin, together with various mutants, complexed with a trisaccharide analogue of sialic acid (sialyllactose). The sialic acid binds to the HA with one face downwards in the depression and 66% of the sialic acid molecule surface is buried upon binding. There is no evidence that conformational changes take place in the HA upon binding. Both the

$\alpha(2,3)$ - and the $\alpha(2,6)$ -linked sialic acid bind in essentially the same way to the haemagglutinin. When leucine at position 226 of HA1 is changed to glutamine then a two-fold reduction in affinity for $\alpha(2,6)$ -linked sialic acid compared to $\alpha(2,3)$ -linked occurred (Weis *et al.*, 1988; Neeve *et al.*, 1984; Rogers *et al.*, 1983). This is thought to be due to small conformational differences rather than altered binding. This conformational change is minor and did not alter the antigenic sites which surround the receptor binding pocket (Daniels *et al.*, 1987). Interestingly if residues 224 to 230 of HA1 were deleted (completely removing the left side of the pocket), the virus was still viable but only had altered binding characteristics (Weis *et al.*, 1988). Also in nature, avian viruses which replicate in the intestinal tract and have a higher affinity for $\alpha(2,3)$ -linked sialic acid, have a glutamine instead of leucine at position 226. If mutants of A/Duck/Ukraine/63 were produced with an affinity for $\alpha(2,6)$ -linked sialic acid, then glutamine was found to be altered to leucine at position 226 (Rogers *et al.*, 1985).

Recently considerable interest has focussed on the point of cleavage between the C-terminal of HA1 and N-terminal of HA2. Wilson *et al.* (1981) showed the ends of the HA1 and HA2 chains are 2.1nm apart which implies a considerable structural rearrangement occurs on cleavage. The N-terminal region of HA2 is highly conserved and is glycine rich. This causes a series of four reverse turns which is an uncommon protein conformation. This region of the HA is therefore strongly non-polar and highly flexible, and has been strongly implicated in the low pH induced fusion of viral and cellular membranes (discussed in more detail later).

Cleavage is also important in the internalization of attached influenza virus by cells. Scholtissek (1986) found that influenza viruses with uncleaved haemagglutinin adsorbed to cells but were not internalized. Viruses with uncleaved HA, in contrast to viruses with cleaved HA, remained accessible to removal or destruction by pH2.5 treatment. Cleavage of HA is also an important determinant of virulence. This has been reported for various HA subtypes (Soech et al., 1979; Rott et al., 1980; Chuchi et al., 1989). Influenza viruses with uncleaved HA were found to be non-pathogenic and although they replicated they failed to spread from the site of infection, unlike those with cleaved HA which spread rapidly and were pathogenic (Rott et al., 1980; Scholtissek et al., 1988). Kawaoka et al. (1987) compared the HA of avirulent A/Duck/113/83(H5N8) and a highly virulent virus strain, A/Turkey/1378/83(H5N8). Genetically the viruses were very similar, with only a 16 base and 4 amino acid differences between the HA's. The HA of the avirulent virus was not cleaved whereas the virulent form was, indicating that cleavage was important in determining virulence. In the connecting peptide region of the HA there was only a single arginine in avirulent virus whereas virulent strains have at least a pair of basic amino acids. They concluded that HA cleavage is determined by the connecting peptide and is important in virulence. Additional factors were also involved in determining the virulence of influenza, because these workers found that if they infected wild ducks with the virulent strain, then replication occurred but no disease. Virulence may be linked to the distribution of trypsin-like enzymes in tissues of the host and it is possible that in wild birds such enzymes have a restricted distribution. The host trypsin-like enzymes may result from co-infection with bacteria. The strain of co-infecting bacteria has been shown to affect the cleavage of HA and influenza virus pathogenicity (Tashiro et al., 1987). Wild

aquatic fowl may act as a 'reservoir' for influenza viruses which are virulent for domestic poultry (Kawaoka *et al.*, 1987; Nestorowicz *et al.*, 1987). It is also worth noting that both of the above viruses occurred very close together in geographic origin and time, and is good evidence of the spread of virus from wild to domestic birds. Also Kawaoka *et al.* (1987) were able to demonstrate a divergence of the H5 subtypes by following either the North American or European flight paths of wild aquatic fowl.

1.5. The antigenic sites on the haemagglutinin.

The antigenic sites on two haemagglutinin subtypes (H3 and H1) have been extensively investigated.

1.5.1. The H3 haemagglutinin subtype.

Using monoclonal antibody generated variants of A/Memphis/1/77(H3N2), Webster & Laver (1980) found at least 3 non-overlapping antigenic areas by comparing haemagglutination-inhibition titres to a panel of 30 monoclonal antibodies. These antigenic sites were designated I, II and III. The frequency of variation within each antigenic site varied but averaged \log_{10} -5.3 and could be as low as \log_{10} -7.3, indicating that differences exist within single antigenic areas. When two monoclonal antibodies directed to different antigenic areas were used in combination, the frequency of escape mutants fell to less than \log_{10} -8.0. Only one escape mutant (in site II) could be differentiated from the parent using polyclonal, hyperimmune sera, suggesting that most of the variants created would not have had a selective advantage in nature. The fact one did occur indicates that a change in a single site may have epidemiological significance. The epidemiology of

natural H3N2 variants was examined, the first changes occurred in site II, then site III (in roughly 1972) and lastly in site I (in roughly 1977). These workers also found two different H3N2 lineages, A/Hong Kong/1/68 which spread to Europe and A/Aichi/2/68 which spread to the USA, but they were uncertain as to whether the viruses originally came from the same source.

Breschkin *et al.* (1981) used A/Memphis/102/72 (H3N2) variants to map the antigenic sites, but instead of mapping the positions of mutation they studied the competition in binding of monoclonal antibodies by RIA. Four distinct antigenic regions were found: (i) There was one major region to which all neutralizing and HI antibodies bound. These had differing strain specific and cross-reactive patterns and therefore they concluded that there were many overlapping/adjacent epitopes. This region agreed with group III site of Webster & Laver (1980). (ii) Two of the other regions were distinct but not remote to that described above, to which non-neutralizing and non-HI monoclonals bound. These would not be found by Webster & Laver (1980) as escape mutants would not have been produced. (iii) The last region was remote from others to which again non-neutralizing, non-HI monoclonals bound.

When the 3D structure was resolved (Wilson *et al.*, 1981), it became possible to map mutations to their position on the HA molecule. Wiley *et al.* (1981) used A/Hong Kong/68 (H3N2) bromelain cleaved HA (BHA) and located 4 distinct antigenic sites by mapping both natural and laboratory generated mutants (Figure 1.3a). Site A was a 'loop' structure (HA1 residues 140 to 146) which protruded 0.8nm from the rest of the HA monomer. This is believed to be the immunodominant site described by Webster & Laver

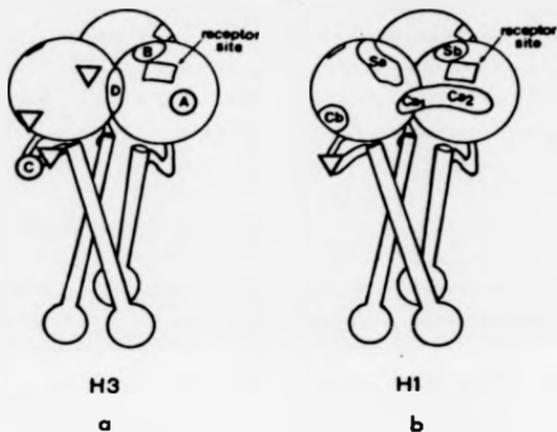


Fig.1.3. Diagram of the neutralizing antigenic sites on the H3 (a) and H1 (b) haemagglutinin trimers. Also shown are the carbohydrate attachment sites (▽) and the receptor binding sites. (Caton et al., 1982).

(1980). Site B was defined only by natural H3 variants and lay on the edge of receptor-binding pocket (HA1-187 to 196). Site C was a bulge 6ms from distal tip, at S-S bond (cysteine residues at 52 and 277). Site D occurred at the trimer interface. A further putative antigenic area was proposed, site E, but this was masked by carbohydrate on the A/Hong Kong/68 virus. The H3 antigenic sites were confirmed to be the actual sites of antibody binding by observation using the electron microscope (Wrigley *et al.*, 1983). It was also found that the angle of the antibody arm and the haemagglutinin was varied according to the site to which it was bound. The H1 sites were mapped using the H3 structure. These were similar but not identical (see below).

1.5.2. The H1 haemagglutinin subtype.

Gerhard *et al.* (1981) used monoclonal antibodies to produce escape mutants of A/IR/8/34 (H1N1) and constructed an operational (rather than physical) antigenic map of the HA using RIA. They found four antigenic sites, two of which were 'strain specific' (designated Sa and Sb) and two that were more 'cross reactive' (designated Ca and Cb) (Figure 1.3b). There was some linkage between Sa and Sb, indicating that they may possibly be close physically. The designation of 'strain specific' versus 'cross reactive' arose because it was thought that antiviral immune mechanisms exert stronger selection pressure on Sa,Sb than on Ca,Cb. There was no greater inherent mutability of these sites but antibodies to Sa and Sb neutralized ten-fold better *in vitro* than those to Cb. They found anti-Ca antibodies neutralized relatively well compared to anti-Sa and anti-Sb, they suggested that man produced an insufficient quantity of anti-Ca antibody, thereby reducing the immune selection pressure on the Ca antigenic site.

Caton et al. (1982), also using A/PR/8/34 compared the positions of mutations to where they lie on the H3 structure found by Wiley et al. (1981). The H1 had low total homology with the H3 but almost all the mutations lay on the predicted surface of the HA. The monoclonal antibody mutants clustered into distinct groups on the $\alpha_1\beta_3$ -D model. Previously Gerhard et al. (1981) had found 4 sites on A/PR/8/34 HA and designated them Sa, Sb, Ca, Cb. Caton split the Ca site into Ca1 and Ca2 but found a strong linkage between them. Other linkages were found between antigenic sites e.g. Sa-Ca1 and possibly Cb-Ca2. Although more than 50% of variants mapped exclusively to distinct sites, it was found that as the number of mutants examined was increased, then sites became less distinct. They concluded that it was best to view the HA head as a large contiguous antigenic area with 5 immunodominant regions.

Lubeck & Gerhard (1982) demonstrated synergistic binding between the Sa and Ca antigenic sites, giving roughly ten-fold enhanced binding. This enhancement was not due to antibody-antibody interaction and was maximal when the other antibody saturated its antigenic site. The synergism was only reflected in antibody binding by RIA and was not apparent by haemagglutination-inhibition, unfortunately they did not assay neutralization. It was concluded that the binding of antibody induced a conformational change in the H1 haemagglutinin. It should be noted that they measured binding to HA immobilised on plastic by RIA. It has been argued that the binding of HA to plastic induces gross conformational changes in the HA, similar to that found induced by low pH (Nestorowicz et al., 1985).

Drescher *et al.* (1987) prepared polyclonal antibodies to A/Brazil/11/78 (H1N1) in chickens and rabbits, and separated the antibodies so that they were either directed against strain specific (SEA) or common (CAA) antigenic sites. These polyclonal antibody preparations seemed to represent the corresponding mixtures of monoclonal antibodies (i.e. pcSEA competed with Sa/Sb and pcCAA competed with Ca/Cb). Also SEA and CAA did not differ in avidity for their respective sites, all were either (SEA,CAA) or (Sa,Sb,Ca,Cb) recognised roughly 1,000 epitopes per virion.

1.5.3. Conformational changes induced in the antigenic sites of haemagglutinin by low pH-mediated membrane fusion.

Daniels *et al.* (1983) treated the X-31 (H3N2) (which is derived from A/Aichi/68) BHA at pH 5.0 which causes fusion of viral and cellular membranes. This also led to a conformational change in the N-terminal region of HA2. The antigenic changes were observed by reaction with monoclonal antibodies with defined specificities. On treatment at pH 5.0, BHA became aggregated and susceptible to tryptic digestion. The latter resulted in two fractions, the 'tops' of HA1 monomers, and aggregates of HA2 plus some HA1 (residues 1 to 27). This would suggest that the HA heads separate at pH 5.0. Daniels *et al.* (1983) found monoclonal antibodies to sites A,C or E still recognised the BHA at pH 5.0 (with or without tryptic digestion). Whereas monoclonals to sites B or D failed to recognise or recognised poorly pH 5.0 BHA. This indicates that a conformational change has altered the antigenicity of these sites. Some antibodies to site B (to HA1 residues 157 and 193) still recognised pH 5.0 BHA and therefore site B can be split into 2 regions (residues 156,158,198 versus residues 157,193).

There was no alteration of site B (site D was not done), when uncleaved EHA was exposed to pH 5.0. Conformational change and antigenic alteration therefore depends on cleavage of the HA.

Webster *et al.* (1983) used both untreated and pH 5.0-treated virus to generate monoclonal antibodies. The virus strains used were A/Memphis/2/71 (H3N2) and X-31 (H3N2) (which is closely related to A/Memphis). The irreversible conformational change in the H3 HA following exposure to pH 5.0 resulted in the generation of new, non-neutralizing antigenic regions. These monoclonal antibodies inhibited haemagglutination only after pH 5.0 treatment of HA. There was a concomitant loss of the previously defined antigenic sites at the tip (site B) and interface (site D), hence conformational changes caused by fusion are primarily located in these regions. The sites at the loop (site A) and bulge/hinge (site C) are not affected by pH 5.0 treatment. The binding of antibody specific for HA at neutral pH was not sufficient to prevent the change in HA taking place following exposure to acidic conditions. Viruses pre-treated at low pH bind to erythrocytes and haemagglutinate, but have no infectivity and do not cause haemolysis. Virions that have attached before the acid-shift do haemolyse. It was surprising that antibodies to neutral pH HA inhibited haemolysis whereas those to acid-treated HA did not. It is possible that the process of conformational change at the erythrocyte surface is responsible for the HA penetrating the lipid membrane. This taken together with the fact that monoclonals to the acid-treated HA did not neutralize indicated that in the normal infection the HA alters its conformation at a location where it is inaccessible to antibody i.e. in endosomes. Therefore virions with acid-modified HA are not capable of initiating infection.

Kostolansky *et al.* (1988) used monoclonal antibodies prepared against either HA1 or HA2 to assess the low pH-induced conformational changes in the HA. The antibodies recognised epitopes outside the major antigenic sites and neither inhibited haemagglutination nor neutralized the virus. They found some of the antibodies, both anti-HA1 and anti-HA2, had improved binding, indicating that both subunits of the HA underwent a conformational change at acid pH. If polyclonal antibodies were used then no differences in binding were observed.

Yedell *et al.* (1983) found pH 5.0 treatment of A/PR/8/34 (H1N1) lead to widespread and irreversible conformational changes of the antigenic sites. Antibodies against Sa and Sb had reduced HI activity whereas those to site Cb had increased HI activity. Antibodies against site Ca varied in their alteration, some with improved activity and others decreased activity. They support the view of Caton *et al.* (1982) that site Ca should be split into two close but distinct sites, Ca1 and Ca2. Comparison of the data using HI haemagglutinin with those using the H3 subtype revealed differences in the pH-induced alterations in conformation, suggesting that each HA subtype maybe affected differently.

1.6 Structure of the neuraminidase glycoprotein.

The N2 neuraminidase structure has been resolved using X-ray crystallography (Varghese *et al.*, 1983). The spikes are mushroom shaped and tetrameric. Each monomer has a slender, flexible stalk and a box-shaped head composed of six anti-parallel beta sheets. There are five potential glycosylation sites on the N2 neuraminidase, four of which are linked to carbohydrate. The tetramer has a four-fold axis of symmetry and is possibly stabilised by

one of the carbohydrate moieties from each monomer. The membrane orientation of the neuraminidase is unusual for a transmembrane glycoprotein as it is anchored via the N-terminal rather than the usual C-terminal.

Neuraminidase (acylnauramyl hydrolase EC 3.2.1.18) enzymatically hydrolyses glycosidic bonds of N-acetylneuraminic acid (sialic acid) to release either D-galactose or D-glucose (Gottschalk, 1957). Each monomer has a large, highly conserved pocket at the end distal to the virus surface and therefore each spike has four catalytic sites (Varghese *et al.*, 1983). The N9 neuraminidase subtype is unusual as it also has a high haemagglutination activity (Air *et al.*, 1987). They suggest that the two activities reside at separate sites on the N9 molecule because an inhibitor reduces the neuraminidase activity but has no effect on haemagglutination. Gene and protein sequence analysis of this neuraminidase showed no significant homology to either influenza virus haemagglutinin or the HN glycoprotein of the paramyxovirus, SV5 (Air *et al.*, 1985).

Neuraminidase mutants can be selected using monoclonal antibodies suggesting a possible role for neuraminidase in disease and selection (Webster *et al.*, 1982). The antigenic sites on the N2 subtype overlap and form a nearly continuous surface covering the top of each monomer, encircling the catalytic pocket (Colman *et al.*, 1983). The N9 subtype has also been analysed and has a very similar structure, it is also one of the few antigens to have been successfully crystallised with bound Fab' antibody fragments (Laver *et al.*, 1987; Colman *et al.*, 1987). Unlike lysozyme (Amit *et al.*, 1985), the N9 neuraminidase undergoes conformational change when Fab' fragments are bound and there is a shift of up to 3Å at the centre of

the epitope (Colman et al., 1987). The Fab' may also alter its conformation and Colman et al. (1987) liken the character of antibody binding to a 'handshake'.

Antibodies to neuraminidase are non-neutralizing (except at very high concentrations) and may attenuate illness (Rott et al., 1974) and give protection against lethal infection (Webster et al., 1988). They inhibit or reduce the substrate activity of the enzyme, possibly by displacing arginine at position 371 from the catalytic pocket (Colman et al., 1987). The size of the substrate influences the antibody effect, the cleavage of large substrates (e.g. fetuin) is inhibited but not smaller substrates and the antibody possibly acts by steric hindrance (Jackson & Webster, 1982). Antibodies also reduce the budding and release of progeny virus from the infected cell surface (Webster & Laver, 1967; Kilbourne et al., 1968; Dowdle et al., 1974). The valency of the anti-neuraminidase antibodies is important because bivalent (Fab')₂ fragments inhibit both NA activity and virus release, whereas monovalent Fab' fragments only inhibit substrate activity (Bacht et al., 1971). These workers argue that anti-neuraminidase antibodies act by forming a lattice between the released virus and the NA in the host cell membrane, thereby reducing the release of virus from the cell. Others believe the anti-NA antibodies act by preventing the NA cleavage of host cell receptors; the progeny virus particles re-attach to these receptors and are not released into the culture medium (Kienk et al., 1970; Sato & Rott, 1966; Compans et al., 1969; Palese et al., 1974).

Anti-NA antibodies may also inhibit influenza virus-induced low pH membrane fusion to BHK cells (Huang et al., 1981) and erythrocytes (Huang et al., 1985). Liposomes only fused if either they contained cleaved HA and NA

together; or if they contained cleaved HA alone plus additional soluble NA (Huang *et al.*, 1980). This indicated an enzymatic role for the NA within the fusion process, and these workers argue that *in vivo* the NA cleaves the HA1-receptor bond, allowing the HA2 to insert into the lipid membrane, resulting in fusion. The anti-NA antibodies sterically block the NA activity and inhibit the membrane-fusion. Fusion activity could be restored by the addition of extra soluble RDE (receptor destroying enzyme, a bacterial neuraminidase from *Vibrio cholerae*). The role of NA in virus-induced membrane fusion is disputed by White *et al.* (1982) who showed that cells transfected solely with the haemagglutinin gene of influenza did fuse efficiently at acid pH and demonstrated that the presence of neuraminidase is not essential.

2. Influenza infection and disease.

2.1. Clinical manifestations of disease in man.

In man, influenza is a predominantly an upper respiratory tract (URT) infection with occasional lung involvement and rarely viraemia (reviewed by Kilbourne, 1987). The severity of the illness varies considerably, some influenza viruses produce subclinical infections whereas others produce acute illness which may prove fatal. The symptoms commonly associated with influenza include nasal discharge, cough, fever, headache, myalgia, anorexia, malaise and depression; occasionally gastrointestinal and neurological effects are seen. Secondary bacterial infections are common and are major contributors to influenza complications and mortality. Influenza infection is frequently associated with acute respiratory disease which can lead to hospitalization (Glezen *et al.*, 1987). In the U.S.A. during the pandemic years 1968-69 the economic impact of influenza virus was estimated as between \$1.7 to \$3.9 billion (Kilbourne, 1987).

The data on the relative virulence of different strains of influenza virus in man mainly come from clinical observation of either natural epidemics or volunteer trials. Interpreting data from epidemics is complicated by socioeconomic factors e.g. the mortality rate of the 1918 pandemic was 16 times higher than that in 1957 which might suggest that the 1918 virus strain was much more virulent (Sweet & Smith, 1980). However this could be equally due to the poor primary health care in 1918, lack of availability of antibiotics to control bacterial pneumonia and the low socioeconomic conditions which prevailed at the end of the Great War. Volunteer trials attempt to overcome the variations in epidemiological studies of morbidity

and mortality by using a defined sample population. Most trials involve a limited number of healthy adults and therefore fail to reflect the sections of society for whom influenza is a serious problem i.e. the old and those with cardio-pulmonary problems (Sweet & Smith, 1980).

2.2. Animal models of influenza infection in man.

The complications associated with interpreting data involving human populations has led many groups to explore the use of animal models. Influenza infection of laboratory animals can be studied at two levels: The in vivo effects on the whole animal and the in vitro effects on isolated respiratory tissue explants i.e. organ cultures.

2.2.1. The in vivo infection of animals by influenza virus.

Most in vivo investigations of influenza virus pathogenesis have utilised the ferret because intranasal inoculation of human influenza virus isolates leads to a non-fatal URT infection with symptoms similar to those in man i.e. sneezing, nasal discharge, listlessness, pyrexia, and anorexia (Sweet & Smith, 1980). Infection is not fatal even though ferrets are slightly more susceptible to infection than man (Campbell et al., 1979). The relative differences in virulence observed in man agree well those observed in ferrets (Tom et al., 1976; 1977; Sweet et al., 1977; Fenton et al., 1977; Campbell et al., 1979).

In-bred strains of mice can be infected and have proved important in the study of the immunological responses to influenza virus infection and this will be discussed later. In-bred mice produce no overt disease when

inoculated intranasally with freshly isolated human influenza viruses although infection of the respiratory tract does occur (Frankova, 1975). Passage of virus through in-bred mice increases virulence (Sugiura & Ueda, 1980; Rudneva *et al.*, 1986). The infection is primarily of the lower respiratory tract (LRT) with gross lung pathology (Frankova, 1975). The infection can become systemic leading to infection of many internal organs and is often fatal (Frankova & Rychterova, 1975). The strain of mouse used also influences the infection, mice bearing the Hx gene survived infection by a neurotropic influenza virus whereas Hx negative mice died (Spriggs, 1986; Krug *et al.*, 1985; Staeheli *et al.*, 1986). Virulence in mice does not correlate well with that found in man (Sweet & Smith, 1980) and therefore mice are not as good a model of human infection as ferrets, although the involvement of the LRT is similar to that occasionally found in man.

2.2.2. The organ culture technique.

The aim of the organ culture technique is to maintain fully differentiated cells in a histologically and functionally intact state. Organ cultures have been prepared from a wide range of tissues e.g. palate, nasal turbinate, trachea, lung, skin, cartilage, fallopian tube, gut, brain etc. Most attention has focused on those tissues that have ciliated epithelium, particularly nasal and tracheal tissue of the URT, because the cilia can be seen beating using light microscopy (Hoorn, 1966) and this provides an easy method of assessing the functional activity of the tissue. The technique used to prepare tracheal organ cultures, the most commonly used source of organ cultures, is discussed later in the thesis (section 6.2.2.) and therefore will not be dealt on here. The range of animal donors from which

organ cultures have been prepared in equally as wide and this is discussed below. Organ cultures have been used for almost a century and their use in virology can be roughly divided into the pre-1970 and post-1970 phases.

The early history and usage of organ cultures is reviewed by Hoorn (1966). Before 1970 organ cultures were used to isolate new virus strains (Hoorn & Tyrrell, 1965; Almeida & Tyrrell, 1967; Harnett & Hooper, 1968) and observe the pathogenic effects of a wide range of respiratory viruses (Hoorn & Tyrrell, 1966a; 1966b). Comparisons were made between unrelated viruses but little attempt was made to compare closely related viruses of differing virulence and this is one of the features that distinguishes pre-1970 work from later work. The second distinguishing feature of the early work is the animal sources used for organ culture preparation. Organ cultures were prepared from human foetal and adult post-mortem tissues and the animals used tended to be either large and expensive or limited in supply e.g. calf, dog, pig and monkeys. Tracheal organ cultures were prepared by longitudinal sectioning (Hoorn, 1966) rather than by transverse sectioning which has been more commonly used since 1970.

The development of live, attenuated influenza virus vaccine strains in the 1970's changed the use of organ cultures. Many groups now used organ cultures, mainly prepared from ciliated respiratory tissues, to assess and compare the virulence of closely related virus strains. Most research has focused on influenza virus (discussed below) but other viruses and pathogens have been studied. Chick embryo tracheal organ cultures were used to study the pathogenesis of infectious bronchitis virus (IBV) (Cherry & Taylor-Robinson, 1970; Darbyshire *et al.*, 1979; Cavanagh *et al.*, 1986). The relative virulence of sexually transmitted diseases has been assessed using

human fallopian tube organ cultures, which also possess ciliated epithelium (McGee *et al.*, 1976). The pathological effects of virulent mycoplasmas have been determined using tracheal organ cultures from ferret (Hu *et al.*, 1975) and chick embryo (Cherry & Taylor-Robinson, 1970).

Many different groups have used tracheal, and to a lesser extent nasal turbinate, organ cultures as model to assess influenza virus virulence. The most striking feature of the data is the lack of a uniform technique. Each group has tended to use different maintenance conditions and different methods to assess pathological damage (discussed in Methods 2.2. and 2.3.). Attempts have been made to resolve the differences concerning medium (Blaskovic *et al.*, 1973) and whether cultures should remain stationary or be kept mobile (Cherry & Taylor-Robinson, 1970), but the results have had little impact on the field of organ cultures. New techniques have been explored and abandoned; for example the combined tissue culture and organ culture system (Blaskovic *et al.*, 1972b) or the use of tetrazolium-trypan blue staining (TT test) to demonstrate histological damage (Herbst-Laier, 1970b).

The animal source used to prepare organ cultures also changed in the 1970's. Herbst-Laier (1970a) compared tracheal organ cultures prepared from a wide range of animal sources (human, monkey, dog, ferret, rat, pig, hamster and mouse) as a model system to evaluate the efficacy of anti-influenza virus drugs. She found the limited supply of human foetal and dog trachea together with their variable quality restricted the potential use of these animals. Pig tracheal organ cultures were also rejected because they had poor susceptibility to influenza virus infection. Hamster and mouse organ cultures suffered necrotic damage during sectioning

because of the small trachea size, which resulted in a shorter effective lifespan for such cultures. It was concluded that the most suitable animal models were the ferret and rat. These findings have been largely either ignored or refuted by others. Human embryo tissue continued to be used (Mostow & Tyrrell, 1973; Hara *et al.*, 1974) but the use of smaller animals became more common. Ferrets were employed predominantly but other animals have also been used successfully.

When comparing the known virulence in man to that on organ cultures, two criteria have been investigated: virus multiplication and destruction of cilia activity. Virus multiplication has proved to be a poor measure of virulence using both human, ferret and hamster tracheal organ cultures, with little or no difference between virulent and avirulent strains (Mostow & Tyrrell, 1973; Hara *et al.*, 1974; Mostow *et al.*, 1979; Abou-Donia *et al.*, 1980; Heath *et al.*, 1983). However the destruction of ciliary activity on tracheal organ cultures has correlated very well with virulence using tissue from humans (Mostow & Tyrrell, 1973; Hara *et al.*, 1974), ferrets (Hara *et al.*, 1974; Fenton *et al.*, 1977; Boudreault, 1979; Diaz-Rodriguez & Boudreault, 1982), rats (Ali *et al.*, 1982a), hamsters (Ali *et al.*, 1982b; Heath *et al.*, 1983), mice (Westerberg *et al.*, 1972) and chick embryos (Blaskovic *et al.*, 1972a; 1972b). Many factors have been found which influence the rate of destruction of cilia activity: increasing the amount of virus in the inoculum accelerates the rate of damage and shortens the time at which virus multiplication is detected (Hoorn, 1966). Hara *et al.* (1974) compared tracheal organ cultures from humans and ferrets. In both systems virulence correlated very well with the destruction of ciliary activity. The destruction of ciliary activity on ferret tissue was considerably slower than found on human tissue (i.e. a 50% reduction in

activity occurred by day 12 in ferret tissue compared to day 4 in human tissue). The age of the donor animal may also influence the rate of damage observed as has been found using hamsters, where cultures from old animals were less susceptible to infection and suffered less cilia damage (Schiff, 1974). The serostatus of the donor has an effect as Edwards et al. (1986) demonstrated using human adenoid organ cultures. The passage history of the particular virus strain may influence the apparent virulence. Virus that had been repeatedly passaging in eggs showed reduced multiplication on organ cultures and damaged cilia less than similar virus that had been passaged a few times through organ cultures (Hoorn, 1966; Gorev, 1975).

2.3. Pathogenicity of influenza viruses.

Pathogenicity describes the mechanism by which the virus produces illness and is a result of complex interactions between the virus and the host organism (reviewed by Sweet & Smith, 1980).

In ferrets, the detection of viral antigens by fluorescent antibody demonstrates that the infection tends to spread down the respiratory tract.

(Sweet et al., 1981; Hussein et al., 1983). Nasal turbinates are the major site of influenza virus replication in ferrets (Sweet et al., 1981). Some *in vivo* studies have demonstrated that avirulent viruses could be distinguished from virulent strains because they multiplied over a longer period and gave lower nasal wash virus titres (Toms et al., 1977; Sweet et al., 1977; Fenton et al., 1977; Campbell et al., 1979; Heath et al., 1986). Other studies have reported no appreciable differences in nasal wash virus titres (Sweet et al., 1981; 1984) and this parameter does not appear to be reliable. The

trachea is also a primary site of infection. Using fluorescent antibodies, influenza antigens were detected within 24 hours of intranasal inoculation and were maximal at 48 to 72 hours (Husseini et al., 1983). It was observed that infection was focal and did not encompass the entire epithelium; goblet cells in addition to epithelial cells were infected. This contrasts with the findings of Dourmashkin & Tyrrell (1970), who did not observe virus attaching to mucus-secreting cells of guinea-pig trachea. No differences in virus multiplication, ciliary destruction or histology were found between the upper/middle/lower sections of the trachea (Hara et al., 1974). The pattern of infection in the bronchi is similar to that of the trachea and both these tissues release virus more efficiently than the nasal turbinates (Husseini et al., 1983). Viruses of differing virulence could be distinguished both by cilia damage and virus multiplication using ferret bronchi (Sweet et al., 1984). In the lungs, both the bronchioles and the alveolar cells show few signs of infection in the ferret (Husseini et al., 1983). The nasal turbinates of ferrets produce 10-fold more virus on a per cell basis than lung alveolar cells and release into the medium a greater proportion of the virus produced (Cavanagh et al., 1979; Husseini et al., 1983). If alveolar organ cultures were maintained for 24 hours before inoculation then higher virus titres were obtained in the medium (Kingsman et al., 1977a; 1977b; Cavanagh et al., 1979), in contrast maintenance of nasal and tracheal organ cultures did not increase virus titres appreciably. Kingsman et al. (1977a) found that maintained lung organ cultures were more susceptible to infection and underwent marked histological changes which included the appearance of necrotic zones and vacuolization. The maintained alveolar organ cultures did not have a different amount of surfactant (Kingsman et al., 1977b). It was observed that 2- to 3-fold more virus particles bound to maintained cells but a

similar observation was made using nasal turbinate organ cultures, suggesting that this is not an important factor in tissue maintenance. Cavanagh *et al.* (1979) found that maintenance did not increase either the number of cells infected or the total virus production but that the amount of virus released by cells had increased by 20- to 100-fold. The reasons why maintained tissue released more virus is unknown and is not due to proteolytic cleavage of HA because both fresh and maintained lung cultures produced virus with cleaved HA. They concluded that the poor virus release in the lungs explained why influenza virus produced an URT rather than LRT infection. It was suggested that the poor virus release may be due to alveolar cells having either small numbers of ribosomes and mitochondria (type I alveolar cells) or little smooth endoplasmic reticulum (type II alveolar cells), both of which are important in the formation of progeny virus.

The first barrier to infection of the URT is the mucociliary blanket which covers the epithelial surface. The virus particles become "entangled" in the mucus blanket by attaching to N-acetyl neuraminic acid residues present in the mucus secretion (Dourashkin & Tyrrell, 1970) and the rate at which the virus penetrates this barrier is affected by its thickness and viscosity (Blaskovic *et al.*, 1972a). These workers suggested that virus attachment to tracheal organ cultures should be studied at physiological temperatures because at 4°C the viscosity of the mucus increased, greatly slowing virus penetration to the epithelial cells. The viral neuraminidase may be important at this stage of infection by releasing virus that has attached to the mucus and possibly also decreasing viscosity (Gottschalk, 1957). Influenza virus may also reduce mucociliary clearance by infecting the subepithelial mucus-secreting glands. Gentry *et al.* (1988) have

demonstrated that primary cultures of feline tracheal glands do undergo a productive non-lytic infection by influenza. When the virus particles have penetrated the mucus blanket they attach to the epithelial cells and in particular adhere to the cilia (Dourashkin & Tyrrell, 1970). Westerberg et al. (1972) observed by EM that virus attachment caused the cilia of mouse tracheal organ cultures to clump and suggested that this might reduce mucociliary clearance of the virus. The significance of these findings is doubtful because every other group that has used ciliated organ cultures have found that the cilia appear to beat normally after inoculation of virus. The virus enters the cells by viropexis and replication proceeds, this will be discussed later. The pathogenic damage caused to the ciliated epithelium of organ cultures is usually recorded as a reduction in ciliary activity and disorganisation of the synchrony of cilia beating, often referred to as ciliostasis. As discussed previously the reduction in ciliary activity correlated with the relative virulence of the virus strain, suggesting that a similar mechanism has a major role in influenza pathogenesis. At the cellular level cytopathic changes precede ciliostasis (Dourashkin & Tyrrell, 1970; Westerberg et al., 1972; Schiff, 1974). Hu et al. (1975) used ferret tracheal organ cultures to examine the destructive effects of virulent mycoplasma on ciliated epithelium. Metabolic changes occurred in the cells prior to ciliostasis, including reduced uptake of galactose and amino acids together with reduced cellular RNA and protein synthesis. Blaskovic et al. (1972a; 1972c) detected influenza viral antigens in the epithelial cells of chick embryo tracheal organ cultures at 12 hours post-infection (p.i.), and budding of progeny virus also started at this time. At 24 hours p.i. considerable vacuolization and dehydration of the epithelial cells was seen, and the epithelial layer progressively became flatter and less columnar in appearance. At 48 hours p.i. the

ciliary activity of the cultures began to fall, large numbers of epithelial cells had a rounded-up morphology and were eventually shed from the culture surface. Cells continue to be shed from the surface of cultures and eventually this strips the organ cultures, revealing the basal membrane. Frequently the cells that had been shed from the cultures still had actively beating cilia. Westerberg et al. (1972) observed very similar cytopathic changes using mouse tracheal organ cultures and in addition found that the infection was restricted to the epithelial layer, the sub-epithelial tissues remained normal in appearance. The appearance of lesions and ciliostasis on tracheal organ cultures leads to a concomitant fall in virus multiplication but low levels of multiplication may persist for up to 2 months indicating that influenza virus can replicate in subepithelial tissue (Hoorn, 1966).

3. Immune responses to infection by influenza virus.

Influenza virus stimulates a full range of immune responses in the infected host (reviewed by Mims & White, 1984; Couch & Kasel, 1983; Mitchell *et al.*, 1985; Yeudell & Heckett, 1987). These responses combine to aid recovery from the illness and prevent re-infection of the host by the same strain of virus. Influenza virus can abortively infect most resting cells of the immune system (Rodgers & Mims, 1982; Kilbourne, 1987; Mims, 1986; van Campen *et al.*, 1989), with various adverse effects e.g. reduced IgG and IgM synthesis (Casali *et al.*, 1984). Immune responses can be conveniently split into cell-mediated and humoral.

3.1. Cellular immune responses.

3.1.1. Polymorphonuclear (PMN) cells and macrophage responses.

As previously discussed influenza virus infection results in considerable damage to URT ciliated epithelium. The infection, together with the resulting cell debris, stimulates PMN and macrophage cells to migrate in large numbers to the site of infection (Tomé *et al.*, 1977; Sweet & Smith, 1980). These cell types are highly phagocytic and actively digest and degrade cell debris and virus particles. Both PMN and macrophage cell membranes have Fc-receptors and if virus-specific antibodies are also present, as found in a secondary rather than primary infection, then phagocytosis is enhanced.

Antibody-dependent cell-mediated cytotoxicity (ADCC), which is also mediated via Fc receptors, plays an important role in influenza infection (Shore *et al.*, 1976). Macrophages, PMN and non-T, non-B (K) lymphocytes are all capable of performing ADCC (reviewed by Fanger *et al.*, 1989). Hashimoto *et al.* (1983) demonstrated that ADCC by human peripheral blood was influenza subtype specific but variant cross-reactive, and was mediated by both anti-HA and anti-NA antibodies. They also found ADCC cell lysis to be greater than that caused by cytotoxic T lymphocytes (Tc or CTL). Very low levels of antibody are required for ADCC which can be detected at dilutions below that required for haemagglutination-inhibition (Greenberg *et al.*, 1977; Hashimoto *et al.*, 1983). Murakami & Matsuyama (1988) found that PMN released reactive oxygen species when influenza infected MDCK cells were encountered. These behaved as strong mutagens on the replicating virus and lead to an increase of 7 to 10-fold in the frequency of production of influenza HA antigenic variants. By increasing the rate of variant production, PMN cells may aid the generation of influenza viruses that can escape neutralization by antibody.

Macrophages release interleukin 1 (IL-1) which is a molecule that is involved in T-cell activation. Soluble factors (cytokines) such as interleukins and interferons (IFN) are important modulators of the immune response and are vital within numerous feed-back loops. During the early stages of influenza virus infection the release of alpha-IFN and beta-IFN is vital, these two IFN species are similar. Probably all infected cells produce and secrete alpha- and beta-IFN, which then binds to specific receptors on other cells close to the site of infection (Marris, 1988). The binding of alpha- or beta-IFN to cells stimulates the expression of class I major histocompatibility complex (MHC) antigens and the importance of this,

especially with respect to the action of Tc cells, is discussed below. Cells stimulated with IFN also become refractory to infection by viruses via induction of enzyme systems which prevent the synthesis of viral proteins (Morris, 1988).

3.1.2. Antigen processing and presentation to MHC-restricted T lymphocytes.

MHC antigens are coded for by the H-2 loci in the mouse and HLA in man, and are divided into class I (H-2 D,K or L in mouse; HLA-A,B or C in man) and class II (Ia in mouse; D in man) (reviewed by Roitt *et al.*, 1985; Mies & White, 1984). MHC antigens are expressed on the surface of cells and their structures suggest that they belong to the immunoglobulin 'superfamily' (Williams, 1984; 1985). T lymphocytes recognise foreign peptide antigens in association with a particular class of MHC antigen on the surface membranes of the other cells, i.e. MHC restriction. Class I MHC antigens are present on most nucleated cells, although in variable amounts. Class II MHC antigens are present on macrophages and lysphoid cells although class II can be induced on other cells, such as astrocytes, by gamma-IFN. The classical view of class I MHC-restriction (as found with Tc cells which are CD8+) was that the intact foreign antigen interacted with the MHC-I antigen and required no processing. This was based on the fact that Tc cells seemed to be specific for membrane glycoproteins of viral origin. Recent work has radically altered this perception and this work is discussed in further detail below. For some time it has been known that foreign antigens presented by class II MHC-bearing cells are processed before being presented to CD4+ T lymphocytes (Schwartz, 1985; Mills, 1986). The processing and presentation functions are performed by antigen presenting cells (APC). There are three requirements for an APC (1) to express MHC-II

antigens (ii) to process foreign antigen (iii) to synthesis and release IL-1. Macrophages are the most studied and possibly most important class of APC and therefore have a central role within the immune system. Dendritic cells, B cells, Langerhans cells and astrocytes can also express MHC class II determinants and act as APC's (Unanue, 1984; Morris, 1988). Dendritic cells have been found associated with the basal membranes of rat tracheal organ cultures and protrude through to the epithelial surface (Holt & Schon-Hegrad, 1987). This would be a favourable position for an APC to be situated during a respiratory tract infection and suggests they may play a role in the immune responses to influenza virus. Antigen processing by class II bearing cells (reviewed by Unanue, 1984; Germain, 1986) involves the internalization of the foreign antigen, which is then degraded in an acidified intracellular compartment; this is the processing stage. (Braciale *et al.*, 1987) have called this the exogenous pathway. It differs from the endogenous processing pathway which presents foreign antigen with class I MHC antigens. A comparison between the two pathways will be made later). Exogenous antigen processing is therefore time-dependent and sensitive to lysosomotropic agents (e.g. chloroquine) which raise the lysosomal compartment pH (Germain, 1986; Eisenlohr *et al.*, 1987). The processed antigen is then cycled to the APC surface where it is recognised in conjunction with the MHC-II antigen. How, and when, the processed antigen interacts with the class II antigen is currently a matter of heated debate (reviewed by Partridge, 1984; 1988). Analysis of small peptides involved in presentation has led to the suggestion of a basic structure for the presented antigen i.e. a region of nonpolar amino acids next to a region of polar amino acids that differ from those of self-proteins. X-ray crystallography of the class I MHC antigen has revealed the presence of an unknown peptide bound in a large groove at the distal end of the MHC

molecule (Bjorkman *et al.*, 1987a; 1987b). They suggest this is the binding site for processed foreign antigen. This evidence would argue for a complex with the MHC molecule rather than a loose association resulting from random collision on the APC surface membrane. The presentation of foreign antigen is modulated by the level of MHC-II expression on the APC surface (reviewed by Unanue, 1984) and APC situated at different locations in the body express differing amounts of MHC-II. Gamma-IFN is produced by lymphocytes, particularly Tc, and up-regulates both class I and II MHC expression (Morris, 1988), whilst prostaglandin E, alpha-fetoprotein and glucocorticoids down-regulate expression (Unanue, 1984).

3.1.3. Helper T lymphocyte (Th) response.

Th cells (CD4+) recognise foreign antigens in conjunction with MHC-II antigen on the surface of APC and are stimulated by IL-1 (Kappler & Murrick, 1976). As their name suggests, the primary role of Th is in aiding other immune responses. Th cells are required for a potent Tc population (Zinkernagel & Doherty, 1979) and they are vital for antibody production by stimulating B lymphocytes (Mims & White, 1984; Riott *et al.*, 1985). Most of the functions of Th cells are mediated by the synthesis and release of lymphokines (including IL2-5 and gamma-IFN) and on the basis of different interleukine synthesis Th cells can be sub-divided into Th1 and Th2 (Mosmann & Coffman, 1987).

During influenza virus infection Th cells are important in the host for both recovery and protection from further infection. Lightman *et al.* (1987) found adoptive transfer of Th cells to monoclonal antibody-Th-cell-depleted mice resulted in the clearance of a persistent X31 (H3N2) infection within

6 days. The type of antigen is important in the stimulation of Th cells. Eisenlohr *et al.* (1987) found UV-inactivated influenza virus stimulated Th proliferation 1000-fold better than either denatured protein, protein fragments or synthetic peptides of HA, NP and M proteins. The fact that Th cells can recognise both internal and surface influenza proteins explains why both sub-type specific and cross-reactive Th cell clones exist (Laab & Green, 1983; Andes *et al.*, 1981). HA-, M- and NP-specific Th cells all stimulate anti-HA antibody production (a Th cell-dependent response) in nude mice equally well upon stimulation using whole virus; the phenomenon of 'cross-help'. With NP-specific Th cells only the IgM isotype was produced whereas with HA- or M-specific Th cells class switching occurs leading to IgA or IgG (Scherle & Gerhard, 1986). Using adoptive transfer of Th cell clones into nude mice, Scherle & Gerhard found an accelerated antibody response and reduced fatalities upon infection by influenza virus. They also demonstrated that the Th cells interacted directly with B lymphocytes (cognitive help) rather than by a general lymphokine release mechanism (non-cognitive help).

Some of the antigenic sites on the HA molecule recognized by Th cells are known (Hackett *et al.*, 1983; 1985). Thomas *et al.* (1987) demonstrated that the HA of X31 (H3N2) with a single mutation which alters glycine to arginine (HA1 residue 135) no longer stimulated proliferation of some Th cell clones. This mutation lies within antigenic site A (Wilson *et al.*, 1981) and therefore in this instance both Th and B cells appear to focus on the same region of the HA molecule. It should be noted that no correlation was found when Th cells were tested with natural mutants in this region. Brown *et al.* (1988) isolated Th cells from spleens of A/Memphis/71 (H3N2) infected BALB/C and C57BL mice. The proliferative responses of the Th cells

were assessed by stimulation with natural and laboratory-generated variants with known amino acid substitutions and monoclonal antibody reactivities. They found 4 overlapping epitopes centred on residues 60 and 63 of HA1 i.e. antigenic site E on H3, and they concluded therefore that this site was also a major antigenic region recognised by Th cells. Also sequence analysis of one HA variant showed that a carbohydrate moiety was linked to the HA at site E, they suggested that this either masked the site or prevented the proteolytic fragmentation of the HA during APC presentation.

Th cells can mediate delayed-type hypersensitivity (DTH) response (reviewed by Liew & Russell, 1980) but this response is more commonly associated with Td lymphocytes which are also MHC-II restricted and express the CD4 marker (Miller & Jenkins, 1985). Adoptive transfer of Td cells into mice increased lung infiltrates and resulted in no reduction in influenza virus titre (Pedson *et al.*, 1971; Couch & Kasel, 1983), suggesting that Td have a deleterious effect during infection. Inactivated influenza gave a greatly enhanced DTH response compared to live influenza. Liew *et al.* (1979) found the DTH response in mice, measured in terms of foot-pad swelling, could be elicited within 24 hours of challenge stimulation. The DTH could be enhanced by pre-treating with cyclophosphamide which is thought to act by inhibiting suppressor T lymphocyte (Ts) saturation. If whole virus was used as the immunizing antigen then the DTH response showed extensive cross-reactivity, responding to influenza B and Sendai viruses. This was thought to be due to host cell components in the viral envelope because allantoic fluid could also elicit a DTH response. If purified M protein was used as an immunogen then the response was type-specific and if

purified HA was used then the response was subtype-specific but variant cross-reactive. This suggested that Td cells recognised different epitopes from those utilised by the humoral response.

3.1.4. Cytotoxic T lymphocyte (Tc) response.

In man the Tc (CD8+ cells) response, detected by MHC-I restricted cytotoxicity against influenza virus-infected target cells, peaked after approximately 1 week during a natural infection or immunization with live vaccine (Greenberg *et al.*, 1978), and between days 3 to 7 using an inactivated vaccine. Cytotoxicity returned to baseline levels after 1 month but persisted for longer if the subject had been pre-exposed (i.e. had a detectable serum antibody titre). The appearance of a Tc response correlated with the onset of recovery. Ennis (1981a) reported similar findings and found that the route of inoculation (i/n or i/m) was not important in generating a Tc response. Interestingly, it was also found that certain of host HLA groups (A1, A9 and B8) generated a lower cytotoxic T lymphocyte response than did others. The significance of this is not clear but MHC dependent presentation has been found for class-II antigen processing (Puri *et al.*, 1986).

The primary role of Tc cells is to lyse virus-infected cells which reduces the amount of virus shed and results in the clearance and recovery from infection (McMichael *et al.*, 1983; Mitchell *et al.*, 1985). Most work has focused on Tc cells isolated from the spleen of mice, a T lymphocyte-rich organ, but the virus-specific Tc cells can also be stimulated and isolated at the local site of infection e.g. pulmonary lymph nodes (Cambridge *et al.*, 1976), Peyer's patches (London *et al.*, 1987), bronchus (Waldman *et al.*,

1972), or peripheral blood (McMichael *et al.*, 1983). The major sites of action for influenza-specific Tc cells are the respiratory surfaces. The cytolytic activity of Tc cells may cause the characteristic lung pathology found in influenza infection of mice but does ultimately lead to virus clearance (Wells *et al.*, 1981). These workers found that influenza virus infection of 'nude' mice (which lack T lymphocytes), caused a high mortality rate, a persistent infection of the lungs and a slower development of lung pathology. A greater proportion of normal mice survived infection, the lung pathology developed more rapidly and the incidence of lung pathology correlated with increased Tc activity in the lungs.

Protection is also partially mediated by cellular immunity and does not solely result from the action of antibodies. Adoptive transfer of influenza-specific Tc cells does protect mice from lethal doses of challenge virus (Sullivan *et al.*, 1976; Yap *et al.*, 1978; Yap & Ada, 1978a), although some have found that Tc cells are not protective (Taylor & Askonas, 1983; Liew *et al.*, 1984). That there is a Tc anamnestic response with a memory ranging from 6 months (Ernis, 1982) to years (McMichael *et al.*, 1983) would suggest a protective role, even if it is short-lived. Adoptive transfer of cloned Tc cells leads to passive protection. This also correlates with the gamma-IFN level (Morris *et al.*, 1982), which is released on contact with infected cells. UV-inactivated virus did not induce gamma-IFN. Gamma-IFN limits virus spread by establishing an antiviral state, modulates other immune responses (particularly NK activity) and up-regulates both class I and class II MHC-expression (Morris, 1988).

The virus-specific Tc cells recognise viral antigen in a class I MHC-restricted manner. It was believed that the influenza virus glycoproteins, in particular the HA, were the target antigens recognised by Tc populations (Cambridge *et al.*, 1976) and that Tc cells recognised intact antigen in association with MHC-I. The work pioneered by Townsend's group has radically altered our understanding of which influenza proteins are antigens for Tc cells and also how antigens are presented in a MHC-I restricted manner to Tc cells (reviewed by Germain, 1986; Long & Jacobson, 1989). It had been long recognised that Tc cells were composed of both type-specific and cross-reactive populations (Zweirink *et al.*, 1977; Doherty *et al.*, 1977; Wraith, 1987) and assumed that Tc were recognising either a common epitope on different HA subtypes which is different to those recognised by antibodies, or an internal viral component (Zweirink *et al.*, 1977; Yap & Ada, 1978b). The second possibility was confirmed when a cross-reactive Tc clone was found which was influenza type-specific but failed to recognise either the HA or NA glycoproteins (Townsend & Skehel, 1982; Townsend *et al.*, 1984a). Comparison of the reactivities of this clone with cells infected with different influenza virus strains found that it recognised a viral determinant which had undergone an antigenic change between 1943 and 1946. The probable candidate was the NP protein. Townsend *et al.* (1984b) used transfected L cells expressing different influenza proteins to investigate the specificities of different polyclonal Tc lines. They found only a minor subpopulation of cells recognised the expressed HA and were subtype-specific, whereas the majority of cells recognised NP and were cross-reactive. Further, NP could not be detected on the transfected cell surface using monoclonal antibodies and this suggested that the NP was not presented in its native form but had undergone processing, possibly similar to that for MHC-II restricted Th cells as previously discussed.

This hypothesis was explored (Townsend *et al.*, 1985; 1986) by using truncated forms of NP expressed in transfected cells. Both the N- and C-termini were independently transported to the cell surface and were recognised by Tc in a MHC-I restricted manner. Anti-NP antibodies were unable to block Tc recognition. It was found that the MHC haplotype helped to define the epitope which was recognised by Tc because Tc cells from C5A (H2k) mice recognised an epitope in region 1-130 whilst C57BL (H2b) recognised an epitope residing in the region 328-386. This selection by the MHC class I haplotype is not absolute as C3H (H2k) Tc cells recognised the region of NP from 328-386. These findings confirmed the processing hypothesis. However chloroquine failed to inhibit recognition indicating that the processing did not involve endosomal degradation. The discovery that immunization using inactivated virus could generate a MHC-II restricted Tc population (Jacobson *et al.*, 1984; Kaplan *et al.*, 1984; Maimone *et al.*, 1986; Mills *et al.*, 1988) has allowed the differences between MHC-I and MHC-II processing to be explored using the same APC system (reviewed by Braciale *et al.*, 1987a). MHC-II processing utilises a chloroquine-sensitive lysosomal route to degrade input antigens; the "exogenous pathway". MHC-I processing requires *de novo* virus protein synthesis and does not involve lysosomal compartments (Wraith & Vessey, 1986) but does present viral antigens in a non-native form.

Although most interest has concentrated on Tc recognition of NP, Tc subpopulations can recognise all other influenza virus proteins, in addition to the HA as mentioned previously e.g. M1 (Gotch *et al.*, 1987). Also the subtype-specific Tc populations that recognise HA are not limited to recognising epitopes in the HA1 polypeptide as antibodies tend to be (Braciale *et al.*, 1984; 1987b; Gould *et al.*, 1987).

3.1.5. Natural killer (NK) cell responses.

NK cells kill virus infected cells and tumour cells by spontaneous cytotoxicity which is not virus-specific (Couch & Kasal, 1983) and is up-regulated by gamma-IFN. When studying influenza infection of man, Ennis *et al.* (1981b) found that NK activity was maximal on day 3 and correlated with the rise in IFN, virus shedding and onset of symptoms. Adoptive transfer into infected 'nude' mice did not result in virus clearance unless MHC-I restricted T lymphocytes⁺ also transferred (Ennis, 1982). NK cells are found in epithelial-associated lymphoid tissues such as Peyer's patches (London *et al.*, 1987), suggesting that they might have an early role in infection.

3.2. Antibody responses.

Infection by influenza virus elicits a humoral response in the host involving the three major immunoglobulin classes: IgG, IgM and IgA. Murphy *et al.* (1982) studied the primary serum and nasal wash anti-HA antibody responses of children inoculated intranasally (i/n) with live attenuated H3N2 and H1N1 influenza virus strains. A serum IgM response was found in all but one of the subjects and peaked at 2 weeks p.i. before declining. Only two-thirds of the subjects developed a serum IgA response; this had a lower titre than that found for serum IgM but followed similar kinetics. All the subjects developed a serum IgG response which reached high titres between 4 to 7 weeks p.i.. In contrast, the predominant immunoglobulin class in the nasal wash samples was IgA which occurred in over three-quarters of the subjects. The nasal IgA response appeared at 2 weeks

p.i. and rose to high titres at 7 to 9 weeks p.i.. A similar number of subjects developed a nasal IgM response, which peaked at 2 weeks p.i.. IgM titres then declined rapidly and were negligible at 7 to 9 weeks p.i.. Only half the subjects developed a nasal IgG response with titres considerably lower than that found for IgA. This peaked at 4 to 6 weeks p.i. and persisted upto 7 to 9 weeks in a few individuals.

During an acute primary influenza infection of a naive host, the virus titre peaks within the first few days of infection and by the end of the first week is declining (Murphy et al., 1973; Dhar & Ogra, 1985). The fact that the detectable humoral response appears during the second week of infection has led to the suggestion that the role of antibody in the recovery from a primary influenza infection is minimal (Mims & White, 1984). The fact that antibody cannot be detected does not mean that antibody is not present during the early phases of infection merely that there is an excess of antigen. In mice at 3 days p.i. there is a rise in the numbers of IgA and IgM plasma cells in the sub-epithelial respiratory tissues and a few days later there is a rise in the number IgG plasma cells (Owens et al., 1981). This indicated that antibody production could be occurring earlier than the findings of Murphy et al. (1982) would suggest. As previously discussed, antibody in low amounts, undetected by other assays is capable of mediating ADCC.

Antibodies are the host's main line of defence against re-infection by the same or closely related influenza virus strain (reviewed by Couch & Kasel, 1983). The mechanisms by which antibody can mediate host resistance to

re-infection include opsonization for phagocytosis, complement fixation, ADCC (discussed previously) and neutralization, which will be discussed later.

Protection is conferred mainly by neutralizing anti-HA antibodies and passive transfer of neutralizing anti-HA antibodies to naive mice protected against a subsequent challenge infection (Virelizier, 1975; Ennis, 1982; Ennis *et al.*, 1982). The role of anti-NA antibodies is less clear. As previously discussed (section 1.4.), anti-NA antibodies are non-neutralizing but inhibit progeny virus release from infected cells. Reports that anti-NA antibody was rarely demonstrable following primary infection may reflect the insensitivity of the neuraminidase-inhibition (NI) test, together with the fact that the NA represents a smaller antigenic mass compared to the HA (Couch & Kasel, 1983). The anti-NA response of immunized subjects is suppressed by the anti-HA response when challenged with a homologous virus strain and is greater when challenged using a virus with a heterologous HA subtype (Johansson *et al.*, 1987a). This suppression operates at the Th cell level, the homologous challenge generates fewer NA-specific Th cells than does the heterologous HA challenge, which suggested that antigenic competition between the virus HA and NA existed (Johansson *et al.*, 1987b). The poor anti-NA response is not due to poor antigenic properties of the NA glycoprotein because Johansson *et al.* (1989) demonstrated that mice inoculated with equal amounts of purified HA or NA gave equivalent antibody responses, indicating that both viral glycoproteins are equally immunogenic. If an HI titre of greater than 1:16 was produced by immunization with purified HA, then the mice were completely protected from challenge infection. Immunization with purified NA did not protect the mice from challenge infection (all mice were

infected) but did reduce the amount of pulmonary virus in proportion to the amount of antigen administered. This confirms the infection-limiting, rather than protecting, role of anti-HA (Schulman, 1969). Humoral responses are also generated against the internal M and NP virus proteins (Cretescu *et al.*, 1978) but these are not protective (Oxford & Schild, 1976).

Although it is clear that anti-HA antibodies protect against re-infection, there is debate how this protection is mediated. Infection by influenza virus stimulates both systemic and secretory immune responses as demonstrated by Murphy *et al.* (1982). Stimulation of the systemic immune system results in a predominantly serum IgG response and some groups have found that this response showed the best correlation with protection (Couch & Kasel, 1983; Potter & Oxford, 1979; Couch *et al.*, 1979). In contrast, others have found protection to correlate better with local antibody levels (Waldman *et al.*, 1968; Liew *et al.*, 1984) and there is a considerable body of evidence (discussed below) which demonstrates that the secretory response are, if not more, important than the systemic response.

Local immunity is mediated by the gut-associated lymphoid tissue (GALT) and the bronchus-associated lymphoid tissue (BALT), both of which are linked and when stimulated produce predominantly IgA antibodies (reviewed by Underdown & Schiff, 1986; Mestecky, 1987). The human IgA immunoglobulin class can be split into two: serum IgA which is mainly (>80%) monomeric and secretory IgA which is mainly (>90%) polymeric (the different immunoglobulin structures are discussed later). Murphy *et al.* (1982) compared the specific activities for each antibody class in nasal wash samples to those in the serum (i.e. ELISA anti-HA titre/RIA antibody concentration) and found that both IgA and IgM antibodies in the nasal wash

samples were secreted locally and did not merely reflect the serum antibody concentrations. The fact that these antibody classes are secreted locally does not necessarily mean that their production occurred at the mucosal surface. The mucosal epithelium actively transports secretory IgA from the basolateral cell surface to the apical cell surface and hence into the external secretions, therefore the increased concentration of IgA in secretions may result from selective transport of serum IgA produced at distant sites of the body. The transepithelial IgA transport pathway is reviewed by Underdown & Schiff (1986). They concluded that very little of the IgA in external secretions came from the circulating pool of serum IgA and that the transepithelial IgA transport pathway was dedicated to transport of IgA from surrounding, local IgA producing cells. There was some inter-species variation: blood to bile transport was found to occur efficiently in the rat, the rabbit and the chicken but not in all other species so far studied, including both man and mouse. Local antibody production (not just the IgA class) has been demonstrated during influenza virus infection. Jones and Ada (1986) intranasally (i/n) inoculated mice with influenza virus and examined different tissues for evidence of antibody secreting cells (ASC). ASCs were found in the mouse lungs that secreted each of the major immunoglobulin classes: IgA, IgM and IgG. More IgA ASCs were found in the lungs than the spleen, and they persisted for a longer period (more than 11 month p.i.) than did the spleen ASCs which were more transient. Edwards *et al.* (1986) isolated lymphocytes from human adenoidal organ cultures and found only those from seropositive donors could be stimulated *in vitro* to produce antibody, with IgM and IgA antibody classes produced in greater quantities than IgG. Tracheal organ cultures from seropositive ferrets required approximately 100-fold more homologous influenza virus to become infected than did those from seronegative animals

(Cogliano & Small, 1978). Once infected the organ cultures continued to shed virus for over 60 days, suggesting that protection from infection was due to local immune mechanisms whilst recovery from infection required factors not present in the tissue explants, presumably originating from the systemic response. Peyer's patches are also a site rich in IgA-committed ASCs and these cells can be stimulated by intragastric (i/g) inoculation of virus (London *et al.*, 1987). Chen *et al.* (1987) i/g inoculated mice with influenza virus and these had 13-fold more IgA than IgG in the lungs. Lymphoid cells isolated from Peyer's patches produced predominantly IgA whilst those isolated from the spleen produced IgG. The presence of IgA in the lungs persisted for 3 to 5 months and protected the mice against a subsequent i/n challenge with a lethal dose of homologous virus. Partial protection was given against antigenic variants of the same HA subtype (Chen & Quinlan, 1988). The ability to generate a BALT immune response by stimulating the GALT demonstrates that the two immune systems are closely linked and others have demonstrated the migration of GALT and BALT-primed ASCs between different mucosal sites (Meatrick, 1987; Dahlgren *et al.*, 1987).

If protection is primarily due to a local immune response, then the immunization regime (local immunization versus systemic immunization) should radically alter the immune response generated by varying the site of antigen delivery and the nature of the antigen presented. Snyder *et al.* (1986) demonstrated that intranasal (i/n) inoculation stimulated an equivalent immune response in squirrel monkeys when compared to aerosol inoculation. In man Johnson *et al.* (1986) found that i/n inoculation with a live attenuated influenza virus vaccine gave protection (determined by amount of virus shed) against a challenge infection that was similar to the

protection found in subjects who had prior exposure to natural infection by a virus of the same subtype. Both groups had detectable pre-challenge nasal IgA titres. In comparison, intramuscular (i/m) vaccination with an inactivated virus gave poor protection and these subjects lacked a pre-challenge nasal IgA titre. Local IgA was produced upon challenge suggesting that i/m vaccination did prime the local immune system. Waldman et al. (1968) demonstrated that i/n inoculation of human volunteers with inactivated virus (H2N2) generated a much greater response, with both serum and nasal wash antibody detected, than did sub-cutaneous (s/c) inoculation which only produced a serum antibody response. IgA was found to be the predominant immunoglobulin class in nasal secretions. When the specificity of the serum and local anti-HA antibodies were compared it was found that whereas the serum antibody was sub-type specific, the local antibody cross-reacted with a heterologous (H1N1 and H3N2) influenza viruses (Waldman et al., 1970). This suggested that the IgA antibody was less specific compared to the serum antibody. Liew et al. (1984) found that mice inoculated using an aerosol of live recombinant A/Rec31 (H3N1) (derived from A/England/939/69 and A/PR/8/34) were completely protected against challenge infection using a different H3 virus strain, A/Victoria/3/75 (H3N2). Protection failed to correlate with either serum IgG antibody levels or numbers of virus-specific Tc cells, instead a good correlation was found with the level of IgA in the lungs. In contrast, if mice were inoculated with either live A/Rec31 intravenously (i/v) or UV-inactivated A/Rec31 s/c, then the mice were protected to a lesser extent against A/Vic challenge although higher serum antibody titres were produced. They suggested that secretory IgA was the most important factor in protection against a homotypic cross-reactive influenza virus infection. The importance of the local IgA response was studied by Sears et al. (1988).

who also demonstrated that live and inactivated vaccines gave different responses. They found i/n vaccination using inactivated virus gave an increased serum antibody titre but a reduced the nasal antibody titre compared to a live attenuated vaccine. They also demonstrated that if IgA was present in nasal washes then the amount of virus shed upon challenge was greatly reduced. Coutelier *et al.* (1987) demonstrated that i/n infection of mice with a panel of viruses, including influenza, resulted in a serum response that was restricted to the IgG2a subclass; this fixes complement efficiently. Intraperitoneal (i/p) inoculation of protein antigens resulted in a predominance of IgG1 antibodies which have poor complement fixing activity. Mocart *et al.* (1988) confirmed the findings of Coutelier *et al.* by investigating the IgG subclass response of mice after either i/n inoculation of live virus or i/a inoculation of purified HA. Upon challenge, mice that received i/n live virus gave a predominantly IgG2a response in both the serum and to a lesser extent, in the lungs and saline. In contrast, mice that received purified HA i/a gave a broad IgG response with no subclass predominating. Immunization by the i/a route did result in an IgA response in the mouse lungs but the size of this response was much reduced compared to that produced by i/n immunization.

To summarize, infection or immunization via the respiratory tract gives a local immune response which is durable, active at the site of infection and may be more cross-reactive between variants of the same HA subtype compared to the systemic response. The i/n immunization efficiently stimulates the systemic immune apparatus to produce virus-specific IgG that fixes complement.

3.3. The structures of the different antibody classes.

Antibodies belong to the immunoglobulin 'superfamily' and share many structural features common to molecules such as the MHC antigens (Williams 1984; 1985). They are multidomain glycoproteins that display very diverse range of binding specificities for epitopes on the surface of molecules that are recognised by the host's immune system as foreign or non-self. Genetically, antibodies achieve this diverse repertoire by a mechanism of somatic selection and expansion from a large but more limited germline (reviewed by Rajewsky *et al.*, 1987). The structure of all antibody classes (reviewed by Roitt *et al.*, 1985; Davies & Metzger, 1983) is based upon a four polypeptide chain building block composed of two light (L) chains and two heavy (H) chains. Each L chain is linked by disulphide bonds to a H chain, the H chains are also linked to each other by disulphide bonds and this results in a Y-shaped structure. Each chain has a variable (V) region and a constant (C) region. The binding of antibody to antigen is mediated by the V regions whereas the C regions mediate many of the biological activities of the antibody molecule e.g. complement activation, ADCC etc. There are two classes of L chain, kappa (κ) and lambda (λ), and five classes of H chain (μ , δ , ϵ , γ , α) which define the five classes of antibody: IgA, IgD, IgE, IgG and IgM. The structures of IgG, IgM and IgA antibody classes are discussed briefly below as these are the major classes generated against influenza infection.

3.3.1. IgG

IgG is the predominant class of circulating antibody and is produced in large quantities during anamnestic immune responses. It has the basic four-peptide structure previously described and is defined by the

incorporation of H chain into its structure. There are four major subclasses in man (designated IgG1, IgG2, IgG3 and IgG4) and in mice (designated IgG1, IgG2a, IgG2b and IgG3). These subclasses differ in sequence, carbohydrate content and disulphide bonds of the H chains and therefore mediate activities such as complement fixation to differing degrees (reviewed by Pumphrey, 1986a; 1986b; Oi et al., 1984).

IgG can be cleaved by proteolytic enzymes into characteristic fragments. Pepsin cleavage of a single IgG molecule produces two Fab' ('fragment antigen binding') fragments and one Fc ('fragment crystallizable') fragment. The Fab' fragments are soluble, they can still combine with antigen and each Fab' contains a complete L chain linked to two domains from the H chain by disulphide bonds. The Fc fragment has no antigen binding capacity and is composed of the remaining C region domains of the two H chains which are linked by disulphide bonds and can be crystallized. Another enzyme, papain, cleaves the Fc fragment from the rest of the IgG molecule, but the two Fab' fragments that were produced by papain remain linked together by disulphide bonds and do not separate; this is termed the (Fab')₂ fragment.

3.3.2. IgM

IgM (reviewed by Davies & Schulman, 1989) is a large molecule (Mr 900,000) and is the first antibody class to appear in the serum during a primary immune response. IgM molecules are polymers of five four-peptide units and have the μ H chain. The polymerization depends on the presence of a J-chain polypeptide whose function is to stabilize the IgM Fc regions during synthesis. In solution IgM molecules are observed to adopt a 'star-shaped'

conformation but upon binding to an antigen the IgM can dislocate into a 'staple' conformation (Feinstein & Munn, 1969). IgM is a powerful activator of complement and the dislocation of IgM molecules is thought to be an important step in complement fixation.

3.3.3. IgA

IgA (reviewed by Underdown & Schiff, 1986) appears in external secretions in elevated concentrations compared to other antibody classes and is also found in the serum. IgA can either be a monomer or polymer of four-peptide units with the α H chain. There are two subclasses of IgA in man, IgA1 and IgA2, whereas the mouse has only one. Human serum IgA is 80% monomeric and 80% of the IgA1 subclass. The IgA in external secretions is 90% polymeric and 40% IgA2 subclass. Like IgM, polymeric IgA has a J-chain and is primarily dimeric composed of two four-peptide units, although trimeric, tetrameric and pentamer polymers are produced in decreasing amounts. The IgA found in external secretions also has a secretory component (SC), derived from the poly-Ig receptor which is a transmembrane protein on the basolateral surface of the mucosal epithelial cells. The poly-Ig receptor binds IgA and transfers it across the mucosal epithelium into the external secretions.

4. The infectious route of influenza virus.

The route of infection followed by viruses has been extensively studied, especially with viruses that utilise the host cells endocytotic apparatus (Reviewed by Helenius & Simons, 1980; Marsh, 1984; Lanard & Miller, 1983; Patterson & Oxford, 1985). ⁴ Virus infection can be divide into six chronological stages:

- (1) attachment
- (2) penetration
- (3) uncoating
- (4) replication and translation to produce viral proteins
- (5) assembly of viral structural components
- (6) release of progeny virus

I shall focus on the first three stages of infection.

4.1. Influenza virus attachment.

Viruses attach to cells via receptors on the host cell membrane (Reviewed by Meager & Hughes, 1977; Lonberg-Holm & Philipson, 1980; Lonberg-Holm, 1981; Crowell, 1987; Roessman, 1988). The best understood and characterised cell receptors are those used by the non-enveloped picornaviruses. These receptors are present in limited numbers on cultured cell surfaces, they can be saturated by virus attachment and have in some cases been isolated e.g. a 90kD protein receptor for human rhinovirus isolated from HeLa cells (Tomassini & Colonno, 1986).

Influenza viruses attach to cells via the binding of the haemagglutinin spike to N-acetylneuraminic acid (sialic acid). The receptor binding site resides in a pocket at the distal end of the haemagglutinin spike (discussed previously) and shares morphological features that appear to be common to many viruses; the receptor binding site is highly conserved, surrounded by antigenic sites but in a depression/pocket/canyon which is inaccessible to antibody binding (Roosman & Palmenberg, 1988). The sialic acid moieties are found as oligosaccharide side chains to a wide range of cell membrane glycoproteins and glycolipids (gangliosides). This results in influenza virus having a very broad range of host cells to which the virus can attach. A degree of specificity is conferred by the sialic acid linkage as strains isolated from man bind to $\alpha(2,6)$ linked sialic acid while those isolated from avian or equine hosts bind $\alpha(2,3)$ linked sialic acid (Pritchett *et al.*, 1987). The influenza virus receptor on erythrocytes is the MN blood group antigen; glycophorin A (Jackson *et al.*, 1973) and in many ways this could be considered as a model cell receptor because the cross-linking of erythrocytes that results from virus attachment can be visualised and simply quantified by measuring haemagglutination. In fact the glycophorin A receptor is far from a perfect model for influenza virus attachment because it only projects 5nm from the erythrocyte cell surface and is buried deep in a web of other carbohydrate moieties (Viitala & Jarnfelt, 1985). Attachment of influenza virus does not lead to a productive infection of nucleated avian erythrocytes (Dimmock *et al.*, 1981) and the haemagglutination inhibition effects of antibodies do not correlate with neutralization of the virus (Dimmock, 1982).

Using organ cultures of respiratory tissue (Dourashkin & Tyrrell, 1970) and cultured epithelial cells (Matlin *et al.*, 1981; Yoshimura *et al.*, 1982) influenza viruses are observed to attach to prominent morphological features, such as cilia and microvilli. Virus attachment is rapid and can occur at low temperatures (Stephenson & Dismock, 1975; Eisenlohr *et al.*, 1987). The addition of DEAE dextran increases binding by electrostatic effect (Lenard & Miller, 1983) and binding is also dependent on the pH conditions (Huang *et al.*, 1974), but this is disputed by Yoshimura *et al.* (1982) who found no pH dependency. The presence of the neuraminidase spikes on the virus surface which cleave sialic acid, means that the binding is reversible but this is thought to be significant only under conditions of vast virus excess where released virus is unable to re-attach because the majority of receptor sites have been destroyed by neuraminidase action (Laver & Kilbourne, 1966). Attached influenza virus can be released from the cell surface by addition of soluble neuraminidase (*Clostridium perfringens*) and this technique has been used to assess virus penetration into cells (Matlin *et al.*, 1981; Richman *et al.*, 1986). Proteinase K has also been used to release attached virus (Taylor & Dismock, 1985a, 1985b), but others have found this enzyme to be ineffective (Matlin *et al.*, 1981).

4.2. Influenza virus penetration.

Viruses that have attached to cells can penetrate by either viropexis or direct fusion with the plasma membrane (Reviewed by Marsh, 1984; Lenard & Miller, 1983; Patterson & Oxford, 1986).

Paramyxoviruses e.g. Sendai virus and NDV, have been observed to fuse at the host cell surface whereas influenza viruses are internalised by viropexis (Dourashkin & Tyrrell, 1970; Russell, 1986). Viropexis utilises the normal cell processes of phagocytosis (the uptake of particles greater than 200nm diameter) and pinocytosis (the uptake of particles less than 150nm diameter) (Marsh, 1984). Pinocytosis is further divided into either fluid phase or receptor-mediated (adsorptive endocytosis), fluid phase pinocytosis being relatively inefficient compared to adsorptive endocytosis.

Influenza virus has been found to penetrate cells by adsorptive endocytosis (Dourashkin & Tyrrell, 1970; Matlin *et al.*, 1981; Marsh, 1984). Coated pits form around attached virus particles, which are then internalised in the form of coated vesicles (Matlin *et al.*, 1981). Adsorptive endocytosis is rapid with a half time of less than 10 minutes (Matlin *et al.*, 1981; Yoshisura *et al.*, 1982) and is dependent on both the virus strain and cell type (Russell, 1986). Influenza viruses with uncleaved haemagglutinin are not internalised (Scholtissek, 1986). Virus has also been seen in smooth vesicles, the functions and origins of which are uncertain (Dourashkin & Tyrrell, 1970; Patterson *et al.*, 1979; Matlin *et al.*, 1981).

There is considerable uncertainty as to whether influenza virus penetration is temperature dependent. Some workers report that internalization of virus is blocked at 4°C and that the majority of the virus remains external to the cells (Matlin *et al.*, 1981; Richman *et al.*, 1986). Only when the temperature is increased towards 37°C does penetration occur. Similar findings have been made with other enveloped viruses e.g. VSV (Superti *et al.*, 1987) and retrovirus (Anderson, 1987). In contrast others report

efficient internalisation occurring at 4°C (Kato & Eggers, 1969; Stephenson & Dismock, 1975; Stephenson *et al.*, 1978; Hudson *et al.*, 1978). The discrepancy is not due to the virus strain as both sets of workers used A/Fowl Plague Virus/Rostock/34 (H7N1) but may be due to the host cell. MDCK and LLC cells were used by those who found no entry at 4°C whereas CEF and BHK cells were used those who did find virus entry.

4.3. Influenza virus uncoating.

In less than 2 minutes after penetration, the coated vesicles lose their clathrin coats becoming prelysosomal or endosomal vesicles (Anderson *et al.*, 1977; Marsh, 1984). These vesicles are devoid of lysosomal marker enzymes (e.g. acid phosphatase) and can be isolated from lysosomes by density gradient centrifugation (Marsh, 1984). Endosomes acidify to at least pH 5.5 via an ATP dependent proton pump (Davey *et al.*, 1985), which may be incorporated into the vesicle membrane at the plasma membrane before the endosome matures. The acidification of the endosome is thought to be responsible for influenza virus uncoating by leading to fusion of the viral envelope and the endosomal membrane. The coming together of membranes is energetically highly unfavourable, therefore a mechanism must exist for overcoming this barrier. The uncoating stage has been investigated using two main approaches:

- (i) Artificially reducing the pH of the medium to induce fusion of virus with the plasma membranes of cells.
- (ii) Treating cells with weak bases and lysotropic agents to raise the endosomal pH and then examining the effects on virus uncoating, genome expression and infectivity. Also amantadine-resistant mutants have been examined to determine the site of mutation.

4.3.1. Antibody mediated membrane fusion.

Lowering the pH of the medium results in the fusion of influenza virus particles to cells. Erythrocytes and liposomes have been most studied. The lipid composition of the target bilayer is important (Doms et al., 1985; Stegman et al., 1985). If liposomes have a high net charge (e.g. composed of brain gangliosides) then fusion can occur at neutral pH (Heywood & Boyer, 1985; 1986). Therefore an acid pH is not vital for membrane fusion. The low pH fusion event is temperature dependent (Huang et al., 1981; Doms et al., 1985; Heywood & Boyer, 1985; 1986) and can be blocked by binding of antibody (Stegman et al., 1985).

The low pH fusion event is a function of the influenza haemagglutinin protein and is dependent on its cleavage into HA1/HA2 (Huang et al., 1981; Yewdell et al., 1983). Daniels et al. (1987) found that the receptor specificity also influenced the fusion activity. Lowering the pH leads to irreversible and major conformational changes in the haemagglutinin spike glycoprotein, as a result of which the haemagglutinin: (i) Becomes sensitive to proteinase K digestion and reduction of the inter-chain disulphide bond by reducing agents (Gething et al., 1986) (ii) Acquires two tryptic digestion sites in the HA1 polypeptide (Skehel et al., 1982; Gething et al., 1986) (iii) Becomes more amphiphilic and binds to liposomes (Gething et al., 1986) (iv) Acquires new antigenic sites and loses other antigenic sites on the native HA as discussed previously (v) Aggregates after bromelain cleavage (Skehel et al., 1982) (vi) Alters its optical properties determined by circular dichroism (Skehel et al., 1982).

Overall, the haemagglutinin is thought to partially dissociate at low pH. The HA1 globular heads lose their trimeric structure (Doms *et al.*, 1985; Gething *et al.*, 1986). The HA1/HA2 stems remain trimeric, possibly adopting a more stable conformation (Ruigrok *et al.*, 1988), and become thinner (Ruigrok *et al.*, 1986a, 1986b). The N-terminus of HA2 becomes exposed and moves out of its 'pocket' in the stem (Ruigrok *et al.*, 1986a, 1986b) and increases in length (Ruigrok *et al.*, 1988). Gething *et al.* (1986) used site specific mutagenesis of the N-terminus of HA2 to produce variants with differing fusion characteristics. If glutamine residues were substituted for glycine residues in this region then the haemagglutinin bound and underwent conformational change but fusion did not occur, indicating that the haemagglutinin itself was not sufficient for fusion. If the length of the N-terminal HA2 region was increased then the size of the conformational change also increased but fusion activity was lessened. If in contrast the N-terminal region was shortened then fusion occurred only with erythrocytes and not other cell types, again highlighting the important role played by the target bilayer composition. It was postulated by Gething *et al.* (1986) that the partial dissociation of the haemagglutinin brought the viral envelope closer to the target membrane. The N-terminus of HA2 inserted into the target bilayer, thus making the haemagglutinin integral to both bilayers. In turn this destabilised the bilayer structure allowing fusion to occur. Doms *et al.*, (1986) also demonstrated that the HA2 polypeptide was involved in fusion by producing a single mutation in the HA2 at position 132 which resulted in an increase in the pH at which fusion occurred compared to the parent virus strain.

4.3.2. Weak bases and lysosmotropic agents.

Weak bases (e.g. ammonium chloride, methylamine) and lysosmotropic agents (e.g. amantadine, rimantadine and chloroquine) all act in the same manner i.e. they raise the pH of endosomal and lysosomal compartments and block influenza virus infection (reviewed by Lanard & Miller, 1983; Hay et al., 1985).

Amantadine does not affect attachment and penetration of influenza virus but does block the endosomal uncoating of the virus (Kato & Eggers, 1969; Skehel et al., 1977). It is concentrated in both the cytosol and nucleus of cells and raises the pH of the lysosomal compartment from 4.7 to 5.5. The anti-viral effects of amantadine are transitory and although the concentration in the cytosol and nucleus remain high, the anti-viral activity is rapidly lost by changing the medium (Richman et al., 1981). Yoshimura et al. (1982) found that if chloroquine-treated infected cells were briefly exposed to an acid environment then virus replication could be restored, demonstrating that the effects were reversible. If amantadine was not present at a time prior to or immediately after infection then no effect was found (Kato & Eggers, 1969; Skehel et al., 1977; Richman et al., 1981). Beyer et al. (1986) found that amantadine inhibited influenza viruses that possessed haemagglutinins with a low pH induced fusion threshold of less than 5.5, but failed to inhibit those with a threshold of more than 6.0. They concluded that amantadine resistance was determined by the haemagglutinin.

4.3.3 A two stage uncoating process.

Recently evidence has emerged indicating that uncoating is a two stage process. Bukrinskaya *et al.* (1980, 1982a, 1982b) found that rimantadine treatment resulted in an accumulation of influenza subviral particles (SVP) or virus cores in the nuclear-associated cytoplasm (NAC) whilst preventing the entry of RNP into the nucleus itself. The infectious virus SVPs were more heterogeneous compared to those from rimantadine treated cells. This resulted from differing amounts of associated matrix protein which suggested a gradual release of matrix protein from the RNPs. The release of the RNP was thought to occur at the perinuclear or nuclear membranes, resulting in an accumulation of matrix protein in the perinuclear membranes. They proposed that uncoating was a two-stage mechanism: (i) endosomal removal of the outer envelope together with the surface glycoprotein spikes (ii) the removal of the matrix protein from the RNPs. These workers also produced a rimantadine resistant variant, which could undergo secondary uncoating in the presence of rimantadine (Bukrinskaya *et al.*, 1982b).

Other groups have also found evidence of a two stage uncoating process. Matlin *et al.* (1981) found low pH fusion at the plasma membrane of cells did not result in a productive infection, this suggests that fusion by itself was not sufficient for complete uncoating of the genome. Richman *et al.* (1981) found amantadine to non-specifically inhibit active cellular processes and suggested that the effects of the agent may not be directly related to its lysosomotropic effects. Richman *et al.* (1986) found that both the haemagglutinin and matrix proteins of infecting influenza viruses were

rapidly degraded whereas nucleoprotein was not. If the cells were treated with amantadine then the haemagglutinin continued to be degraded but matrix became resistant to degradation. They concluded that fusion of viral and cellular membranes had been inhibited, but an alternative explanation would be that fusion occurred and that secondary uncoating was blocked by amantadine. Belehe et al. (1988) examined rimantadine-resistant variants. The sole mutation was in the M2 matrix protein gene (position 30/31) which lay in the transmembrane domain of this protein. This would again support a two stage uncoating mechanism. In contrast to the findings of Beyer et al. (1986) described above, other workers have found amantadine resistance to be conferred not by the haemagglutinin but instead by a protein coded for by RNA segment 7 (Hay et al., 1979; Lubeck et al., 1978; Scholtissek & Faulkner, 1979). This work was performed before M2 was discovered in the virion structure (Zabedee & Lamb, 1988) and led to the conclusion that the mutation resided in M1. The findings of Belehe et al. (1988) would indicate that the mutation affected M2 and not M1. Hudson et al. (1978) found the matrix protein migrated to the nucleus together with the RNP segments whilst the virus envelope lipids and glycoprotein spikes remained cytoplasmic, indicating that the matrix protein remained associated with the RNPs following endosomal fusion.

4.4. Influenza virus replication.

Following uncoating the virus cores migrate to the nucleus, the route to the nucleus is not known (Stephenson & Dismock, 1975). Not all virus particles successfully uncoat, some nucleocapsid protein is degraded by lysosomes and this together with other degraded virus material appears in the medium roughly 30 minutes after infection (Metlin et al., 1981).

Influenza virus has negative (non-message) sense genomic RNA and the virus contains RNA-dependent enzymic activities (transcription and replication) which are carried out by PB1, PB2 and PA (reviewed by Krug, 1983; Laab & Choppin, 1983; McCauley & Mahy, 1983). Replication is also dependent on an active host cell nucleus both for viral transcription and for the processing of viral mRNAs (Kelly *et al.*, 1974). In fact all virus-directed RNA synthesis is nuclear (Shapiro *et al.*, 1987). Primary transcription synthesizes viral mRNA species that are shorter than the template, capped at the 5' terminal and polyadenylated at the 3' terminal. Secondary transcription results in full-length non-polyadenylated viral RNA transcripts which act as templates for the synthesis of vRNA species which are incorporated into progeny virus particles. Viral mRNA species are transcribed from the vRNA and are translated into virus proteins (Shapiro *et al.*, 1987).

4.4.1. Synthesis of viral mRNA.

Viral mRNAs are produced by primary and secondary transcription, and can be detected less than 30 minutes after infection and their rate of synthesis reaches a peak after approximately 2 hours (Stephenson & Dimmock, 1975; Bean & Simpson, 1973; Hay *et al.*, 1977). Synthesis of mRNAs occurs in the nucleus (Shapiro *et al.*, 1987), a unique site for non-oncogenic RNA virus transcription, and requires an intact DNA-dependent RNA polymerase II system (Laab & Choppin, 1977; Jackson *et al.*, 1982). Transcription of the influenza virus genome to produce viral mRNA utilises newly synthesised host cell capped mRNA fragments which serve as primers for viral transcription (Plotch *et al.*, 1979; 1981; Krug *et al.*, 1979; 1980; Bouloy

et al., 1978; 1980; Stridh et al., 1981). The PB2 viral protein generates the primers by recognising and cleaving methylated host mRNAs at a purine residue 10-13 bases downstream from the mRNA cap (Herz et al., 1981; Ulanen et al., 1983). The host oligonucleotide then forms the 5' end of the viral mRNA. Primers may also stimulate the virion polymerase by allosteric modulation of the complex (Pann & Mahy, 1984; Kawakami et al., 1985). Transcription is initiated by the PB1 protein which is also involved in chain elongation (Romanos & Hay, 1984; Krug, 1983).

Viral secondary mRNA is synthesized from *de novo* vRNA and its synthesis is regulated (McCauley & Mahy, 1983; Shapiro et al., 1987; Barrett et al., 1979). Early in infection NP and NS1 predominate whereas later in infection the amounts of these species fall whilst the synthesis of HA, NA and M1 species increase. The P protein mRNAs are synthesized throughout the infection. Not all the influenza virus mRNAs are transcribed from colinear nucleotide sequences. The M1 and M2 proteins are derived from overlapping reading frames on RNA segment 7, NS1 and NS2 are derived from overlapping reading frames on RNA segment 8.

4.4.2. Synthesis of template RNA.

Full length copies of each segment of the virus genome are synthesized in equimolar amounts. The synthesis is not regulated and represents 5% of total viral RNA transcription (Hay et al., 1977). Like mRNA synthesis, the synthesis of template RNA occurs in the cell nucleus (Herz et al., 1981; Shapiro et al., 1987) and uses the same viral enzyme complex as described above (Beaton & Krug, 1984). Unlike mRNA synthesis there is no requirement for primer sequences and the transcripts are not polyadenylated, suggesting

that other proteins are involved which modify the activity of the transcriptase complex (Beaton & Krug, 1986). The inhibition of secondary transcription by cycloheximide confirms that other viral proteins are necessary (Taylor *et al.*, 1977; Barrett *et al.*, 1979).

4.4.3. Synthesis of progeny vRNA.

Progeny vRNA is synthesised from the full length template RNA transcripts. Polypeptide PA is involved and the event is nuclear (Shapiro *et al.*, 1987). The characteristics of influenza virus replication can vary depending on the host cell (Smith & Hay, 1982) and virus strain (Laab & Choppin, 1977; Hay *et al.*, 1977). Sub-genomic RNAs which interfere with subsequent virus replication are sometimes synthesised and are termed defective-interfering (DI) RNAs (reviewed by Nayak *et al.*, 1985). The generation of DI RNAs is favored by high multiplicities of infection and the host cell type (Choppin & Pons, 1970; Janda *et al.*, 1979).

4.4.4. Synthesis of influenza virus proteins and assembly of progeny virus.

Influenza virus utilises the normal host cell processes to synthesise viral proteins. Approximately 1 hour after infection NS1 and NP proteins are detected in the host cell and this is followed by a gradual reduction in host cell protein synthesis which is not completely inhibited (Skehel 1972; 1973). Later in infection HA, NA and M1 proteins are produced, although the host cell type can influence viral protein synthesis (Minor *et al.*, 1979). Viral glycoproteins (HA and NA) are synthesised on membrane-bound polyribosomes and are contranationally inserted into the rough endoplasmic reticulum (RER). The HA and NA proteins are glycosylated and

modified as they progress from the RER through the Golgi apparatus to smooth vesicles. These vesicles migrate to and fuse with the apical plasma membrane of the infected cells. Other viral proteins are synthesized on free ribosomes in the cytoplasm and migrate to different sites of the cell by diffusion (Smith *et al.*, 1987). The P proteins become tightly associated with the cell nucleus, though PA is also found in the cytoplasm. The NP protein is initially found associated with the nucleus, later it is found in the cytoplasm before it migrates to the plasma membrane prior to progeny virus release.

Progeny virus particles are produced by budding from the infected cell membranes. The HA and NA glycoproteins concentrate in the apical plasma membrane and displace host cell proteins. The M1 (and M2) proteins migrate to the plasma membrane where they may interact with the viral glycoproteins, although this is not proven (see section 1.). The RNPs form in the cytoplasm before also migrating to the plasma membrane. The mechanism of how the eight RNPs are incorporated into each virus particle, and how the progeny influenza viruses assemble is not known.

5. Mechanisms of neutralization of viruses.

The binding of antibodies, both neutralizing and non-neutralizing, can inactivate virus infectivity in conjunction with accessory factors e.g. complement (reviewed by Lachmann, 1985; Davies & Metzger, 1983; Beebe *et al.*, 1983; Mathiesen *et al.*, 1988; Vasantha *et al.*, 1988), rheumatoid factor (Coutelier & van Shick, 1988) or antibody-dependent cytotoxic cells (section 3.1.1.). In this section I will focus on neutralization resulting from the binding of antibody alone. This type of neutralization depends on the interaction of three components: the virus, the antibody and the cell (reviewed by Dimmock, 1987).

Neutralization (reviewed by Dimmock, 1984; 1987; Mandel, 1985) results from the binding of one or more antibody molecules to a particular protein on the virus particle leading to a loss of infectivity. For influenza virus, antibodies directed against the HA glycoprotein are neutralizing whereas those directed against the NA glycoprotein are non-neutralizing, although they do limit infection (Sato & Rott, 1966; Webster & Laver, 1967). In order to neutralize influenza virus, antibodies have to bind not only to the HA but to specific epitopes (critical sites) on the molecule (section 1.5.). Antibodies that bind to the HA at epitopes outside these antigen regions (non-critical sites) fail to neutralize the virus (Breschkin *et al.*, 1981).

Neutralization can be mediated by all three major immunoglobulin classes, IgG, IgM and IgA (Ogra *et al.*, 1975; reviewed by Mandel, 1984). The class of antibody is a major factor in determining the mechanism of influenza virus neutralization and this will be discussed below. Most of the work on the

neutralization process for other viruses has involved the use of the IgG and unless stated otherwise, it is this class of antibody to which I am referring. The virus occupies a central position when considering neutralization. The diversity of virus structures means that the findings pertaining to one type of virus (e.g. the non-enveloped picornavirus, polio) cannot be automatically interpreted as applying to others (e.g. the enveloped orthomyxovirus, influenza). Considering the diversity of different virus structures it is not surprising there is no single mechanism of neutralization for all viruses. The target cell type has received comparatively little attention, although some studies, using influenza and other virus systems, have demonstrated a clear and important role for the target cell in the neutralization process and will be discussed below.

The mechanisms by which the binding of antibody can result in a loss of virus infectivity can be divided into four categories:

- (i) Aggregation
- (ii) Inhibition of virus attachment to cells
- (iii) Inhibition of virus penetration into cells
- (iv) Inhibition of subsequent intracellular stages of infection

Neutralization may not necessarily result from the inhibition of just one of these but be due to a combination of these events.

5.1. Aggregation as a mechanism of neutralization.

An infectious unit can comprise either a single virus particle or a virus aggregate. Therefore aggregation by antibody will reduce the total number of infectious units within a virus population and result in neutralization.

Neutralization by aggregation does not necessarily involve an intrinsic loss of virus infectivity, each virus particle may retain full infectivity but the infectivity of the population will be reduced.

Aggregation appears to be a major mechanism of neutralization for poliovirus. Using a panel of polyclonal and monoclonal antibodies, Thomas *et al.* (1985; 1986) found that the neutralization of poliovirus correlated with the loss of single virus particles and an increase in aggregation. The poliovirus aggregates were separated using sedimentation velocity gradients and occurred even when low virus concentrations (<1000 pfu/ml) were used. These workers failed to demonstrate the shift in isoelectric point (pI) that Mandel (1976) had previously found and which will be discussed later. Thomas *et al.* (1985) treated poliovirus aggregates with papain which disaggregated the virus and restored the infectivity, demonstrating that aggregation had not intrinsically damaged virus infectivity. Addition of an anti-Ig antibody to the resulting poliovirus-Fab' complexes caused a return of both aggregation and neutralization. Interestingly, Thomas *et al.* (1986) found that the amount of aggregation fell when the concentration of antibody was in excess of that required for saturation of the virus but there was no return of infectivity, indicating that aggregation was not the sole mechanism of poliovirus neutralization. Aggregated poliovirus was stable and could be separated using sedimentation velocity gradients (Icanogle *et al.*, 1983). Wetz *et al.* (1986) also observed a rise in aggregation as the concentration of neutralizing anti-VPI antibody was increased. However single virions, separated from the aggregated neutralized virus preparations, had lost 22% infectivity. These had an average of one antibody molecule bound per five virus particles, indicating that a single antibody molecule could mediate neutralization in the

absence of aggregation. Metz *et al.* suggested that two mechanisms of neutralization operated (i) an antibody-mediated conformational change in the viral capsid proteins, similar to that described by Mandel (1976), and (ii) aggregation as described by Thomas *et al.* (1985; 1986). Neutralization of adenovirus type 2 by polyclonal anti-fibre antibodies correlated with the amount of aggregation (Wohlfart *et al.*, 1985; Wohlfart, 1988). Neutralizing anti-hexon and anti-penton antibodies did not aggregate adenovirus, except when used in high concentrations, suggesting that the protein to which the antibody bound was an important determinant of aggregation.

The relevance of aggregation to influenza virus neutralization has not been extensively studied. Taylor (1986) found aggregation of influenza virus, A/Fowl Plague Virus/Roostock/34 (H7N1), was negligible when saturating amounts of antibody were used for neutralization. When sub-saturating amounts of antibody (either IgG, IgM or sIgA) were used, aggregation did occur but the effects of aggregation were not investigated. Taylor *et al.* (1987) used the same influenza virus strain and quantified the amount of aggregation at various monoclonal IgG concentrations. At high IgG concentrations they found no aggregation but as the antibody was diluted the majority of virus became aggregated. Surprisingly aggregation of virus was observed when the antibody was diluted to sub-neutralizing concentrations, and they proposed that aggregation was occurring on the EM grid. The rise and fall of influenza virus aggregation dependent on antibody concentration is very similar to that discussed above for poliovirus, which would suggest that it is a common phenomenon.

5.2. Inhibition of virus attachment to cells.

The steric inhibition of virus attachment to cells resulting from the binding of antibody is conceptually the simplest mechanism of neutralization.

Lee *et al.* (1981) demonstrated that antibodies against the sigma 1 capsid protein of reovirus inhibited virus attachment to L cells by up to 99%. For Epstein Barr virus (EBV), monoclonal neutralizing antibodies against the gp220 and gp350 glycoproteins inhibited virus attachment to lymphoblastoid cell lines by more than 96% (Miller & Hutt-Fletcher, 1988). In contrast, they found neutralizing antibodies against the gp85 glycoprotein failed to reduce EBV attachment. The inhibition of attachment to cells for other viruses appears to be a less important as a mechanism of neutralization. Anti-gD antibodies only partially inhibited (by up to 30%) the attachment of herpes simplex virus type 1 (HSV-1) to Vero cells (Highlander *et al.*, 1987) and HEp-2 cells (Fuller & Spear, 1985). The small reduction in HSV-1 attachment did not account for the amount of neutralization observed, which implied that other neutralization mechanisms were also operating. Similar findings were obtained using rabies virus neutralized by two different anti-G monoclonal antibodies (Dietzschold *et al.*, 1987). They found the attachment of rabies virus to BHK cells was reduced by 33% and 60% whilst infectivity was "completely neutralized". Roehrig *et al.* (1988) studied the neutralization of Venezuelan equine encephalomyelitis (VEE) virus by monoclonal antibodies against the E2 glycoprotein. They showed that the inhibition of VEE attachment to either Vero or IMF-90 cells was influenced by the epitope-specificity of the anti-E2 antibodies. Antibodies against

E2c and E2h epitopes inhibited attachment by 80-90%, antibodies against other E2 epitopes reduced attachment only by 10-30% whilst those against the E2f epitope actually enhanced attachment by 50-100%. The reduction in VEE attachment was not due to aggregation because Fab' fragments also neutralized the virus (although less efficiently) and inhibited virus attachment, and the addition of an anti-immunoglobulin failed to increase either effect. Joklik (1964) found that polyclonal serum reduced the amount of rabbitpox virus attaching to HeLa cells by 40% but again this did not account for 92-97% loss of infectivity.

The relevance of inhibition of attachment of poliovirus by antibody to neutralization is less clear. Mandel (1967) found poliovirus neutralized by polyclonal hyperimmune serum attached to HeLa cells in similar amounts as infectious virus. In contrast, Emini *et al.* (1983a) found that monoclonal anti-VP1 antibodies did reduce poliovirus attachment to HeLa cells by 30-60% but this was not sufficient to account for neutralization (99%). Inhibition of attachment increased in proportion to antibody concentration up to saturating amounts. One monoclonal antibody and a polyclonal hyperimmune serum enhanced attachment to HeLa and L cells by more than three-fold, which is surprising, because L cells lack poliovirus receptors. It was not known if the binding of antibody had altered the virus-receptor specificity as had been found for vesicular stomatitis virus (VSV) (Schlegel & Wade, 1983), or whether the increased attachment was mediated by the antibodies themselves. All the neutralizing antibodies induced a pI shift from pH 7 to pH 3.6-4.1 which had previously been implicated as a mechanism of neutralization (Mandel, 1976). Emini *et al.* (1983a) also used a neutralizing polyclonal anti-VP4 serum which inhibited virus attachment

by more than 50% depending on the antibody concentration used. This antibody did not induce a pI shift and the inhibition of virus attachment by this antibody may be its predominant mechanism of neutralization.

Neutralization of transmissible gastroenteritis virus (TGEV) by polyclonal IgG or secretory IgA (sIgA) antibodies failed to inhibit virus attachment to swine testis or pig kidney cells although the virus was neutralized by 99.99% (Nguyen *et al.*, 1986). Interestingly, increasing the concentration of sIgA resulted in a three-fold enhancement of virus attachment. The attachment of TGEV to cells did not appear to be mediated by Fc receptors and they suggested that the enhanced attachment of sIgA neutralized virus was due to an aggregation effect. Skinner *et al.* (1986) examined the effects of neutralizing antibodies against human immunodeficiency virus (HIV) type 1 on the binding of purified gp120 to CD4 positive MOLT-4 cells. Polyclonal antibodies raised against recombinant HIV envelope components were primarily directed towards a hypervariable region of the gp120 glycoprotein and failed to inhibit gp120-CD4 binding. Two monoclonal antibodies against the same hypervariable region gave similar results. In contrast, human serum from infected patients did inhibit gp120-CD4 binding and reasonable correlation (0.92) was found between the titres required for neutralization and for inhibited gp120-CD4 binding. These data support the suggestion that two different mechanisms of HIV neutralization can operate dependent on the epitope to which the antibody molecules bind.

For influenza virus, some groups have claimed that neutralization results mainly from the inhibition of virus attachment to cells (Weis *et al.*, 1986). This suggestion is based solely on the fact that the antigenic sites on HA glycoprotein are in close proximity to the receptor binding pocket.

No data were presented to demonstrate a reduced attachment of influenza virus to cells. Contrary to the suggestion of Weis *et al.*, others have found the ability of antibody to inhibit attachment to cells depends on class of antibody and the cell type used. Possee *et al.* (1982) demonstrated that polyclonal anti-HA antibodies from hyperimmune rabbit serum against A/Fowl Plague Virus/Rostock/34 (H7N1) (A/FPV/R) did not prevent virus attachment to CEF cells although the virus was neutralized by 99.999%. This finding was confirmed using BHK cells and A/FPV/R neutralized by saturating amounts of monoclonal IgG antibodies directed against different antigenic sites on the H7 haemagglutinin (Taylor, 1986; Taylor & Dismock, 1985a). When polyclonal IgM anti-HA antibodies were used to neutralize A/FPV/R then a reduction in virus attachment to BHK cells was found (Taylor & Dismock, 1985b). The reduction in attachment was temperature dependent; at 4°C the amount of virus attaching was negligible, but as the temperature was raised more virus attached and at 37°C the amount of IgM-neutralized virus attaching had increased to approximately 50% of non-neutralized virus levels. These workers suggested that the larger molecular size of IgM antibodies sterically hindered virus attachment and that thermal agitation was responsible for bringing the cell receptor and haemagglutinin into the correct juxtaposition. Taylor & Dismock (1985a) found that neutralizing polyclonal sIgA antibody also inhibited A/FPV/R attachment to BHK cells in a manner very similar to that found using IgM. Monomeric IgA, produced by differential reduction of the sIgA, did not prevent A/FPV/R attachment but continued to neutralize the virus. Therefore the molecular size of the antibody influences its ability to inhibit the attachment of influenza virus to cells; large polymeric antibodies (IgM and sIgA) reduce attachment, whilst smaller monomeric antibodies (IgG and monomeric IgA) do not.

Although neutralizing anti-HA IgG antibodies at saturating concentrations do not reduce the attachment of A/FPV/R to CEF or BHK cells (as discussed above), they can inhibit virus attachment to chicken erythrocytes (Possee, 1981). Dimmock (1987) proposed that the difference was due to the length of the cell receptors. The erythrocyte receptor for influenza virus is probably glycophorin (Jackson *et al.*, 1973) which only extends 5nm from the erythrocyte surface (Viitala & Jarnesfelt, 1985). Dimmock argues that the bound IgG molecule is sufficiently large enough to prevent the virus haemagglutinin making contact with the glycophorin. Although the receptor for influenza virus on cultured cells has not been identified, Dimmock suggests that the receptor is long enough to penetrate the fringe of IgG molecules bound to the virus allowing the haemagglutinin to make contact. It is possible that glycophorin (or a similar size of molecule) is the influenza virus cell receptor on all haemopoietic cells, because Eisenlohr *et al.* (1987) found that a neutralizing monoclonal IgG2a anti-HA antibody inhibited the attachment of A/PR/8/34 (H1N1) to A20 murine B-lymphoma cells by more than 90% and (Fab')₂ fragments were equally effective.

The attachment of influenza virus to chicken erythrocytes does not result in a productive infection (Cook *et al.*, 1979). The attachment of virus particles cross-links erythrocytes and is visualized as haemagglutination. The inhibition of attachment of influenza virus to erythrocytes probably accounts for the inhibition of haemagglutination (HI) but this does not necessarily reflect neutralization. Polyclonal (Yoden *et al.*, 1982) and monoclonal (Kida *et al.*, 1982) anti-HA antibodies can exert a neutralizing effect on influenza virus in the absence of HI activity. These workers suggested that position of the antigenic site on the HA molecule may

determine whether antibodies had HI activity or not. Gitelman et al. (1986) produced monoclonal antibody escape mutants which were not neutralized by the generating antibody but the antibody still had HI activity against the variant. They suggested that this may result from altered specificity and/or avidity of the virus variant for cell receptors because the pattern of haemagglutination titres using erythrocytes from different animal species was also altered. Cesino et al. (1986) reported that a monoclonal IgM antibody had HI activity but did not neutralize. They suggested that "either the epitope for neutralization overlaps with, but is smaller than, the epitope for haemagglutination, or that it only partially overlaps with the latter epitope" but this interpretation is open to question (section 10.3.5.). The fact that neutralization and haemagglutination-inhibition are not closely linked suggests that the erythrocyte is a poor model on which to study neutralization of influenza virus by antibody. Escape mutants of poliovirus have been produced to which the generating antibody continues to bind (Thomas et al., 1986).

5.3. Fc receptor-mediated antibody-dependent enhancement (ADE) of infectivity.

The enhanced attachment of neutralized poliovirus and TGEV to cells described above did not appear to be mediated by Fc receptors (FcR). The binding of sub-neutralizing amounts of IgG1 or IgG2a monoclonal antibody to the flavivirus, West Nile virus (WNV), resulted in almost a 200-fold enhancement of infectivity using the macrophage cell line, P388D1 (Parris et al., 1981; 1982). ADE could be abolished by pre-treating the cells with anti-FcR antibody. If WNV was treated with (Fab')₂ fragments prepared from the neutralizing antibodies then ADE did not occur but the (Fab')₂

fragments could neutralize infectivity if their concentration was increased. The WNV, with sub-neutralizing amounts of antibody bound, attached to and entered P388D1 cells using the same endosomal pathway as virus with no antibody bound (Gollins & Porterfield, 1984; 1986). Similar ADE have been found using macrophage cell lines and other viruses including alphaviruses (Parris & Porterfield, 1982), respiratory syncytial (RS) virus (Gimenez et al., 1989) and Sindbis virus (Chanas et al., 1982).

Ochiai et al. (1988) demonstrated ADE for influenza virus A/WNS (H1N1) treated sub-neutralizing amounts of IgG antibody and grown on P388D1 macrophage cells. The virus attached to and multiplied in neuraminidase-treated cells; ADE was not mediated by Fab fragments. Treatment of cells with ammonium chloride abolished multiplication of both the normal and ADE virus which demonstrated that they were internalized and uncoated by a similar endosomal pathway. If the antibody concentration was increased to that required for neutralization (determined by plaque assay using MDCK cells) then ADE also decreased, demonstrating that neutralization could occur in P388D1 cells.

5.4 Inhibition of virus penetration in-to cells.

Joklik (1964) demonstrated that the neutralized-rabbitpox virus that had attached to HeLa cells was not internalised and the majority eluted from the cell surface. Similarly IgG and sIgA neutralized TGEV which had attached normally to swine testis and pig kidney cells was not internalized (Nguyen et al., 1986). Internalization was determined by treating the cells

with proteinase K at various times after the virus attached. After 45 min. p.i. 90% of infectious virus was resistant to removal by proteinase K but there was no increase in resistance of neutralized virus.

Highlander et al. (1987) found anti-gD antibodies slowed, rather than blocked, HSV entry into Vero cells. Anti-gB antibodies did block HSV entry, again suggesting that the mechanism of neutralization is dependent on the protein to which the antibody is directed. Emini et al. (1983a) used a panel of polyclonal and monoclonal antibodies against poliovirus and found they all inhibited penetration by approximately 50%. Hyperimmune sera completely abolished poliovirus entry but complete inhibition of entry could not be achieved using a cocktail of monoclonal antibodies against 5 different poliovirus epitopes. Surprisingly a non-neutralizing polyclonal antibody preparation inhibited virus penetration to similar extent as found using neutralizing monoclonal antibodies, suggesting that the inhibition of virus entry was not an important mechanism of neutralization for poliovirus.

Influenza virus (A/FPV/R) neutralized by either IgG or monomeric IgA antibodies was internalized by CEF and BHK cells with kinetics indistinguishable from those of infectious virus (Poesse et al., 1982; Taylor, 1986; Taylor & Dimmock, 1985a). The IgM or sIgA neutralized virus that attached to BHK cells was not internalized and remained sensitive to removal by proteinase K (Taylor & Dimmock, 1985b).

5.5. Inhibition of intracellular stages of virus infectivity.

Gollins & Porterfield (1986) demonstrated that polyclonal IgG antibodies neutralized MNV on P388D1 macrophage cells when used in concentrations greater than required for ADE. They found that neutralization correlated with a block in uncoating, defined as increased resistance of the virion RNA to degradation by RNase. Also the antibody dilution required for neutralization correlated with that required to inhibit fusion of MNV with liposomes at pH 6.6. They concluded that neutralizing antibody inhibited MNV-endosome fusion and neutralization resulted from the degradation of virus in lysosomal vesicles. Miller & Hutt-Fletcher (1988) measured the membrane fusion activity of EBV by using a self-quenching fluorescent rhodamine probe. Monoclonal anti-gp85 antibodies inhibited fusion whereas antibodies against gp220 and gp350 (previously shown to inhibit virus attachment) did not. Neutralization of adenovirus type 2 by polyclonal anti-hexon and anti-penton antibodies did not inhibit virus attachment to or internalization by HeLa cells (Wohlfart et al., 1985; Wohlfart, 1988). The majority of neutralized virus appeared to be trapped in intracellular vesicles suggesting that virus-endosomal fusion had been inhibited.

The binding of neutralizing antibody to poliovirus has been shown to shift the pI of the virus from pH 7 to roughly pH 4 (Mandel, 1976; Eain; et al., 1983a). These workers found a good correlation between the pI shift and virus neutralization and suggested that the binding of antibody induced an irreversible conformational change in the viral capsid protein, manifested as the pI shift, which blocked the successful uncoating of the virus particles. Mandel (1976) found Fab' fragments caused a pI shift and

neutralization similar to intact antibody. Bains *et al.* (1983b) found cleavage of attached anti-VP1 antibodies using pepsin restored both poliovirus infectivity and reversed the pI shift. Addition of anti-immunoglobulin to Fab'-virus complexes resulted in a return of neutralization and pI shift. In contrast to Mandel, these workers concluded that bivalent antibody binding was necessary for neutralization. Bains *et al.* (1983b) also found the neutralized virus was not aggregated by the neutralizing antibody. Others dispute the pI shift hypothesis (Brioen *et al.*, 1985b) and claim that neutralization of poliovirus occurs in the absence of a pI shift. Neutralization instead correlated with aggregation (Thomas *et al.*, 1985; 1986). Others suggest that both mechanisms may operate depending either on the antibody concentration (Wetz *et al.*, 1986) or specificity (Loonagle *et al.*, 1983). Brioen *et al.* (1985a) demonstrated yet another mechanism of poliovirus neutralization. They found that using low ionic strength conditions the binding of neutralizing monoclonal antibody 35-If4 resulted in disruption of the virions leaving empty viral capsids which were no longer recognized by the antibody. The bound antibody was released and could neutralize other virions, therefore they termed this a "hit and run" mechanism. If isotonic conditions were used 35-If4 neutralized poliovirus by aggregation.

Similar findings to those for poliovirus have been made using a different picornavirus, human rhinovirus type 14 (HRV14) (Colonna *et al.*, 1989). Eight IgG2a monoclonal antibodies against four different antigenic sites on VP1, VP2 and VP3 were tested. All the antibodies caused a pI shift and pepsin treatment of intact antibody already bound to virions reversed this shift. Interestingly Fab' fragments prepared before their addition to HRV14 did neutralize, although a 13- to 61-fold increase in Fab' concentration

was required compared to intact antibody. Antibodies to three of the four sites aggregated the virus but because Fab' fragments could neutralize in the absence of aggregation the importance of this is not clear. Fab' fragments prepared from each antibody group inhibited HRV14 attachment to membranes prepared from HeLa cells. The inhibition by intact antibody was confused by the effects of aggregation and were difficult to assess. These data suggest that the mechanism of neutralization of HRV14 is complex and involved aggregation, pI shift and inhibition of attachment to cells.

For influenza virus the inhibition of virus uncoating can be sub-divided into inhibition of membrane fusion and inhibition of secondary uncoating.

Four non-overlapping antigenic sites (I, II, III, IV) on the H7 haemagglutinin of A/Seal/Mass/1/80 (H7N7) were defined by mapping of monoclonal antibody escape mutants (Kida *et al.*, 1985; Yoden *et al.*, 1986). Antibodies against each site neutralized the virus and inhibited low pH haemolysis. At pH 7, antibodies against sites I and II had HI activity whilst those against sites III and IV did not. At this pH antibodies against each site were capable of inhibiting the haemagglutination by HA rosettes (HIR). At pH 5.9 neither of these activities were apparent for antibodies against any site. If the antibody reacted with the virus HA at pH 7 before being shifted to pH 5.9, then the HI and HIR activities were unaffected. These findings indicated that the binding of antibody was interfering with the conformational change that occurs in the HA molecule at low pH (section 1.5.3.). This was further examined by spin-labelling the HA molecule and detecting changes (L and S components) in the electron spin resonance (esr). The binding of one antibody, 86/1 (site IV), suppressed both the L and S components while another antibody, 58/6 (site I),

suppressed only the S component. These workers suggested that the fact that 86/1 had no HI activity but did neutralize was due to interference with the low pH endosomal fusion event. Also Fab' fragments from antibodies against sites I and II neutralized the virus, although only poorly. Fab' fragments from antibodies against sites III and IV failed to neutralize the virus unless anti-Fab' was added. This indicated that bivalency was important in the neutralization by site III and site IV antibodies but no mechanism was proposed.

In contrast to the findings reported above, Possse et al. (1982) demonstrated that radiolabelled virion RNA of A/FPV/R neutralized by 99.8% by anti-HA polyclonal IgG antibody accumulated in the nucleus of CEF cells with the same kinetics as infectious virus but there was ^{no} primary transcription of the virus genes. This implied that viral and endosomal membrane fusion had occurred and that the antibody block occurred at a later stage. Examination of the in vitro neutralized virion transcriptase activity revealed a 7-fold reduction compared to infectious virus. Using the X49 (H3N2) strain neutralized by 99.999% by monoclonal IgG antibodies a reduction of 9-fold was found in the virion transcriptase activity. In vivo a good correlation was found between the increase in virion RNA resistance to RNase digestion and neutralizing antibody concentration. It was suggested that the binding of neutralizing antibody to the influenza virus HA directly or indirectly inhibited transcription of virion mRNA. The binding of antibody changed the conformation of the HA molecule and this was communicated to the underlying proteins via the C-terminus of HA2 (Dimock, 1984). Later Rigg et al. (1989) found that the RNA of neutralized virus recovered from cell extracts was resistant to RNase whereas that of infectious virus became sensitive. This was interpreted as showing that

neutralization caused a block in secondary uncoating of the virus core. The failure of transcription is thus considered to be an incidental effect and it is predicted that if secondary uncoating was achieved artificially the transcriptase would be functional. Antibody is known to induce conformational changes in viral proteins, including the HA (Lubeck & Gerhard, 1982) and NA (Coleman *et al.*, 1987), and non-viral proteins (reviewed by Celada *et al.*, 1983). Taylor *et al.* (1987) developed this model of influenza virus neutralization by studying the kinetics and stoichiometry of the reaction, and this will be discussed later. Shimizu *et al.* (1985) found a 50-63% reduction in virion transcriptase activity following neutralization of A/Udorn/72 (H3N2) with polyclonal IgG. One neutralizing monoclonal anti-HA antibody, 22/1 (site B), reduced the transcriptase activity by 76% but seven other monoclonal antibodies had no effect. They confirmed that for some antibodies the inhibition of transcriptase activity was a mechanism of neutralization. The fact that most monoclonal antibodies failed to inhibit the *in vitro* transcriptase activity implied that it was not a common or the sole mechanism of neutralization and was dependent on the antigenic site against which antibody was directed.

2.6. Quantitation of the neutralization reaction.

The question of how many bound antibody molecules are required for neutralization is a contentious subject (reviewed by Mandel, 1985) and will only be briefly discussed here. Two models have been proposed; single-hit and multi-hit. The single-hit model argues that the binding of one antibody molecule to a virus particle results in neutralization. The multi-hit model

argues that more than one antibody molecule has to bind to result in neutralization. The kinetics of the neutralization reaction can be defined by the equation:

$$(1) \quad \frac{V_t}{V_0} = 1 - (1 - e^{-kt/D})^n \quad (\text{Mandel, 1985})$$

where V_t and V_0 are the infectivity titres at time t and 0, k is the reaction rate constant, D is the dilution factor of the serum and n is the number of 'hits' required for neutralization.

If $n=1$ (the single-hit model) then a plot of \log_{10} remaining infectivity with respect to time (using a constant antibody dilution) will give a straight line. Infectivity will fall immediately with no lag period.

If n is greater than 1 (the multi-hit model) a plot of \log_{10} of remaining infectivity against time results in a curve. During the early stages of the reaction, relatively little or no loss of infectivity occurs and this is termed the lag period.

For poliovirus, Mandel (1976) found single-hit kinetics of neutralization but this model has been challenged and the arguments for a multi-hit model are reviewed by Della-Porta & Westaway (1977). The ability of monoclonal antibodies to enhance virus infectivity at sub-neutralizing concentrations (as described above) is clear evidence of a multi-hit mechanism because molecules must have bound before the neutralization effect was observed. Synergistic neutralization between monoclonal antibodies against different epitopes also demonstrates a multi-hit mechanism of neutralization (e.g.

rubella virus, Gerna et al., 1987). No neutralization was detected using monoclonal antibodies against the E1 envelope protein, but when two different antibodies were pooled neutralization was detected. The pooling of antibodies also increased the HI activity by 2- to 8-fold. Individually most of the monoclonal antibodies neutralized rubella virus if anti-immunoglobulin was also added, suggesting that cross-linking of antibodies on a single virion or aggregation of virus was important. Synergistic neutralization⁴ also been demonstrated for RS virus (Anderson et al., 1988), Newcastle disease virus (Russell, 1986; Iorio & Bratt, 1984) and La Crosse virus (Kingsford et al., 1983). Synergistic binding of antibodies has been shown for Sindbis virus (Clegg et al., 1983), tick-borne encephalitis virus (Heinz et al., 1984), VSV (LaFrancis & Lyles, 1982) and influenza virus (Lubeck & Gerhard, 1982). The problem of quantifying neutralization can be approached in another way. The stoichiometry of the neutralization reaction can be examined by comparing the amount of neutralization observed with the number of antibody molecules bound per virion. Icenogle et al. (1983) demonstrated that although poliovirus had single-hit kinetics, the binding⁴ of four antibody molecules were required for neutralization. They proposed cross-linking of capsid pentamers by the bivalent antibody molecule was important for neutralization. They suggested two hypotheses⁴ to explain the both kinetic and stoichiometric data; 'stepwise' neutralization and 'critical site' neutralization. The first hypothesis proposes that the binding of each successive antibody reduces the virus infectivity by a factor of 3/4. The second hypothesis proposes that only antibody binding to 'critical' antigenic sites (in this case about 1 out of 4) results in neutralization, the binding of antibody to 'non-critical' sites does not affect infectivity. The 'critical' and 'non-critical' sites are equivalent in

terms of antibody binding but are not equivalent in terms of function. They suggested that possibly the 'critical' sites were internally associated with crucial regions of the virion RNA and that the binding of antibody prevented the successful uncoating of the viral genome. The data reported were not precise enough to distinguish between the two models.

Taylor et al. (1987) quantified the neutralization reaction for a monoclonal antibody to influenza virus using kinetic and stoichiometric analysis. At three temperatures, the neutralization of influenza virus by 0.1 μ g of IgG followed single-hit kinetics i.e. there was no lag period. Lowering the IgG concentration 10-fold did produce a lag period and linear regression analysis and extrapolation of the slope indicated that 2- or 3-hits were required for neutralization. Lafferty (1963) had previously demonstrated that the neutralization of influenza virus was multihit when low antibody concentrations were used at low temperatures (4°C) but interpretation is difficult because he was using a polyclonal, polyspecific antiserum. Taylor et al. (1987) then determined the stoichiometry of neutralization using two methods; the attachment of radiolabelled IgG of known specific activity, and the detection of attached IgG by anti-IgG labelled with colloidal gold. Comparison of the number of radiolabelled IgG molecules bound with the amount of neutralization demonstrated that at 50% neutralization there was approximately 50 IgG molecules bound per virion. A similar result was obtained using colloidal gold detection of IgG bound to virions. The presence of bound IgG molecules was detected using an antibody dilution 100-fold less than that required for neutralization of the virus. Therefore, the kinetic data which suggested a single (or limited number) of 'hits' failed to agree with the stoichiometric data which demonstrated that many antibody 'hits' were necessary to achieve neutralization. These

workers reconciled the difference between the kinetic and the stoichiometric data by suggesting the existence of neutralization 'relevant' and 'irrelevant' HA spikes. This hypothesis proposes that antibody binding to 'irrelevant' HA spikes, which are in the majority (49/50), fails to neutralize the virus and that only antibody binding to 'relevant' (1/50) HA spikes causes neutralization. This proposal satisfies both the kinetic and stoichiometric data, neutralization would result from a single antibody 'hit' but an average of 50 antibodies would have to bind before the 'hit' could be made. The mechanism of how the binding of antibody to 'relevant' spikes might mediate neutralization has been discussed previously.

5.7. The role of the target cell in the neutralization process.

As previously mentioned, the effects of antibody binding to influenza virus can be influenced by whether cultured or haemopoietic cells are used. The potential role of the target cell in the neutralization process has also been demonstrated using other viruses.

Grady & Kinch (1985) found that one monoclonal anti-G1 antibody, 3G8, neutralized La Crosse (LAC) virus when assayed using BHK cells but not on mosquito cells. A second monoclonal anti-G1 antibody, ZB9, had the converse effect, showing neutralization on mosquito cells but having no effect using BHK cells. They suggested the difference may have a significance in the life-cycle of LAC which interacts with mosquitoes and small mammals. Kjellen & von Zeipel (1984) demonstrated that echovirus type 4 was more effectively neutralized on RD cells compared to GMK cells. Aggregation was not responsible for the difference between the two cell types, or for the non-neutralizable fraction. Kjellen (1985) found that the kinetics and

amount of neutralization by polyclonal hyperimmune serum of enterovirus 71 differed according to whether the assay was performed using RD cells, which resulted in greater neutralization, or GMK cells. The differences in kinetics were not due to aggregation and were not due to variation in rates of virus attachment or entry into the two cell types. The antibody population that neutralized on RD cells appeared to bind to the virus only after the virus had attached to the RD cells, indicating that "new" antigenic sites were revealed during interaction of the virus with the cell receptor. Unfortunately these workers did not attempt to remove the free antibody before inoculating the cells which would have proven the post-attachment neutralization. Kennedy-Stoskopf & Narayan (1986) demonstrated the mechanism of neutralization of visna virus by polyclonal hyperimmune serum differed according to whether fibroblast or macrophage cell lines were used. On both cell types the antibody prevented transcription of the viral RNA and resulted in 99% neutralization by plaque assay using monolayers of fibroblasts. Using fibroblasts, the antibody inhibited the binding of virus to cells by more than 80%. The virus that had attached was not internalized by the cells. Using macrophages, virus attachment was enhanced by 50%, and the attached virus was internalized and uncoated. They suggested that the mechanism of neutralization using macrophages involved a block between the uncoating and transcription of the virion RNA. The kinetics of neutralization was slow compared to the rate of virus attachment and entry into cells. They suggested that this may be an explanation for the persistence of visna virus infection of macrophages in vivo.

MATERIALS
AND METHODS.

6.1. Suppliers of reagents.

(Amersham)	Amersham International plc, Amersham, U.K.
(Amicon)	Amicon Ltd, Stonehouse, U.K.
(BDH)	BDH Chemicals Ltd., Poole, U.K.
(Difco)	Difco Ltd., London, U.K.
(Fisons)	Fison Scientific Apparatus, Loughborough, U.K.
(Flow)	Flow Laboratories, Irvine, U.K.
(Gibco)	Gibco-BRL Ltd., Paisley, U.K.
(Hyllys)	Hyllys Rabbits Ltd., Northwich, U.K.
(Nordic)	Nordic Immunology, Tilburg, Netherlands.
(Searle)	G D Searle Ltd., High Wycombe, U.K.
(Sigma)	Sigma London Chemical Co., Poole, U.K.
(Sterilin)	Sterilin Ltd, Feltham, U.K.

6.2. Cells.

6.2.1. Tissue culture cells.

Primary chick embryo fibroblast (CEF) cells were prepared as described by Morser *et al.*, (1973). Plastic Petri dishes, 5cm diameter (Gibco), were seeded with either 9×10^6 or 3×10^4 cells per dish in 3ml of medium and incubated at 37°C in an atmosphere of 5% CO_2 until confluent. Roller bottles were seeded with 2×10^8 cells in 200ml of medium and grown to confluence. The medium used was 199 medium (Flow) containing 100U/ml penicillin, 100ug/ml streptomycin, 1mM glutamine and 5% v/v newborn calf serum (NCS) (Gibco).

Baby hamster kidney (BHK-21) cells were propagated in Glasgow Modified Eagles Medium (GEM) (Gibco) containing 100U/ml penicillin, 100ug/ml streptomycin, 4mM glutamine and 5% v/v NCS.

C3H10T1/2 mouse fibroblast cells were grown in GEM supplemented with non-essential amino acids (GEM-NEAA) (Gibco), 100U/ml penicillin, 100ug/ml streptomycin, 4mM glutamine and 5% v/v NCS.

Medin-Darby canine kidney (MDCK) cells were propagated in Dulbecco's Modified Eagles Medium (DMEM) (Gibco) containing 100U/ml penicillin, 100ug/ml streptomycin, 4mM glutamine and 5% v/v NCS.

6.2.2. Preparation of tracheal organ cultures.

5-10 week old C3H-Hs-Mg mice of both sexes were killed by cervical dislocation and the trachea removed as rapidly and aseptically as possible and placed in a vial of 199 medium. The tracheas were then taken back to the laboratory where extraneous tissue was removed using a sharp scalpel blade and organ cultures prepared. Tracheal organ cultures can be prepared either using longitudinal or transverse sectioning (Figure 6.1.).

Early organ culture work (reviewed by Hoorn, 1966) used the longitudinal method of sectioning and the cultures were observed microscopically using reflected light. I initially attempted this method but found the sectioning difficult due to the very small diameter of the mouse trachea and the highly elastic nature of the tissue. I also found the reflected light examination technique unsatisfactory and time-consuming.

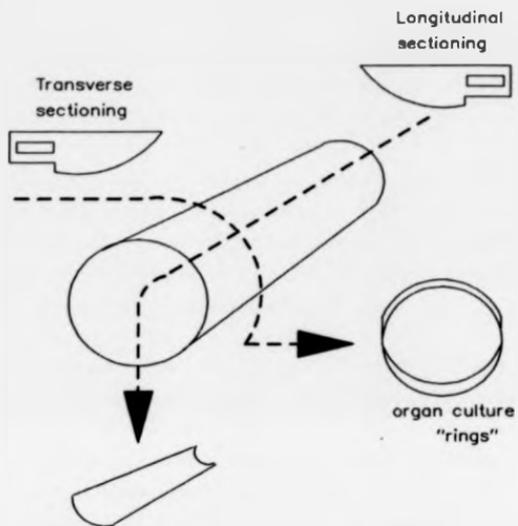


Fig.6.1. Different techniques used to prepare tracheal organ cultures; longitudinal and transverse sectioning.

Most organ culture work after 1970 has used the transverse method of sectioning and produces trachea "ring" cultures which can be observed using transmitted light microscopy. It is important that each section contains at least one cartilage ring, otherwise the culture rapidly sheds the epithelial lining and dies (Hoom, 1966; Hussein *et al.*, 1983). I have found the transverse method of sectioning relatively fast, reliable and reproducible, and is the method I have used throughout my work.

Transverse organ culture sections were cut using a sterile scalpel blade and placed in 199 medium supplemented with 0.1% bovine serum albumin (BSA) (Sigma), and maintained at 37°C (5% CO₂ atmosphere) overnight before use. This medium containing dead cells and debris, was removed and replaced with fresh medium before the cultures were used.

The choice of medium in which to maintain the cultures presented a problem because each research group has tended to use a different medium supplemented with various buffers and additives. I used 199 medium buffered with bicarbonate which has been used throughout organ culture work, from the early days of research (e.g. Hoom & Tyrrell, 1965a) to more recent times (e.g. Heath *et al.*, 1983). Others have used either L15 medium (e.g. Boudreault, 1979) or Eagles MEM (e.g. Darbyshire *et al.*, 1979). Attempts have been made to assess the effects of different media and buffers on the performance of chick embryo tracheal and human fallopian tube organ cultures (Blaskovic *et al.*, 1973; McGee *et al.*, 1976). The results were inconclusive but HEPES or TRIS buffers were preferred to the use of bicarbonate buffer. This has not prevented the successful use of bicarbonate by other workers and I found bicarbonate buffering satisfactory.

When studying virus growth, batches of organ cultures were inoculated and then seeded into 96 well, flat bottomed, tissue culture trays (Sterilin) with one culture and 50ul medium per well. The medium was harvested daily and replaced with fresh medium, the cultures were then examined microscopically. The samples of harvested media were centrifuged to remove cell debris before storing at -70°C .

When studying virus attachment, organ cultures were grouped into batches of 20-30 cultures and maintained at 37°C in 1ml of medium.

6.2.3 Determination of ciliary activity.

The condition of the tracheal organ cultures can be determined by assessing the level of activity of the ciliated cells making up the luminal lining of the cultures. The "ring" organ cultures were observed by transmitted light using an inverted microscope (Olympus) with the X40 objective and X10 eyepiece. The cilia were clearly seen and the whole of the trachea ring was visible in a single field of view.

A system of scoring ciliary activity was developed; various workers have used different methods of determining the condition of organ cultures e.g. percentage surface area beating (e.g. Mostow *et al.*, 1979), vigour of beating (Cherry & Taylor-Robinson, 1970), mapping of individual sections (e.g. Edwards *et al.*, 1986) or histologically using the tetrazolium trypan blue (TT) test (Herbst-Laier, 1970). The scoring technique I developed is shown in Table 6.1.. Such a scheme is subjective but this is an inherent

Table 6.1. Method used to determine the amount of ciliary activity of mouse tracheal organ cultures.

<u>SCORE</u>	<u>DESCRIPTION</u>
0	No observable activity
1	Patchy, but definite activity
2	Activity covering <50% area
3	Activity covering >50% area
4	Strong activity covering 100% area

problem of all such schemes. In practice, it is fast and easy to use; with experience intermediate scores can be given as well as those listed and, to reduce any bias, each section is scored "blind".

6.2.4. Removal of epithelial cells.

The method used was kindly supplied by Dr. C. Sweet (University of Birmingham, personal communication) as is described below:-

The organ cultures are digested with 1ml of Hank's balanced salt solution (HBSS) (Flow) containing 7mM Mg⁺⁺, 0.02% w/v EGTA (Sigma), 10U/ml protease XIV (Sigma), 10U/ml protease XXI (Sigma). The digest was carried out at 37° C for 20 min., with occasional gentle shaking at approximately 5 min. intervals. At the end of the digestion the cultures were vigorously agitated to free the cells, this was done by tapping the vial containing the cultures firmly on a hard surface. The supernatant was removed and kept on ice. This procedure was repeated four times, the organ cultures were then washed with 1ml of 199 media to ensure all the available cells were collected. At the end of the procedure the four digests and the wash were pooled and the cells pelleted by centrifuging for 10 min. at 650g. The pelleted cells were resuspended in 1ml 199 medium containing 0.1% BSA by gently sucking up and down with a Pasteur pipette.

6.3. Viruses.

6.3.1. Influenza strains and their propagation.

The avian strain of influenza A/Fowl Plague/Rostock/34 (H7N1) (A/FPV/R) was grown up from #533 stock preparation (University of Warwick) in the allantoic cavity of 10 day old embryonated hen eggs and incubated for 20 hours at 37°C. The allantoic fluid was collected after chilling the eggs at 4°C overnight, clarified by low speed centrifugation, aliquoted, snap frozen and stored at -70°C.

A mouse-adapted derivative of the above strain, designated adpFPV/R (H7N1), was prepared by serial passaging in C3H10T1/2 cells, followed by plaque purification (further details given in section 7.2.4.). The virus was grown up in eggs in an identical manner described above.

The avian strain A/Fowl Plague/Dutch/27 (H7N7) (A/FPV/D) was grown up from #336 stock preparation (University of Warwick) and was propagated in a manner identical to A/FPV/R.

The infectivities of A/FPV/R, adpFPV/R and A/FPV/D were determined by plaque assay on CEF cell monolayers under 0.9% agar (Difco) in 199 medium containing 100U/ml penicillin, 100ug/ml streptomycin and 5% v/v NCS. The cell monolayers were incubated at 37°C (5% CO₂ in air) for 3 days before staining with 0.02% neutral red (Flow) in PBS. The infectivity was expressed as plaque forming units (pfu) per ml (Dimmock & Watson, 1969).

The human influenza strain A/Wilson Smith Neurotropic/40 (H1N1) (A/WSN) was grown up from #320 stock preparation (University of Warwick) in the allantoic cavity of 10 day old embryonated hen eggs and incubated for 36 hours at 33°C. The allantoic fluid was collected and treated as before. The infectivity was determined as previously described except 0.15% w/v BSA replaced the NCS, and 0.02% w/v DEAE/dextran (Sigma) was added to the overlay medium.

The human strains A/Puerto Rico/8/34 (H1N1) (A/PR/8) and A/Japan/305/57 (H2N2) (A/Jap) were derived respectively from #593 and #572 stock preparations (University of Warwick). The human strain A/Colorado/231/83 (H3N2) (A/C1r) was kindly donated by Dr. P.D. Minor (N.I.B.S.C., South Mims, U.K.). The avian strains A/Chicken/Scotland (H5N1) and A/Turkey/Ontario/6118/68 (H5N4) were derived respectively from #251 and #260 stock preparations (University of Warwick). The type B influenza virus B/Lee/40 was kindly donated by L. McLain (University of Warwick). These strains were grown up in the allantoic cavities of 10 day old eggs and incubated for 36 hours at 33°C before being harvested and stored at -70°C.

The infectivities of these strains were determined by plaquing on MDCK cell monolayers rather than CEF cells. The overlay contained 0.15% w/v BSA, 0.02% w/v DEAE/dextran and 1.25ug/ml trypsin TPCK (Sigma). The monolayers were then incubated at 33°C (5% CO₂ in air) for 3 days before staining. Trypsin was required in the overlay to ensure the cleavage of the haemagglutinin necessary for infectivity.

The haemagglutination titre of all the influenza strains was determined by making doubling dilutions in PBS and adding chicken erythrocytes to 0.5% v/v. This is equivalent to 7.3×10^8 cells per well. The titre was taken as the 50% end-point between full agglutination and zero agglutination after 30 min. incubation at room temperature. The titres were expressed as haemagglutination units (HAU) per ml.

6.3.2. Purification of influenza virus.

The procedure described by Kelly & Diamond (1974) was followed and all steps were carried out at 4°C. Allantoic fluid containing the virus was clarified by spinning at 1,500g for 30 min. The supernatant centrifuged at 75,000g for 90 min. The pellets were soaked overnight in 0.5ml PBS, then resuspended, vigorously vortexed and clarified at low speed. The supernatant was loaded onto a 50ml 15-45% w/v linear sucrose gradient in 150mM NaCl, containing 10mM Tris/HCl pH 7.4 (TN) and spun at 60,000g for 90 min. The visible band was harvested and diluted to 25ml with PBS and loaded onto a 30-70% w/v linear sucrose gradient in TN and centrifuged at 60,000g for 16 hours. The virus band was collected and diluted to 12ml with PBS and spun at 110,000g for 90 min. The resulting pellets were soaked overnight in 200ul PBS, then resuspended and stored as aliquots at -70°C.

6.3.3. Radiolabelling of influenza virus.

6.3.3.1 A/FPV/R influenza strain.

Confluent CEF monolayers in roller bottles were incubated for 24 hours with phosphate free GMEM (GMEM-P) containing 4mM glutamine, 100U/ml penicillin, 100ug/ml streptomycin and 1% v/v NCS. The monolayers were then washed with

phosphate free saline and 10ml of GMEM-P containing 1 pfu/cell of virus was added to each bottle. The cells were incubated for 60 min to allow the virus to attach before adding a further 35ml of medium plus 2.5mCi 32 P-orthophosphate (Amersham) to each bottle, incubation was continued for a further 20 hours. The cells were then examined for evidence of cytopathic effect (CPE) and the tissue culture fluid was harvested and clarified by centrifuging at 1,000g for 10 min. at 4°C. All the following procedures were carried out at 4°C. The virus in the supernatant was precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to give 60% saturation whilst stirring on ice, and purified by sucrose velocity centrifugation (Dimmock *et al.*, 1977) except that no unlabelled carrier was added. The precipitate was pelleted by centrifuging at 20,000g for 20 min, resuspended in 5ml PBS and loaded onto a 55ml 15-45% linear sucrose gradient in TN containing 0.1% w/v BSA. Following centrifugation at 90,000g for 90 min, the gradient was fractionated into 2ml aliquots and assayed for radioactivity and HAU activity. Where the peaks of activity coincided the fractions were pooled. The virus in this pooled peak was diluted with PBS to 30ml and precipitated with 60% saturating $(\text{NH}_4)_2\text{SO}_4$ on ice with fetuin (gift from A. Carver, University of Warwick) added as a carrier. The virus was again pelleted by centrifuging at 20,000g for 20 min., resuspended in 0.5ml PBS and dialysed overnight against PBS. The resulting preparation was tested for radioactivity, infectivity and HAU titre before being aliquoted and stored at -70°C.

6.3.3.2. A/PR/8 influenza strain.

Confluent MDCK cell monolayers in roller bottles were incubated for 24 hours at 33°C with phosphate free GMEM (GMEM-P) (Gibco) containing 4mM glutamine, 100U/ml penicillin, 100ug/ml streptomycin and 0.1% w/v BSA. The

monolayers were then washed with phosphate free saline and 10ml of GEMH-P containing 1 pfu/cell of virus was added to each bottle. The cells were incubated for 60 min. to allow the virus to attach before adding a further 35ml of medium plus 2.5mCi 32 P-orthophosphate (Amersham) and 125ug trypsin TPOX (Sigma) to each bottle. The incubation was continued for a further 40 hours. The cells were then examined for evidence of cytopathic effect (CPE) and the tissue culture fluid was harvested and clarified by centrifuging at 1,000g for 10 min at 4°C. All the following procedures were carried out at 4°C. The virus in the supernatant was precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to give 60% saturation whilst stirring on ice, and pelleted by centrifuging at 20,000g for 20 min. The pellet was resuspended in 5ml PBS and loaded onto a 30-70% w/v sucrose gradient in TN plus 0.1 % BSA and spun at 60,000g for 16 hours. The visible virus band was collected and diluted to 12ml with PBS and spun at 110,000g for 90 min. The resulting pellet was soaked overnight in 200ul PBS, then resuspended, assayed for radioactivity, infectivity and HAU titre, then stored as aliquots at -70°C.

6.3.4. Beta-propiolactone inactivation of influenza virus.

Beta-propiolactone (EPL) (Sigma) was added to the purified virus to give a final concentration of 1/1000 (v/v). The mixture was incubated for 60 min at room temperature and then overnight at 4°C. The inactivated virus was dialysed against PBS to remove any remaining EPL and the residual infectivity determined by plaque assay.

6.4. Antibodies.

6.4.1. Sources.

6.4.1.1. Monoclonal antibodies.

Ascitic fluid containing neutralizing murine monoclonal IgG antibodies against the haemagglutinin of A/FPV/R (H7N1) were kindly provided by A.R.Douglas and J.J.Skehel (N.I.M.R., London, U.K.). These are designated HC2; HC10; HC58; HC51. These monoclonal antibodies have been extensively studied by Taylor (1986) and have been shown to bind to different antigenic sites on the H7 haemagglutinin (P.Garner & N.J.Dimock, University of Warwick, personal communication). Recently Dr.A.Hay (N.I.M.R., London, U.K.) has confirmed that the antibodies map to different antigenic sites by sequencing escape mutants produced by each of the monoclonal antibodies (personal communication).

Ascitic fluid containing monoclonal IgG antibody against the haemagglutinin of A/Texas/1/77 (H3N2), designated 185/1, was kindly provided by Dr.R.G.Webster (St. Jude Childrens' Research Hospital, Memphis, U.S.A.).

Murine hybridoma cells producing neutralizing IgA antibodies against the haemagglutinin of A/PR/8 (H1N1) were kindly provided by Dr.W.Gerhard, (Wistar Institute, Philadelphia, U.S.A.). These cells were grown up by E.Green (University of Warwick) to provide tissue culture fluid (tcf#37-66-1). The hybridoma cells were also seeded into Balb-c mice to provide ascitic fluid (WR131).

6.4.1.2. Polyclonal antibodies.

Polyclonal IgG hyperimmune sera to the H7 haemagglutinin was prepared in 2kg half-lop rabbits (Hylyne). This was done by inoculation via the ear vein of 10^6 HAU of EPL-inactivated, purified A/FPV/D (H7N7) boosted at day 22 with a similar inoculum. Blood was collected at days 5, 6 or 7 post-boost and serum prepared. The sera from 2 rabbits were produced, designated WR120 and WR121.

Polyclonal IgM sera to the H7 haemagglutinin was produced by tail vein inoculation of 7-12 week old C3H/He mice. Each mouse received 10^8 HAU of EPL-inactivated, purified A/FPV/D (H7N7) and the blood collected on day 6 post-inoculation from the heart. Serum from each mouse was prepared individually before being pooled into batches of between 100-150 samples. Each mouse yielded on average 0.25ml of serum.

6.4.2. Purification.

Purified preparations of the IgG monoclonal antibodies were kindly donated by H.P.Taylor (University of Warwick). They had been purified by affinity chromatography using a protein A-Sepharose CL-4B column (Sigma).

The IgM was purified from the day 6 post-inoculation mouse serum by gel filtration using a Sephacryl S-300 (Sigma) column. A polyethylene glycol 6000 (PEG) (BDH) precipitation step was carried out as a preliminary concentration step. 5ml of serum was dialysed overnight at 4°C against 2M phosphate buffer pH 6. The pH of the serum was raised to pH 8 with 2M Tris and PEG added to give 6% w/v whilst stirring on ice. After 60 min. the

sample was centrifuged at 2,000g for 10 min. at 4°C. The precipitate was resuspended in 2ml TN and applied to the S-300 column (length 60cm, diameter 1cm) and eluted with TN containing 0.08% Na₂S₂O₄ at 4°C. Fractions were collected at 15 min. intervals (volume approximately 1ml) over a period of 12 hours. The fractions were assayed for haemagglutination inhibition (HI) (section 6.4.3.) and protein concentration using the Biorad method (section 6.4.8.). The peaks containing the highest HI activity were pooled and concentrated using Centricon-10 (Amicon) tubes.

The IgA monoclonal antibody was purified using a technique similar to that used for the IgM except the PEG precipitation step was not used because this failed to precipitate a significant amount of the antibody. Again the peak containing the HI activity was pooled and concentrated.

Monomeric IgA was prepared from purified IgA using the method of Taylor & Dismock (1985) which involved differential reduction using 0.01% w/v beta-mercaptoethanol (BME) (Sigma) in 0.3M Tris/HCl pH 8 plus 0.12M iodoacetamide for 60 min. at 25°C. After dialysis overnight at 4°C against TN the antibody was loaded onto a S-300 column and eluted with TN containing 0.08% Na₂S₂O₄ and fractions collected. These were assayed for HI activity and run on a non-reducing SDS-PAGE gel to determine if reduction had occurred.

The IgG antibodies were stored at -70°C, whilst the IgM and IgA were stored at 4°C because freezing and thawing was found to be deleterious to polymeric antibodies.

6.4.3. Haemagglutination-inhibition assay.

The titres of anti-HA antibodies were determined by making doubling dilutions in 100ul PBS using round bottomed microtitre trays (Sterilin). To these dilutions 4 HAU/well of virus was added, mixed and incubated for 60 min. at room temperature. 25ul of 2% chick erythrocytes was added to each well, mixed and left for 30 min. The 50% end-point was estimated in a manner similar to the HA assay. The titre is expressed as haemagglutination-inhibition units (HIU) per ml.

6.4.4. Anti-neuraminidase assay.

In a capped boiling tube, 50ul of virus with a known neuraminidase activity was mixed with 50ul of serial dilution of the serum under study, plus appropriate controls. These were incubated for 60 min. at 25°C before the addition of 100ul of 12.5mg/ml fetuin (Gibco) in 0.2M phosphate buffer pH 6. The tubes were incubated for 90 min. at 37°C and then cooled to room temperature before adding 100ul 4.28% w/v sodium periodate (BDH) in 62% v/v orthophosphoric acid (BDH). The tubes were left for 20 min. at room temperature. 1ml filtered arsenite solution (10% w/v sodium arsenite (BDH); 7.1% w/v sodium sulphate (BDH); 0.28% v/v sulphuric acid (Fisons)) was added and vigorously mixed until all evolved iodine had redissolved. 2.5ml of 0.6% w/v thiobarbituric acid (BDH) in 7.1% w/v sodium sulphate was added and the tubes were boiled for 15 min., a red colour developed which faded as the solution was cooled to room temperature. 4ml of N-butanol (Fisons) was added, mixed and the tubes were centrifuged for 10 min. at 1500g to

separate the two phases. The butanol phase was removed and read on a spectrophotometer at 549nm. The anti-neuraminidase titre was that which caused a 50% reduction in neuraminidase activity of the standard virus.

6.4.5. Ouchterlony analysis.

2.5ml molten 1% w/v "Noble" agar (Difco) containing 0.08% NaCl was poured into 5cm diameter plastic petri dishes. The agar was allowed to set at room temperature before wells were cut into the agar using a sterile size 1 cork borer. Six wells surrounding a central well were cut using a template to ensure all the surrounding wells were equidistant from the centre. Each well held 15 μ l. Into the central well was placed the test serum and the surrounding wells were charged with a panel of specific goat anti-mouse immunoglobulin subtype (GAM) sera (Nordic). The agar plates were then placed into a humidified container and left at room temperature until precipitation bands appeared. In practice it took approximately 1 week before the bands were visible using dark-ground illumination. The agar plates were then fixed and stained using 0.025% w/v Coomassie Brilliant Blue R (Searle) in 50% v/v methanol:7% v/v acetic acid, followed by repeated destaining with solvent alone. The Ouchterlony plates were then washed extensively with distilled water before being dried in a 37°C incubator.

6.4.6. Gel electrophoresis.

6.4.6.1. General.

Proteins from purified immunoglobulins were analysed by :-

- (i) Polyacrylamide (PAA) gel electrophoresis.
- (ii) Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

The protein bands were located using 0.025% w/v Coomassie Brilliant Blue R (Searle) in 50% v/v methanol:7% v/v acetic acid, followed by repeated destaining with solvent alone. The gels were then washed extensively with water before being dried.

6.4.6.2. PAA gels.

Non-denaturing gels were prepared using a method supplied by S. van de Vries (University of Warwick). 4-30% gels were prepared using the solutions described below:

4% acrylamide solution:-

4% w/v acrylamide (BDH), 0.2% w/v bisacrylamide (BDH), 0.4M Tris/HCl pH 8.8 (BDH). Immediately prior to use 0.05% w/v ammonium persulphate (APS) (BDH) and 0.6ul/ml TEMED (Biorad) was added.

30% acrylamide solution:-

30% w/v acrylamide, 0.14% w/v bisacrylamide, 0.4M Tris/HCl pH 8.8, 11% v/v glycerol (BDH). Immediately prior to use 0.025% w/v APS and 0.3ul/ml TEMED was added.

Sample buffer:-

The sample buffer (50% sucrose plus bromophenol blue (Searle)) was added to the samples in a ratio of 1 to 4; the sample was then vortexed before application to the gel.

Running buffer:-

28.8g/l glycine (BDH) containing 6g/l Tris.

6.4.6.3. SDS-PAGE gels.

Reducing and non-reducing, denaturing gels were prepared as either 10-30% gradients or 20% single strength gels (combining the 10% and 30% acrylamide solutions before pouring the gel) (Laemmli, 1970). The ingredients used are listed below;

10% acrylamide solution:-

10% w/v acrylamide (BDH), 0.05% w/v bisacrylamide (BDH), 0.1% w/v SDS (BDH), 0.375M Tris/HCl pH 8.6 (BDH). Immediately prior to use 0.015% w/v APS (BDH) and 1.3ul/ml TEMED (Biorad) was added.

30% acrylamide solution:-

30% w/v acrylamide, 0.136% w/v bisacrylamide, 0.1% w/v SDS, 7.8% w/v glycerol (BDH), 0.375M Tris/HCl pH 8.6. Immediately prior to use 0.007% w/v APS and 1.3ul/ml TEMED was added.

Stacker gel:-

4.5% w/v acrylamide, 0.12% w/v bisacrylamide, 0.1% w/v SDS, 0.125M Tris/HCl pH 6.8. Immediately prior to use 0.03% w/v APS and 3.0ul/ml TEMED was added.

Sample buffers:-

Either (i) Reducing buffer: 10mM Tris/HCl pH 7.4 containing 2% w/v SDS, 5% w/v BME (Sigma), 5% w/v glycerol and 0.01% w/v bromophenol blue (Searle), or (ii) Non-reducing buffer: as previous except the BME was omitted. The sample buffers were added to the samples in a ratio of 1 to 4, then the samples were vortexed and boiled for 5 min. before application to the gels.

Running buffer:-

13.95g/l glycine (BDH) containing 3g/l Tris and 1g/l SDS.

6.4.7. Iodination of antibodies.

15ul 100mM H_2SO_4 and 15ul 1.5mM chloramine T (BDH) were mixed with 15ul 1mM KI and 250uCi sodium [^{125}I] iodide (Amersham), and incubated for 30 seconds at room temperature. After this time 15ul 1M phosphate (KH_2PO_4) buffer pH 7.9 was added to neutralize the mixture. 150ul of antibody preparation was added and left for 2 min. before being applied to a G-25 sephadex (Pharmacia) column. The antibody was eluted with PBS and fractions collected. The fractions were assayed for radioactivity and HI activity, the peak in which these activities coincided was pooled and the protein concentration, HI and radioactivity determined. BSA was then added to the fraction to a concentration of 1% w/v to stabilise the antibody, and the fraction stored at 4°C.

6.4.8. Protein assay.

The concentrations of proteins were determined using a commercial kit manufactured by Biorad. Absorbances were taken at 595nm and the protein concentrations read off a standard curve prepared using a range of BSA concentrations up to 2mg/ml.

6.5. Neutralization of influenza virus by antibodies.

6.5.1. Plaque reduction.

Virus (10^8 pfu/ml) was incubated with an equal volume of purified immunoglobulin (IgG, IgM or IgA) for 1 hour at 25°C for 60 min. The residual infectivity was determined by plaque assay on CEF or MDCK cell monolayers (section 6.3.1.).

A second form of neutralization assay was performed using virus diluted to 10^8 HAU/ml before addition of antibody. This was the inoculum used in virus attachment experiments. The neutralized virus needed to be diluted in PBS before being assayed for residual infectivity.

6.5.2. Assay of remaining haemagglutination activity.

Considering the number of virus attachment assays performed it was impractical to measure the degree of neutralization by plaque reduction in every experiment; this was usually done in the first of a series of

experiments. In all experiments, neutralization was determined by measuring the residual haemagglutination activity (HA) of the fractions as previously described (section 6.3.1.).

6.5.3. Study of the aggregation of influenza virus by electron microscopy.

The procedure used was described by Taylor *et al.* (1987). Virus preparations were mixed with an equal volume of 3% sodium silicotungstate (Agar Aids, Stanstead, U.K.), pH 6.5. Sul samples were placed on Formvar coated copper grids. After 5 min. the liquid was removed using filter paper and the grids were examined using a JEOL JEM-1005 transmission electron microscope. Areas of the grid were scanned sequentially, noting each single virus particle and the number of virion in each cluster. The total number of virions counted was divided by the number of single virions plus the number of clusters. The resulting figure was termed the average clump size.

6.5.4. Attachment of influenza virus to cells.

The procedure followed is outlined diagrammatically (Figure 6.2.) and general details are given below. More specific experimental details are supplied where relevant in the text of the thesis.

³²P labelled virus and dilutions of the antibody under study, were incubated for 60 min at 25°C before being inoculated onto PBS washed preparations of cells. Cells were washed once with PBS to remove dead cells and debris. Aliquots of neutralized virus were also tested for the degree of neutralization by plaque reduction (section 6.5.1.), remaining haemagglutination activity (section 6.5.2.) and aggregation (section

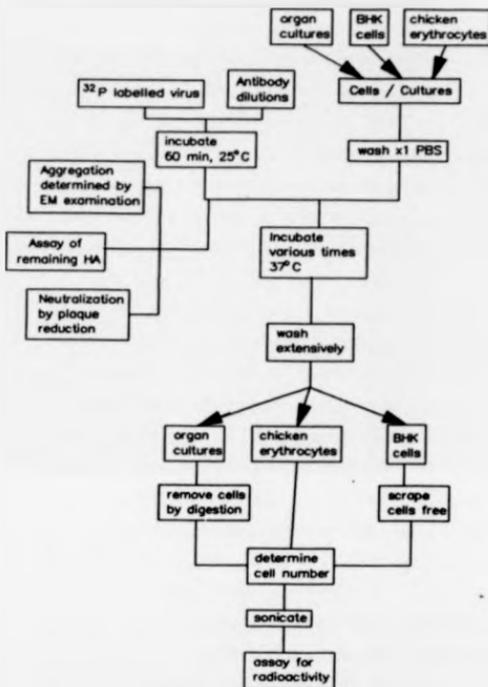


Fig.6.2. Method used to determine the amount of radiolabelled virus attached to various cell types. Details of the method are given in the text (section 6.5.4.).

6.5.3.). The inoculated cells were incubated at 37°C for various times before being extensively washed with chilled PBS. The number of washes required to free the excess, unattached virus from the cells varied according to the cell type under study; chicken erythrocytes required 3 washes, BHK cells 4 washes and organ cultures 5 washes.

When organ cultures were used, epithelial cells were released by protease digestion (section 6.2.4.) and the number of isolated cells was determined using a Neubauer counting chamber. The epithelial cell preparations were then vortexed, sonicated and the levels of cell-associated radioactivity assayed.

BHK cells were removed from the plastic Petri dishes by scraping into 1ml PBS using rubber "policesmen" and the cells vortexed and sonicated. The cells obtained by scraping were too clumped to enable efficient cell counts to be performed, therefore cells were removed from a parallel set of plates with trypsin and counted as described above; this enabled the average number of cells per plate to be determined. The protein content of the sonicated cell preparations were then assayed (section 6.6.1.), and an average value determined. Using these figures, the average protein concentration per cell could be calculated. Assuming this figure to be constant within an experiment then the cell number for each individual sonicated cell preparation could be calculated from the known protein concentration value. This procedure was important to check the efficiency of cell scraping because this could be variable. Finally the sonicated preparations were assayed for cell-associated radioactivity.

Adult chicken erythrocytes (1×10^9 cells) were inoculated. No appreciable haemolysis was seen and it was assumed that 1×10^9 cells remained at the end of procedure. The cells were sonicated as before and the level of radioactivity determined.

6.5.5. Internalization of influenza virus by cells.

The method is essentially the same as that described for the study of virus attachment (section 6.5.4.) with the following additions. After allowing the infectious or neutralized virus to attach, the cells were washed and treated with either PBS or 0.5U of neuraminidase, *Clostridium perfringens* (NAase) (Sigma) in PBS at 37°C for 60 min. The cells were washed 3 times with PBS to remove any virus released, before further treatment.

In other experiments, the effects of NAase pre-treatment of cells on virus attachment was studied. The method used was similar to that previously described (section 6.5.4.) except that before inoculation with the virus preparations, the cells were treated with either PBS or 0.5U NAase for 60 min. at 37°C. The cells were washed twice with PBS to remove the NAase before addition of the inoculum.

6.5.6. Detection of influenza antigens using fluorescent antibody.

Confluent BHK cells on coverslips were washed with PBS before 20ul of an appropriate dilution of A/WSN was applied. The coverslips were gently rocked to ensure all the cells were infected and incubated for 60 min at room temperature. Then the coverslips were extensively washed with PBS to remove unattached virus. The cells were incubated for various periods of

time at 37°C in a Petri dish containing the 4ml MEM medium. The coverslips were again washed and allowed to dry before fixing with acetone at -20°C for 15 min. The cells were stored at -20°C until required.

Organ cultures were infected with the A/WSN before the epithelial cells were removed by protease digestion. These cells, resuspended in 199 medium containing 20% v/v NCS, were then dried onto coverslips. The cells were then fixed using acetone as described above.

Infected and non-infected cells fixed on coverslips were air dried before adding 20ul of 1/10 WR16 anti-WSN HA rabbit antiserum (kindly donated by L. McLain, University of Warwick). The coverslips were gently rocked to ensure an even exposure to the antibody and incubated at 37°C for 25 min. in a humidified container. The cells were washed extensively with PBS and allowed to dry. 20ul of 1/40 fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit (GAR) serum (Nordic) was then added to the coverslips and incubated for 25 min. at 37°C in the humidified container. The cells were again washed extensively in PBS, then washed with distilled water before being air dried. The coverslips were mounted onto microscope slides with 80% glycerol v/v in PBS and examined using a Nikon UFX II microscope with illumination provided by a Nikon Hg 100W lamp.

It proved difficult to fix isolated organ culture cells to the coverslips and a large number of the cells detached during the staining procedure. Increasing the NCS concentration to 40% or substituting 5% w/v BSA for the NCS in medium in which the cells were resuspended before fixation proved unsuccessful.

RESULTS 1.
Development of the tracheal
organ culture technique.

2.1. Introduction.

This chapter describes: (1) The preparation and maintenance of tracheal organ cultures from the mouse. Earlier workers found that organ cultures derived from the mouse trachea were difficult to maintain (e.g. Cherry & Taylor-Robinson, 1970) and for this reason they have been largely ignored as a model system. Despite these earlier difficulties, the mouse was chosen for my work since a primary aim was to create a homogeneous experimental system where the target cells, antibody and virus were all derived from the same host species. (2) An investigation as to whether or not mouse tracheal organ cultures could support the multiplication of the influenza strain, A/FPV/R. This avian strain of influenza virus was chosen because there was a set of well characterised neutralizing monoclonal IgG antibodies to this strain in the laboratory (i.e. HC2, HC10, HC38 and HC61). Also, previous work on the mechanisms of neutralization of influenza virus had been done using this strain (Ponsee *et al.*, 1982; Taylor & Dimmock, 1985a & 1985b; Taylor, 1986; Taylor *et al.*, 1987). (3) An attempt to specifically remove the epithelial cells lining the lumen of the trachea from the organ cultures. This would allow later investigation at the cellular level of the mechanisms of neutralization of influenza virus. (4) An investigation as to whether or not cells infected and isolated from tracheal organ cultures are those which are infected by intranasal inoculation in vivo.

7.2. Results.

7.2.1. Preparation of tracheal organ cultures.

"Ring" organ cultures were prepared by transverse sectioning of tracheas removed from 5-10 week old C3H/He-Mg mice (section 6.2.2.). Each trachea yielded on average 6-7 cultures which compared well with numbers produced by other workers using mice (Cherry & Taylor-Robinson, 1970). Tracheas from larger animals yield a greater number of cultures e.g. hamster, 8-12 (Heath *et al.*, 1983; Hu *et al.*, 1975), human foetus, 8-15 (Hara *et al.*, 1974; Mostow & Tyrrell, 1973), chick embryo, 17 (Cherry & Taylor-Robinson, 1970) and ferret, 40-50 (Hara *et al.*, 1974).

No attempt was made to separate the organ cultures prepared from the upper and lower trachea. Others have found no difference in the ability of different parts of the trachea to sustain an infection of influenza virus (Hara *et al.*, 1974; Kingman *et al.*, 1977a).

At approximately 24 hour intervals the medium was changed and the 'ciliary score' noted for each culture (section 6.2.3.). The 24 hours post-dissection value is assumed to represent maximal (100%) activity (Blaskovic *et al.*, 1972c; Mostow & Tyrrell, 1973; Heath *et al.*, 1983) and all subsequent scores are related to this initial value. Figure 7.1. shows that cultures could be maintained for approximately 150 hours post-dissection before an appreciable reduction in activity was observed. No out-growth of dedifferentiated cells was seen similar to observations of others (Hoorn, 1966), although Mostow & Tyrrell (1973) observed fibroblast outgrowth from human embryo organ cultures at 12-14 days post-dissection.

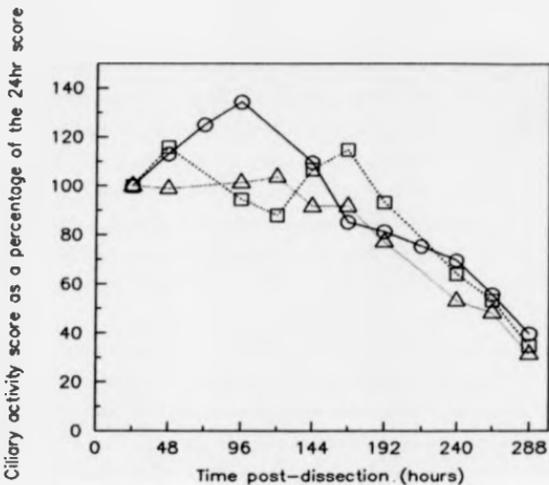


Fig.7.1. Ciliary activity of non-infected mouse tracheal organ cultures.

Results from 3 experiments;

- Average score from 13 organ cultures
- Average score from 18 organ cultures
- △...△ Average score from 18 organ cultures

7.2.2. Attempt to propagate A/FPV/R on tracheal organ cultures.

Organ cultures were prepared and infected with A/FPV/R (H7N1) for 60 min. at 37°C. The organ cultures were extensively washed with PBS (5 washes, each 2.5ml PBS) and then placed individually into 96 well flat bottomed, tissue culture trays. These were incubated at 37°C and the medium harvested daily and replaced. The harvested medium was centrifuged to remove cell debris before being frozen and stored at -70°C. The cultures were also observed daily and the ciliary activity score measured (section 6.2.3.). At the end of the experiment the infectivity of each sample of harvested medium was determined (section 6.3.1.).

A range of inocula, from 10^8 to 10^6 pfu/ml, was used (results not shown) but A/FPV/R failed to multiply on the organ cultures. Some residual virus remained associated with the organ cultures and most of this eluted during the first 24 hours of incubation. A/FPV/R caused no appreciable reduction in the ciliary activity of the cultures when compared to the non-infected controls. This would be expected if no infection had been established. To overcome the fact that A/FPV/R failed to multiply I attempted (a) to propagate a panel of different influenza virus strains on mouse tracheal organ cultures and (b) to adapt A/FPV/R to mouse cells by repeated serial passaging in a mouse tissue culture cell line (i.e. C3H10T1/2 fibroblasts).

7.2.3. Propagation of a panel of influenza strains on tracheal organ cultures.

A panel of influenza virus strains available within the laboratory was tested for their ability to multiply on mouse tracheal organ cultures and the effects on the ciliary activity of the cultures were also investigated (Figures 7.2. to 7.8.). Results are summarized in Table 7.1.. The infectivity (pfu/ml) at 96 hours p.i. has been used as a criterion of infection (Kingsman et al., 1977b). The pathogenic effect of the virus is determined by taking the time at which 50% ciliary activity inhibition (CAI₅₀) occurred (Diaz-Rodriguez & Boudreau, 1982).

The human influenza strains (A/PR/8; A/WSN; A/Jap; A/Cir) all multiplied on mouse tracheal organ cultures. Virus titres of between $10^{6.4}$ - $10^{6.8}$ pfu/ml were obtained at 96 hours p.i. (Table 7.1.), although there were higher titres at approximately 48 hours p.i.. It would appear that repeated passages of these viruses in fertile eggs of hens had not prevented them from infecting respiratory tissue from a mammalian source. The pathogenicity differed between strains: A/Cir caused a rapid loss of ciliary activity, A/WSN and A/Jap also caused a loss of activity but at a slower rate and A/PR/8 caused no reduction in the ciliary activity.

The influenza strains isolated from chickens (A/Ch/Scot; A/FPV/D) failed to multiply on mouse tracheal organ cultures. The influenza strain isolated from turkeys (A/Tky/Ont) did multiply but growth was delayed and there was a no appreciable multiplication before 72 hours p.i.. Such a delay in

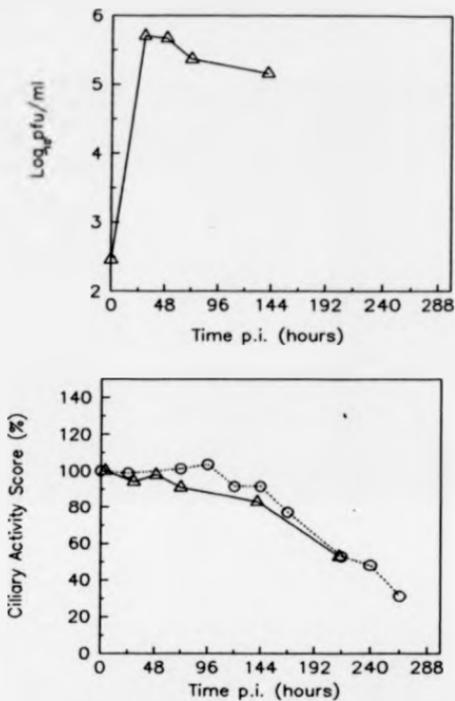


Fig. 7.2. Multiplication and effect of A/PR/8 on mouse tracheal organ cultures. Cultures were inoculated with $10^{7.3}$ pfu/ml (Δ - Δ); 11 o.c. were used in this experiment and the supernatants pooled. Non-infected controls (O-O).

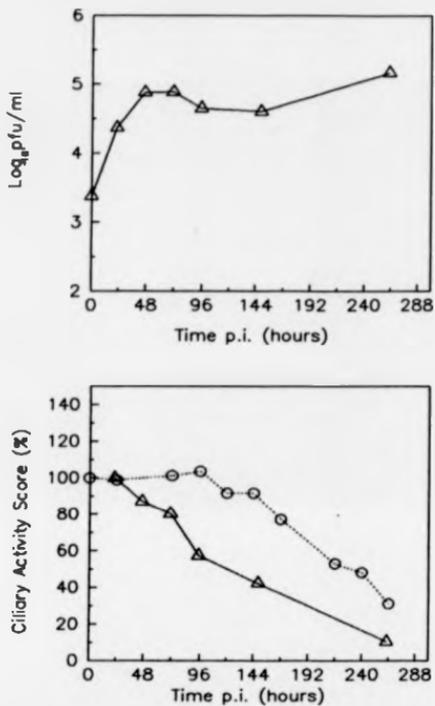


Fig.7.3. Multiplication and effect of A/WSN on mouse tracheal organ cultures.

Cultures were inoculated with $10^{7.7}$ pfu/ml (Δ - Δ). 35 o.c. were used in this experiment and the supernatants pooled. Non-infected controls (\circ - \circ).

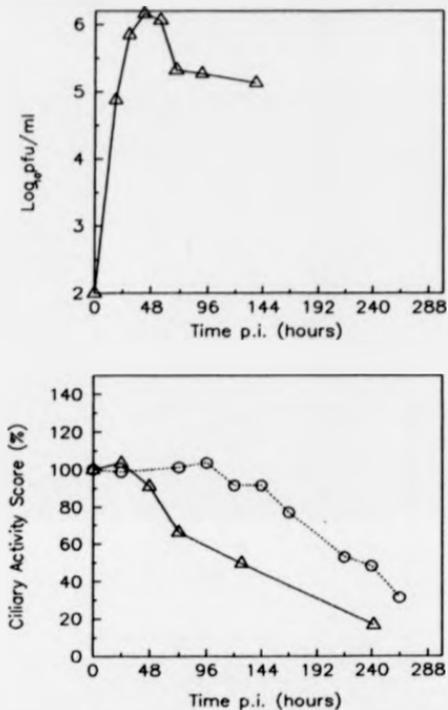


Fig.7.4. Multiplication and effect of A/Jap on mouse tracheal organ cultures. Cultures were inoculated with $10^{5.3}$ pfu/ml (Δ-Δ). 18 o.c. were used in this experiment and the supernatants pooled. Non-infected controls (O-O).

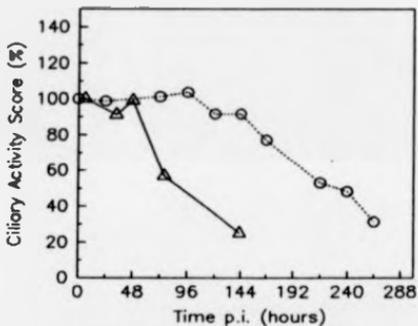
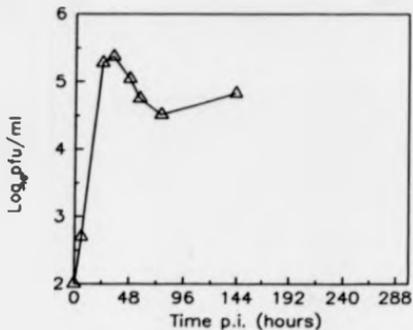


Fig.7.5. Multiplication and effect of A/Cir on mouse tracheal organ cultures.

Cultures were inoculated with 10^5 pfu/ml (Δ - Δ). 20 o.c. were used in this experiment and the supernatants pooled. Non-infected controls (O-O).

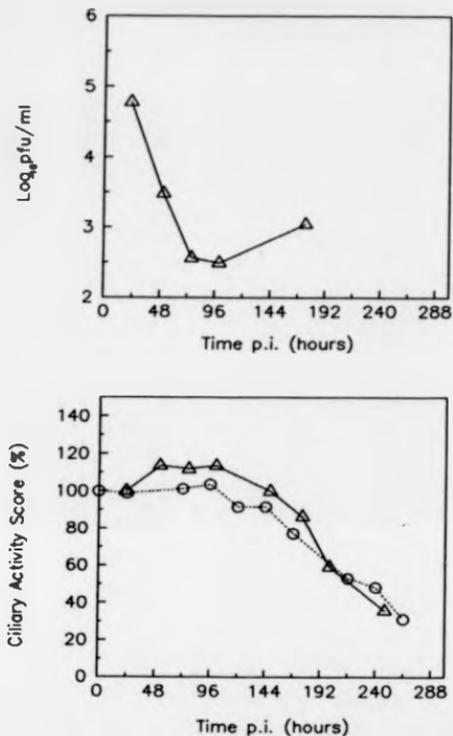


Fig. 7.6. Multiplication and effect of A/FPV/D on mouse tracheal organ cultures.

Cultures were inoculated with $10^{8.3}$ pfu/ml (Δ — Δ). 14 o.c. were used in this experiment and the supernatants pooled. Non-infected controls (\circ --- \circ).

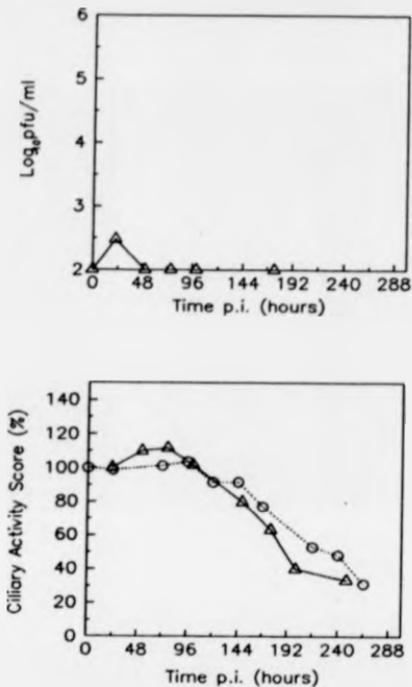


Fig.7.7. Multiplication and effect of A/Ch/Scot on mouse tracheal organ cultures. Cultures were inoculated with $10^{5.1}$ pfu/ml (Δ - Δ). 14 o.c. were used in this experiment and the supernatants pooled. Non-infected controls (O---O).

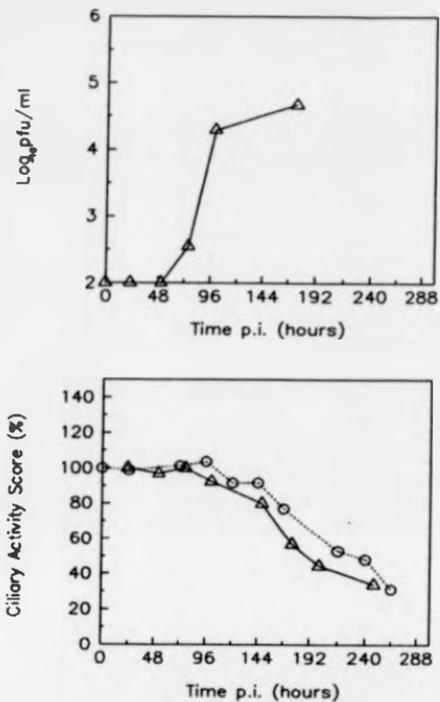


Fig.7.8. Multiplication and effect of A/Tky/Ont on mouse tracheal organ cultures.

Cultures were inoculated with $10^{7.2}$ pfu/ml (Δ — Δ). 14 o.c. were used in this experiment and the supernatants pooled. Non-infected controls (\circ — \circ).

Table 7.1.

Summary of the multiplication characteristics of a panel of influenza viruses on mouse tracheal organ cultures.

<u>Influenza strain</u>	<u>Infectivity at 96 hours p.i. (Log₁₀ pfu/ml)</u>	<u>Time at which a 50% reduction in ciliary activity score (hours p.i.)</u>
Control (non-infected)	N/A	240
A/FPV/R (H7N1)	< 2.0	170
A/PR/8 (H1N1)	5.3	>200
A/WSN (H1N1)	4.7	120
A/Jap (H2N2)	5.2	130
A/Cir (H3N2)	4.6	90
A/Ch/Scot (H5N1)	< 2.0	190
A/Tky/Ont (H8N4)	3.7	190
A/FPV/D (H7N7)	2.5	220

growth was not seen for any of the influenza strains isolated from man. No reduction in ciliary activity was seen in the organ cultures infected with these avian influenza strains.

7.2.4. Adaptation of A/FPV/R.

An attempt to adapt the avian influenza strain A/FPV/R to mouse cells was made by serially passaging in a mouse fibroblast cell line, C3H10T1/2.

The C3H10T1/2 monolayers (approximately 10^8 cells) were inoculated with a known amount of infectious virus in 20ml medium and incubated for 60 min. at 37°C. The cells were then washed twice with 20ml PBS and 20ml fresh medium added. The monolayers were incubated at 37°C for 48 hours or until a cytopathic effect (CPE) was observed. At the end of this period the medium was harvested and the infectivity measured.

Initially A/FPV/R multiplied poorly, or failed to multiply, on C3H10T1/2 cells (Figure 7.9.). Less virus was recovered than was applied and some of the recovered virus may be due, in part, to elution of residual inoculum from the monolayer. The multiplication was so poor, on occasions, that the harvested virus had to be boosted by growing in fertile hens eggs (section 6.3.1.) before continuing to passage in C3H10T1/2 cells. The recovered virus was diluted and used to infect more C3H10T1/2 monolayers in a similar manner. The multiplicity of infection (MOI) was always adjusted by dilution to 0.1 or less in order to reduce the incidence of defective interfering (DI) influenza particles (Nayak *et al.*, 1985; Janda *et al.*, 1979).

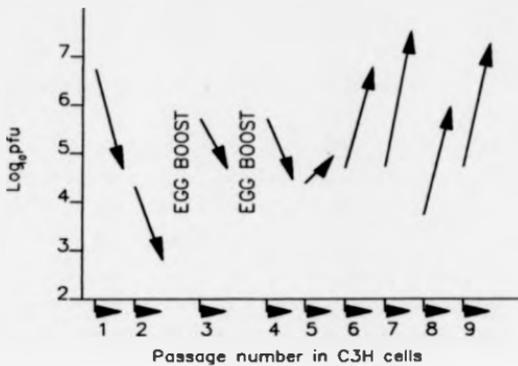


Fig.7.9. Adaptation of A/FPV/R by serial passaging in C3H10T1/2 cells

pfu applied —————> pfu recovered

A/FPV/R began to show signs of adaptation on the 5th passage when more virus was recovered than was used to inoculate the cells. Good growth was seen in the 6th passage and this pattern of growth was repeated in subsequent passages. The virus produced by the 7th passage was plaque purified and grown up in fertile hens eggs (section 6.3.1.). This was used as a working stock preparation and designated adpFPV/R.

7.2.5. Propagation of adpFPV/R on tracheal organ cultures.

Mouse tracheal organ cultures were prepared and infected with the mouse adapted A/FPV/R influenza virus (adpFPV/R). Virus multiplication was determined and the effect on the ciliary activity observed (Figure 7.10.).

The adpFPV/R strain multiplied to high titres, with 10^6 pfu/ml at 48 hours p.i.. The 96 hours p.i. titre of $10^{6.5}$ pfu/ml was the highest found for any influenza strain (Table 7.1.). The adpFPV/R was also highly pathogenic to the organ cultures, the CAI₅₀ occurred at 65 hours p.i.. This was more rapid than that found for the human A/Clr strain, and the adpFPV/R strain could be designated as being virulent on this basis. The virus was treated with caution because of its potential virulence for mammalian hosts.

7.2.6. Antigenic changes caused by adaptation of A/FPV/R to mouse cells.

It was of interest to see if the process of adaptation of A/FPV/R had altered the antigenic nature of the H7 haemagglutinin. This was done by examining the haemagglutination-inhibition (HI) and neutralization caused by a panel of monoclonal IgG antibodies available to A/FPV/R (i.e. HC2; HC10; HC58; HC61).

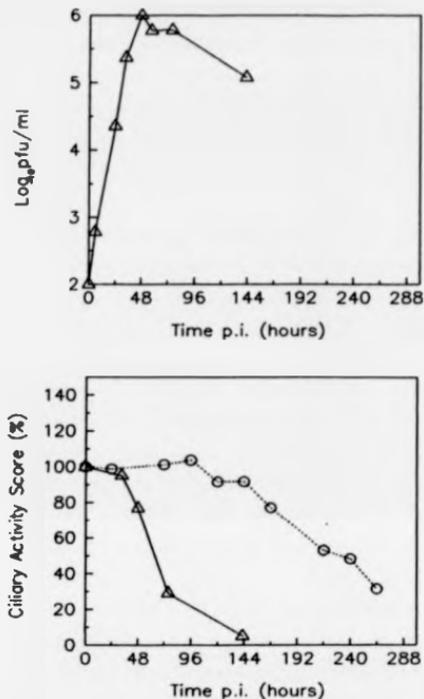


Fig. 7.10. Multiplication and effect of adpFPV/R on mouse tracheal organ cultures.

Cultures were inoculated with 10^5 pfu/ml (Δ - Δ). 20 o.c. were used in this experiment and the supernatants pooled. Non-infected controls (\circ - \circ).

The HI profile of the adpFPV/R (Table 7.2a.) had changed with an increased titre against HC2 and HC58; the HI titres were largely unchanged against HC10 and HC61. The adpFPV/R strain was also more sensitive than the unadapted A/FPV/R to neutralization irrespective of the monoclonal antibody used (Table 7.2b.).

These results suggest that antigenic alteration(s) of the A/FPV/R had been forced by its adaptation to the mouse host. It was of interest to determine if the change resided in the H7 haemagglutinin, and if so, where it was located. A sample of adpFPV/R was sent to Dr. J.W. McCauley (Pirbright, U.K.) who sequenced the haemagglutinin gene of adpFPV/R and compared it to the original A/FPV/R. The sole change was a single residue at position 37 of HA2 from Lysine to Glutamine (personal communication). No change occurred at or close to the antigenic sites or the virus attachment site previously mapped on the HA1 of other haemagglutinins (sections 1.4. and 1.5.).

7.2.7. Removal of ciliated epithelial cells from tracheal organ cultures.

Attempts to remove the epithelial cells from organ cultures have been made using a loose-fitting glass homogeniser (Almeida & Tyrrell, 1967), trypsin (Cavanagh *et al.*, 1979) and EDTA (Kingman *et al.*, 1977b). The latter two groups employed a degree of mechanical force as well. I removed the epithelial cells from the organ cultures using an enzymatic treatment method supplied by Dr. C. Sweet (University of Birmingham), which he had developed to remove epithelial cells from organ cultures of ferret nasal turbinates (methods 2.4). Dr. C. Sweet reported that the technique yielded approximately $1-1.5 \times 10^8$ cells per trachea Between 10-20% of the

Table 7.2. Antigenic changes of adpFPV/R following adaptation to mouse cells.

a) determined by HI activity*

<u>IgG mcAb</u>	<u>A/FPV/R</u>	<u>adpFPV/R</u>	<u>increase</u>
HC2	10,600	35,500	X 3.4
HC58	12,600	25,100	X 2.0
HC10	10,750	14,950	X 1.4
HC61	4,950	4,900	X 1.0

* values expressed as HIU/ml

b) determined by plaque reduction**

<u>IgG mcAb</u>	<u>A/FPV/R</u>	<u>adpFPV/R</u>	<u>increase</u>
HC2	6,900	12,150	X 1.76
HC58	3,150	4,250	X 1.35
HC10	12,900	21,900	X 1.70
HC61	17,000	26,300	X 1.55

** reciprocal of the dilution required to cause 50% neutralization of 1,000 pfu/ml virus when plaqued on CEF monolayers

isolated cells were obviously ciliated and 60% of these possessed beating cilia. The remaining cells were morphologically similar except that they lacked cilia (personal communication).

Using mouse tracheal organ cultures, I found this technique yielded 1.6×10^6 (± 0.4) cells per trachea, or 2.4×10^6 (± 0.6) cells per organ culture ring. 30-40% possessed actively beating cilia, a value higher than that reported by Dr. C. Sweet.

Cavanagh *et al.* (1979) reported a yield of 7.3×10^6 cells from ferret nasal turbinate organ cultures, while Kingsman *et al.* (1977b) found a yield of 1.7×10^6 cells from similar tissue. My results therefore compare favourably with previous findings.

Examples of the cells obtained by this technique are shown in Figure 7.11. The majority of the ciliated cells (Figure 7.11a/b) had actively beating cilia, making the cells highly motile and difficult to study under the microscope. The motility could be reduced by chilling the cells to 4°C . Ciliated cells were often seen in pairs or small groups as their cilia became entwined. Cells with very similar morphology and behaviour were found by Hoorn & Tyrrell (1965). The non-ciliated cells (Figure 7.11c/d) appear to be morphologically similar by light microscopy and they are often seen in small clusters, frequently attached to cells bearing cilia. They may have physically lost their cilia or are possibly immature epithelial cells. The epithelial cell preparations had very little contamination with erythrocytes or cell debris, but some contamination can be seen in Figure 7.11d. The quality of a preparation was highly dependant on the degree of care taken when dissecting the trachea from the mouse.

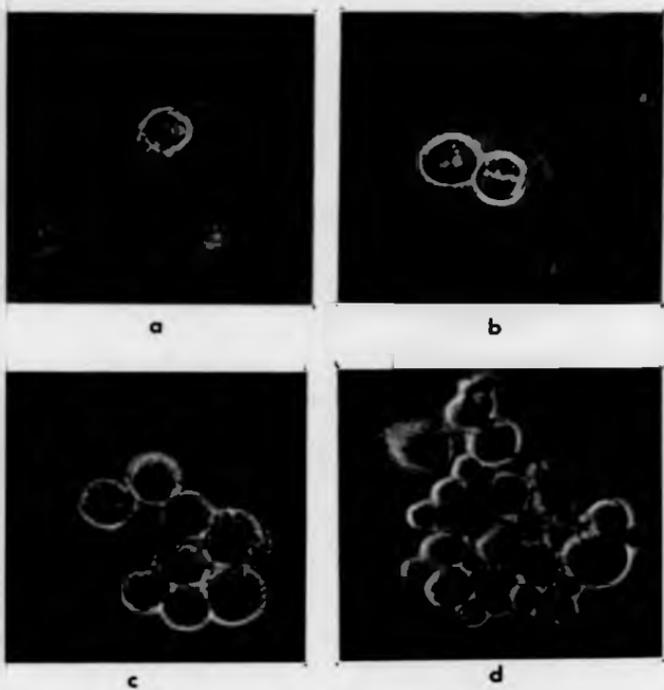


Fig.7.11. Cells isolated from mouse tracheal organ cultures visualized by phase contrast ($\times 720$).

The ciliary activity of the isolated epithelial cells could be maintained for short periods of time (up to 6 hours) at room temperature or 4°C. Attempts to maintain these cells overnight, either at 4°C and 37°C, proved unsuccessful. The cell number dropped, the level of cell debris became marked and the cells that survived lacked actively beating cilia.

Epithelial cells were examined using the transmission electron microscope by Dr. S.J. Armstrong (University of Warwick). She found approximately 50% of the cells displayed evidence of cilia; either actual cilia or cilia base plates (Figure 7.12.) (personal communication). The cilia display the 2 singlet: 9 doublet microtubule structure that has also been observed by others using tracheal organ cultures (Blaskovic *et al.*, 1972a). Dr. S.J. Armstrong also confirmed that the non-ciliated cells were morphologically similar to those displaying cilia and presumably were also originally from the same epithelial layer or were the same cells. Most of the cells were vacuolated suggesting a degree of trauma had been introduced by the isolation technique. Vacuolation has been found by others in epithelial cells removed from organ cultures by asking an 'impression' on a glass slide (Hoorn & Tyrrell, 1965; Blaskovic *et al.*, 1972a).

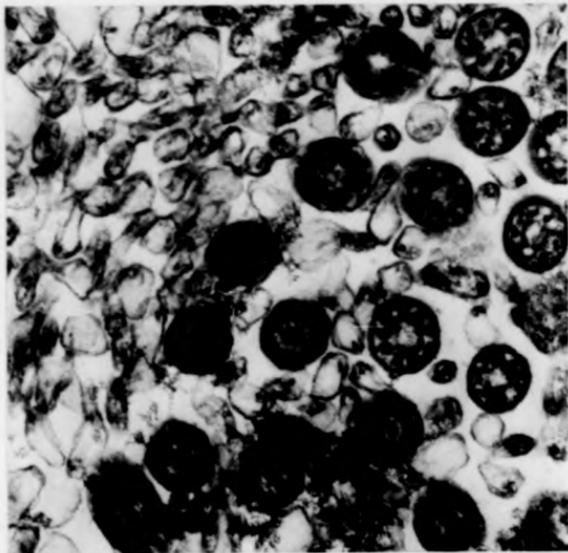


Fig.7.12. Cross-section of cells isolated from mouse tracheal organ cultures visualized using a transmission electron microscope (x100,000).
Courtesy of Dr.S.J.Armstrong.

7.2.8. In vivo infection of the trachea with A/WSN.

It was important to investigate whether or not the ciliated epithelium of the trachea was infected in vivo. Therefore I infected mice before subsequently preparing the tracheal organ cultures. The influenza strain A/WSN was used in preference to adpFPV/R because our laboratory A/WSN strain is pathogenic for mice (Dimmock et al., 1986) and tracheal organ cultures (section 7.2.3.), and because A/FPV/R is restricted to the controlled laboratory area.

When ether-anaesthetised mice are inoculated, A/WSN may primarily infect the lower respiratory tract rather than the trachea. I therefore dissected A/WSN-infected mice at various times post-infection so that if the infection proceeds in an upward direction, the samples prepared immediately post-infection would show less infection, than those prepared later (Figure 7.13.).

Organ cultures prepared from mice at 2 hours p.i. (Figure 7.13a.) initially showed low levels of multiplication at 96 hours p.i. with a maximum titre of $10^{4.6}$ pfu/ml. No reduction in ciliary activity was seen.

Organ cultures prepared from mice dissected at 24 hours p.i. (Figure 7.13b.) shed virus at 48 hours p.i. and gave a maximum titre of nearly 10^6 pfu/ml. There appeared to be some impairment of ciliary activity initially but by 120 hours p.i. the infected and non-infected organ cultures were indistinguishable.

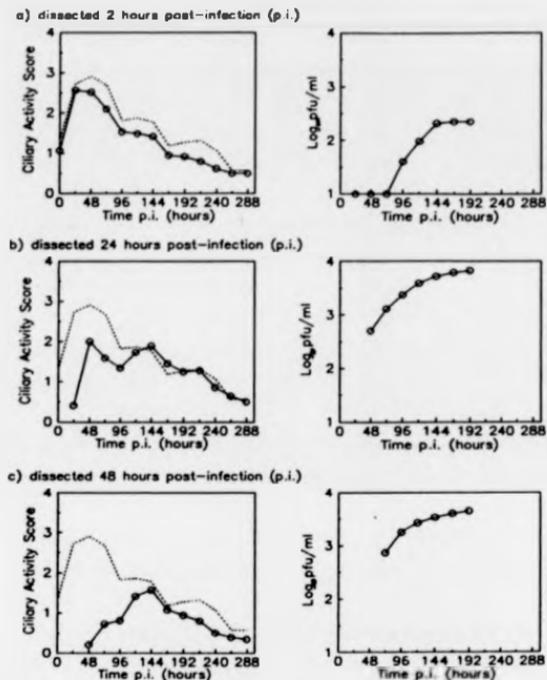


Fig. 7.13. Growth and effect of A/WSN on mouse tracheal organ cultures taken from mice infected in vivo.

Organ cultures prepared from mice dissected at 48 hours p.i. (Figure 7.13c.) showed virus multiplication at the first time interval (72 hours p.i.), with maximum virus titres of $10^{3.0}$ pfu/ml. At first the organ cultures had very little ciliary activity and the respiratory surface of the organ cultures was covered with cell debris and rounded cells which lacked cilia. There were bare patches on the organ cultures where all the epithelial cells had been shed. The ciliary activity score gradually increased as overlying debris and cells were removed by harvesting and replacing of the medium. By 144 hours p.i. the score obtained was the same as that of the non-infected control organ cultures.

7.2.9. Demonstration of A/WSN antigens in epithelial cells of organ cultures infected in vivo.

Mice were infected with A/WSN by intranasal inoculation as previously described. At 24 hours p.i. the tracheas were removed and organ cultures prepared; from these the epithelial cells were immediately removed. The cells produced were fixed to coverslips and stained for A/WSN antigens using a fluorescent antibody technique (section 6.5.6.).

BHK cells were also infected with A/WSN and used as controls. The results (Figure 7.14.) demonstrate that good specific staining was achieved with little non-specific background.

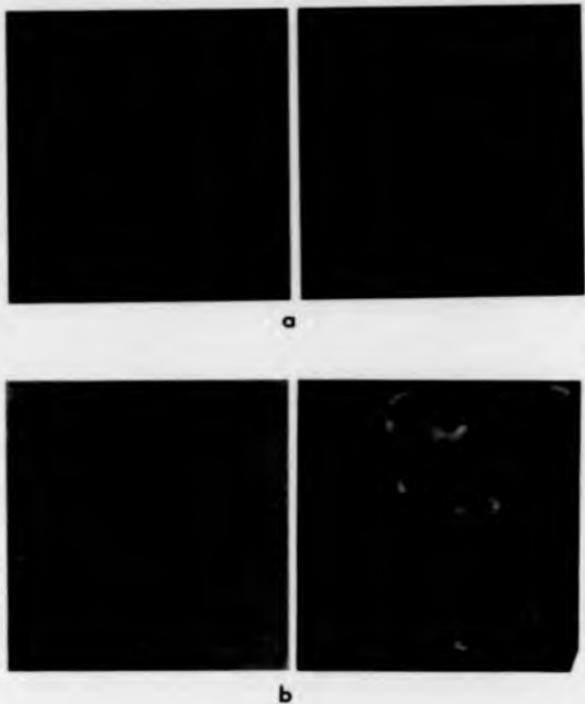


Fig.7.14. Immunofluorescent staining of A/WSN antigens in (a) non-infected, and (b) A/WSN infected BHK cells. Method described in section 6.5.6.. (x700)

The majority of epithelial cells show positive staining for A/WSN antigens (Figure 7.15.) but the intensity of staining was weaker than that found using BHK cells (Figure 7.14.). It is also of interest to note that all evidence of cilia was lost during this procedure, indicating their fragile nature.

The results confirm that the majority of the cells removed from organ cultures taken from mice infected in vivo are infected and are presumably epithelial cells.

7.3. Discussion.

The data show that the maximum time organ cultures from mouse trachea can be maintained in good condition is 150 hours post-dissection, as judged by ciliary activity. This is a shorter period than found by others and may be due to the small size of the mouse trachea which makes it more susceptible to damage incurred during sectioning which leads to a reduced lifespan (Herbst-Laier, 1970). Westerberg et al. (1972), using mouse tracheal organ cultures, found no decline in ciliary activity over a 15 day period. Histologically the epithelium appeared healthy except, ^{and} the nuclei seemed to move towards the apical surface instead of remaining at the base of the cells. Westerberg sectioned the trachea longitudinally (section 6.2.2.) and this may account for the differences in the maintenance results.

Organ cultures from larger animals have been successfully maintained for longer periods, again indicating that the size of the trachea may influence the longevity of the organ cultures produced. Ferrets, the most common source of tracheal organ cultures, yield cultures which can be maintained

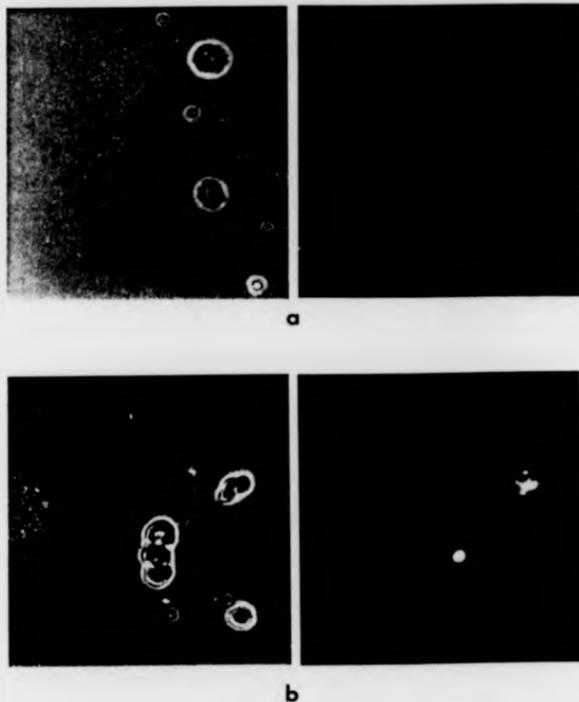


Fig. 7.15. Immunofluorescent staining of A/WSN antigens in cells isolated from tracheas of (a) non-infected mice, or (b) mice infected in vivo with A/WSN 24 hours prior to dissection. (x700).

for 10 days post dissection (Hara et al., 1974). A similar period of maintenance was found for hamster tracheal organ cultures (Heath et al., 1983; 1986). Chick embryo tracheal organ cultures can be maintained for approximately 14 days post dissection (Blaskovic et al., 1972b), but some spontaneous degeneration of the ciliated epithelium was occasionally observed, resulting possibly from secondary infection (Blaskovic et al., 1972a). Organ cultures that are kept in motion remain in better condition than those kept stationary (Cherry & Taylor-Robinson, 1970). Organ cultures from monkeys can be maintained for 24 days with full activity (Hoorn & Tyrrell, 1965b). Human foetus appears the best source of tracheal organ cultures and these have been successfully maintained for up to 6 weeks post dissection (Hoorn, 1966; Mostow & Tyrrell, 1973). Very little decline in ciliary activity occurred during this period though the epithelial layer becomes progressively more thin and flat, and some loss of ciliated cells was also noted. Human nasal turbinate organ cultures could be maintained for at least 22 days (Hoorn & Tyrrell, 1965b).

The first ciliary activity score was made at 24 hours post dissection rather than immediately after dissection (section 7.2.1.), because the organ cultures appear to enter a period of 'post-operative' shock where little or no ciliary activity is observable (Hoorn, 1966; Mostow & Tyrrell, 1973). All workers agree that organ cultures are best maintained for at least 24 hours or overnight after sectioning before use (e.g. Mostow & Tyrrell, 1973), but some workers do maintain them for longer periods (e.g. Boudreault, 1979; Harnett & Hooper, 1968). Maintained ferret tracheal organ cultures gave a more reliable growth pattern for influenza virus (Kingsman et al., 1977a; Cavanagh et al., 1979). Kingsman et al. (1977a) found no major histological changes between fresh and maintained ferret tracheal organ

cultures and that further maintenance (48 hours post dissection) did not improve the performance of such cultures. No mucus covering of the epithelial lining was observed but the goblet cells appeared to empty on maintenance (Herbst-Laier, 1970). The presence of a mucus covering has been reported by others (Blaskovic *et al.*, 1972a) using chick embryo tracheal organ cultures. Considering my findings, and those reported by others, I decided to maintain the tracheal organ cultures overnight before use.

Comparison of the ability of different influenza strains to multiply on tracheal organ cultures (section 7.2.3.), with that found by others, is complicated. The passage history of the influenza strains and the donor animal used to provide the cultures may influence the results obtained. I found A/PR/8 multiplied well (Figure 7.2.) on mouse tracheal organ cultures. This agrees with data from others who have used tracheal organ cultures from mouse, hamster, rat, dog, ferret and man (Westerberg *et al.*, 1972; Schiff, 1974; Herbst-Laier, 1970). Hara *et al.* (1974) reported better growth on human rather than ferret tissues, with maximal titres occurring 1-2 days *p.i.*. Schiff (1974), used hamster tracheal organ cultures and found the older the donor animal then the slower the multiplication of virus. Cogliano & Small (1978) also found the serostatus of the ferrets affected the susceptibility of the organ cultures to infection. Seropositive animal donors required 130-fold more inoculum to infect the cultures. The ability of A/PR/8 to reduce the ciliary activity of organ cultures from different species is less clear. My results show that A/PR/8 caused no appreciable reduction in ciliary activity (Figure 7.2.), but Westerberg *et al.* (1972) found A/PR/8 destroyed the ciliary activity of mouse tracheal organ cultures by 7 days *p.i.*. The A/PR/8 virus I used had been repeatedly passaged in hens eggs, but the virus used by Westerberg *et*

al. had been passaged a total of 593 times in mice and this may have increased its pathogenicity to this animal host. Herbst-Laier (1970) found that A/PR/8 reduced the ciliary activity of ferret and dog tracheal organ cultures but not those of vervet monkey or pig. Mostow & Tyrrell (1973) found damage occurring in ferret but not human tracheal organ cultures. Hara et al. (1974) described A/PR/8 as avirulent on the basis of its low pathogenicity on both human and ferret tracheal organ cultures.

A/WSN (Figure 7.3.) has not, to my knowledge, been propagated on organ cultures before. A/WSN causes a lower respiratory tract (LRT) infection with considerable pathology in the lungs (Dimmock et al., 1986).

A/Jap (Figure 7.4.) has also not been grown on organ cultures although other H2N2 influenza strains have been successfully propagated (Kingsman et al., 1977a; Herbst-Laier, 1970). Heath et al. (1983) found the A/Ann Arbor/6/60 influenza strain grew on hamster tracheal organ cultures and gave a CAI₅₀ by day 6 p.i.. Mostow & Tyrrell (1973) used tissue from human sources and found that attenuated variants of the A/England/501/68 strain caused a variable amount of damage and that this correlated with the in vivo virulence in man.

The A/Clr influenza strain multiplied well on the mouse tracheal organ cultures and rapidly caused a considerable reduction in ciliary activity (Figure 7.5.). This may be due to the fact that the virus has been grown only in MDCK cells and never passaged in eggs. Many other H3N2 influenza strains have been successfully grown on tracheal organ cultures derived from various animal sources. All show good growth with maximal titres by day 3 p.i.. The wild-type and virulent recombinants all rapidly destroy the

ciliated epithelium with the CAI_g occurring within 7 days p.i. (Blaskovic et al., 1972c; Herbst-Laier, 1970; Sweet et al., 1981; Heath et al., 1986; Mostow & Tyrrell, 1973). The A/England/939/69 strain was unusual since it rapidly destroyed the ciliary activity on human tracheal organ cultures but caused little (Hara et al., 1974) or no damage to ferret tissue (Mostow & Tyrrell, 1973). Mostow & Tyrrell (1973) also found that low levels of virus continued to be produced human organ cultures even when they were devoid of visible ciliated cells. Tracheal organ cultures have frequently been used to measure the degree of attenuation of temperature-sensitive and cold-adapted H2N2 strains. The successfully attenuated strains multiplied but failed to produce significant damage to the ciliated epithelium (Mostow et al., 1979; Heath et al., 1986). Some workers have chosen the A/Hong Kong/1/68-ts-[E] influenza virus as a reference strain against which attenuation can be measured (Boudrieux, 1978).

To my knowledge no other workers have attempted to grow avian influenza strains on either mammalian or avian tracheal organ cultures. My results suggest that the initial source of the influenza strain may play a crucial role in its ability to grow on mouse tracheal organ cultures. Three isolates from chickens did not grow, whilst a strain isolated from turkeys did grow. The reasons for this difference are not clear. The turkey influenza virus caused no loss of ciliary activity and was non-pathogenic on mouse tracheal organ cultures. By repeated serial passaging in a mouse tissue culture cell line A/FPV/R can be adapted to multiply on mouse tracheal organ cultures (Figure 7.9.). The virus produced, adFPV/R, caused a very rapid reduction in ciliary activity and was handled with care because of its potential virulence to other mammals, including man.

The antigenic changes in the H7 haemagglutinin caused by the adaptation were small (section 7.2.6.). Surprisingly, no change was seen at residue 226, the key residue associated with the attachment of influenza to different sialic acid receptors (section 1.4.).

The intranasal inoculation of mice with A/WSN before preparation of tracheal organ cultures (section 7.2.8.) demonstrated that the epithelial cells of the trachea were infected *in vivo*. The trachea was not the major initial site of infection since there was little virus multiplication at 24 hours p.i. but multiplication occurred later. By 48 hours p.i. considerable damage to the respiratory surface of the trachea had occurred which may have resulted from virus multiplication or from an immune-mediated response to the infection by the host. Tom et al. (1976) carried out similar experiments in ferrets using H3N2 reassortants. There was little damage to the ciliated epithelium. Virulent reassortants multiplied in organ cultures prepared days 2-6 p.i., whilst attenuated reassortants only multiplied in cultures prepared on day 4 p.i., and to lower titres. An apparent recovery of ciliary activity was also found when cell debris was removed during maintenance. Hussein et al. (1983) demonstrated that the trachea was an initial site of influenza virus infection *in vivo* in ferrets. Sweet et al. (1983) confirmed that virus replication was occurring in this tissue and that the virus found did not result from residual inoculum. Kingman et al. (1977b) estimated that less than 1% of the original inoculum remained 24 hours p.i.

The distribution of viral antigens detected by fluorescent antibody labelling (section 7.2.9.) in cells removed from organ cultures of intranasally infected mice confirmed that tracheal epithelial cells were

infected in vivo. Blaskovic et al. (1972c) demonstrated a similar degree of fluorescent antibody staining in cells obtained from smears of influenza infected ferret tracheal organ cultures with maximal staining at 2-3 days p.i. Hussein et al. (1983) also showed that fluorescence was maximal 2-3 days p.i.. Hussein found between 10-50% of the cells showed positive staining and stated that ciliated cells were predominantly infected. Kingman et al. (1977b) detected influenza virus antigens by fluorescent antibody staining using cells obtained from an organ culture impression on a slide. They found that only 26% of the cells from ferret tracheal organ cultures were infected. If a low inoculum was used the number of cells infected fell to 2-5% but the viral antigen was apparent by 8-10 hours, again indicating that the trachea was an initial site of in vivo infection.

To conclude, my data demonstrate that mouse tracheal organ cultures are a viable experimental model system on which to study influenza virus infection.

RESULTS 2.
Attachment of influenza virus
to various cell types.

8.1. Introduction.

The attachment of influenza virus to ciliated cells of organ cultures from various species has been observed by electron microscopy. Influenza virus attaches strongly to the cilia (Blaskovic *et al.*, 1972a), but this does not mean that the virus enters the cells via the cilia (Dourmashkin & Tyrrell, 1970). The attachment of the virus to cilia may play an important role in vivo by clumping the cilia, inhibiting the ciliary action and preventing elimination of virus in the mucus blanket (Westerberg *et al.*, 1972). Few quantitative data relating to the attachment of virus to ciliated cells of organ cultures in situ are available due to the lack of a suitable method for isolating such cells. The previous chapter described the use of protease digestion to isolate epithelial cells from mouse tracheal organ cultures. This technique enables a quantitative study of the attachment of radiolabelled influenza virus to differentiated epithelial cells to be attempted.

The attachment of radiolabelled influenza virus to dedifferentiated tissue culture cells and erythrocytes has been studied previously (Possee, 1981; Possee *et al.*, 1982; Taylor, 1986; Taylor & Dimmock, 1985a 1985b; Taylor *et al.*, 1987). These studies were performed at 4°C. I chose to investigate attachment at 37°C in preference to 4°C because:

(i) The effects of low temperatures (i.e. 0-4°C) on the early events of influenza virus infection are not clear. Influenza virus does attach to cells at this temperature (Dourmashkin & Tyrrell, 1970) and it has also been shown that temperature does not affect the rate of influenza virus attachment (Taylor & Dimmock, 1985a). Some workers found that the virus was internalised and uncoated but that no viral macromolecular synthesis

occurred (Stephenson & Dismock, 1975), whereas others found that virus entry was blocked and virtually no internalization took place (Matlin *et al.*, 1981; Richman *et al.*, 1986). At physiological temperatures all agree that influenza virus does attach and is internalized.

(ii) One of my primary aims is to study the attachment of influenza virus to the ciliated epithelium of tracheal organ cultures prepared from mice. These epithelial cells have actively beating cilia, the vigour of which is greatly diminished by chilling (section 7.2.7.). Blaskovic *et al.* (1972a) studied the attachment of influenza virus to chick embryo organ cultures and stated that when organ cultures were held at 4°C "the migration of virions through the mucus blanket was very slow ... ciliary activity diminished to a standstill resulting in slower movement of the mucus blanket, which, in addition became more viscous. These conditions, it is assumed, also interfered with the normal activity of neuraminidase, which further contributed to the retardation of virion migration ... inoculated cultures should be held during the adsorption period at 36°C rather than 4°C for at least 30 minutes."

This chapter describes the inoculation of tracheal organ cultures with infectious ³²P adpFPV/R and determination of the amount of radioactive virus attaching to epithelial cells subsequently isolated from such cultures. The amount of attachment is compared with the attachment of virus to other types of cell.

Also described in this chapter are preliminary findings regarding the effect of IgG neutralization on the ability of the virus to attach to cells. Neutralization by polyclonal IgG antibodies directed against the haemagglutinin had previously been shown to cause a reduction in attachment

of A/FPV/R to chicken erythrocytes (Possee, 1981; Dimmock, 1984) but did not reduce the levels or rates of A/FPV/R attachment to CEF cells (Possee *et al.*, 1982) and BHK cells (Dimmock, 1984; Taylor, 1986). I will describe the effects of IgG neutralization on attachment of adpFPV/R to tracheal organ culture cells and compare this with the attachment to other cell types.

8.2. Results.

8.2.1. Effect of the proteases on the attachment of adpFPV/R to cells.

Attachment of adpFPV/R to epithelial cells isolated from mouse tracheal organ cultures was studied by the application of radiolabelled virus to the whole organ cultures, followed by the detachment of the epithelial cells by protease digestion (section 6.5.4.) and the measurement of attached radioactivity. These proteases may release virus from the cell surface and give a falsely low level of virus attachment. Proteinase K released attached A/FPV/R from the surface of BHK cells (Taylor & Dimmock, 1985a, 1985b), however others found proteinase K, trypsin and chymotrypsin failed to release A/FPV/R from the surface of MDCK cells (Matlin *et al.*, 1981).

It was not possible to directly test the effects of the protease XIV and XXI on virus attached to organ cultures since their use is essential to the process of isolating cells from organ cultures. Instead, two mammalian tissue culture cell lines, BHK and C3H10T1/2, were used. ³²P adpFPV/R was inoculated onto confluent monolayers and after incubation at 37°C for various times, the cells were washed to remove the excess unattached virus. The monolayers were then treated with either medium alone or medium plus proteases. The proteases freed considerable numbers of cells from the

plastic Petri dishes; these cells were harvested between each digestion and held at 37°C to ensure continued protease action for the full time course of digestion. The results (Figures 8.1. and 8.2.) demonstrate that the level of adpFPV/R attachment was identical irrespective of whether the cells were treated with proteases or not. Therefore, the proteases do not appear to release attached virus from either of these tissue culture cell lines. I have assumed on the basis of these results that the proteases XIV and XXI do not release attached adpFPV/R from tracheal organ culture cells and that any release of attached adpFPV/R from such cells will represent a minimal value.

8.2.2. Attachment of infectious adpFPV/R to various cell types.

Different amounts of ³²P-labelled adpFPV/R were inoculated onto a range of cell types. These were incubated at 37°C for 60 min. before determining how much virus had attached (section 6.5.4.).

A good correlation (>0.95) by linear regression analysis was found, within each cell type, between the amount of virus inoculated and the amount of virus attached per cell (Figures 8.3. to 8.7.). The correlation remained constant within different assays on the same cell type. The relationship between the inoculum and the amount of adpFPV/R attached was approximately proportional for all cell types. Linear regression analysis gave a slope of between 0.86 and 1.13. Total proportionality would give a slope with a value of 1.

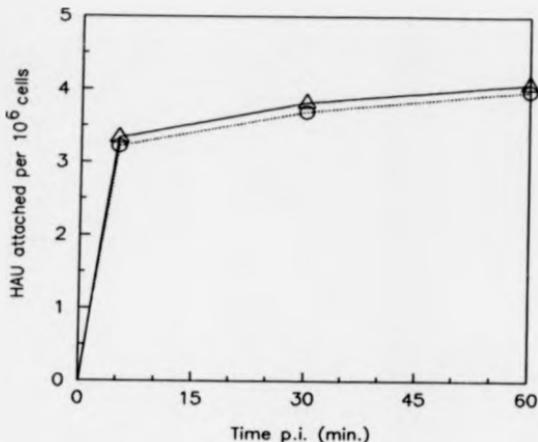


Fig.8.1. Protease treatment does not release virus that is attached to BHK cells.

Confluent monolayers of BHK cells (3.8×10^6 cells) were inoculated with 50 HAU adpFPV/R and incubated for various times at 37°C . The cells were then washed with PBS ($5 \times 2\text{ml}$) before treatment with either medium alone (\circ), or medium plus 10U/ml proteases (\triangle). The conditions used were the same as for removing epithelial cells from tracheal organ cultures. The cells were then harvested and the associated radioactivity determined.

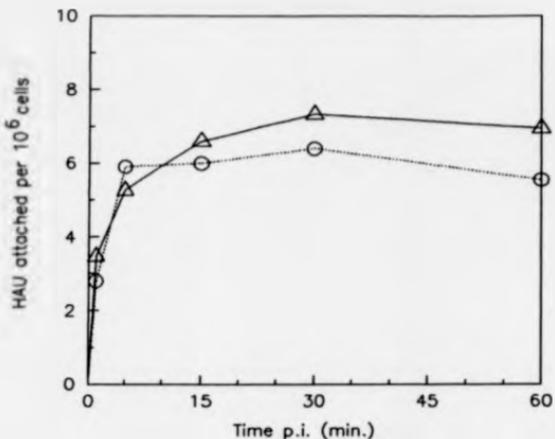


Fig.8.2. Protease treatment does not release virus that is attached to C3H10T1/2 cells.

Confluent monolayers of C3H10T1/2 cells (3×10^6 cells) were inoculated with 50 HAU adpFPV/R and incubated for various times at 37°C . The cells were washed with PBS ($5 \times 2\text{ml}$) before treatment with either medium alone (---○---), or medium plus 10U/ml proteases (---△---). The conditions used were the same as for removing epithelial cells from tracheal organ cultures. The cells were then harvested and the associated radioactivity determined.

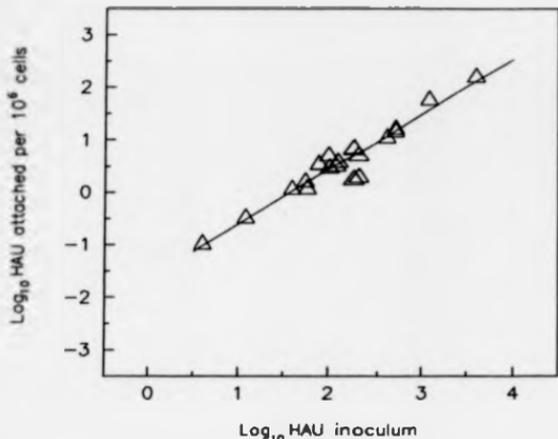


Fig.8.3. Attachment of non-neutralized adpFPV/R to epithelial cells isolated from organ cultures.

Various amounts of ³²P adpFPV/R were inoculated onto tracheal organ culture preparations. After incubation at 37°C for 60 min. the organ cultures were washed 5 times with PBS to remove unattached virus. The epithelial cells were removed from the organ cultures by protease digestion, resuspended in 1ml PBS, sonicated and the amount of radioactivity determined. The number of counts associated with the cells were converted to give the amount of virus attached by dividing by the CPM:HAU ratio (see text).

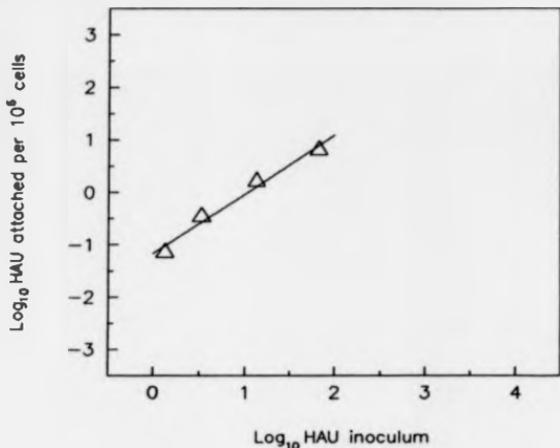


Fig.8.4. Attachment of non-neutralized adpFPV/R to baby hamster kidney (BHK) cells.

Various amounts of ³²P adpFPV/R were inoculated onto confluent monolayers of BHK cells (5×10^6 cells). After incubation at 37°C for 60 min. the cells were washed 4 times with PBS and the cells were scraped from the Petri dish, resuspended in 1ml PBS, sonicated and the amount of radioactivity determined. The number of counts associated with the cells were converted to give the amount of virus attached by dividing by the CPM:HAU ratio (see text).

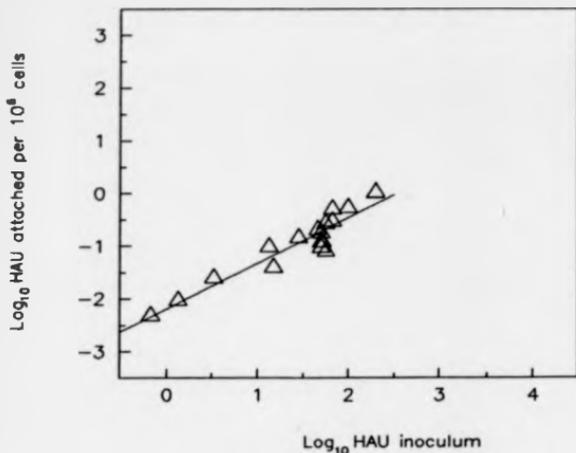


Fig.8.5. Attachment of non-neutralized adpFPV/R to chicken erythrocytes.

Various amounts of ³²P adpFPV/R were inoculated into suspensions of chicken erythrocytes (1×10^8 cells). After incubation at 37°C for 60 min. the cells were washed 3 times with PBS to remove unattached virus. The cells were resuspended in 1ml PBS, sonicated and the amount of radioactivity determined. The number of counts associated with the cells were converted to give the amount of virus attached by dividing by the CPM:HAU ratio (see text).

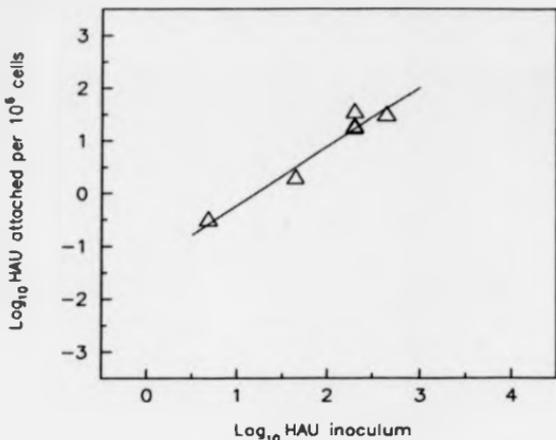


Fig.8.6. Attachment of non-neutralized adpFPV/R to chick embryo fibroblast (CEF) cells.

Various amounts of ³²P adpFPV/R were inoculated onto confluent monolayers of CEF cells (2.3×10^6 cells). After incubation at 37°C for 60 min. the cells were washed 4 times with PBS and scraped from the petri dish. The cells were resuspended in 1ml PBS, sonicated and the amount of radioactivity determined. The number of counts associated with the cells were converted to give the amount of virus attached by dividing by the CPM:HAU ratio (see text).

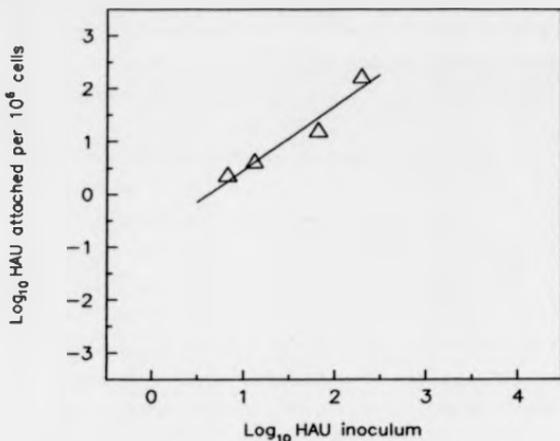


Fig.8.7. Attachment of non-neutralized adpFPV/R to mouse fibroblasts (C3H10T1/2) cells.

Various amounts of ³²P adpFPV/R were inoculated onto confluent monolayers of C3H10T1/2 cells (2.6×10^5 cells). After incubation at 37°C for 60 min. the cells were washed 4 times with PBS and scraped from the Petri dish. The cells were resuspended in 1ml PBS, sonicated and the amount of radioactivity determined. The number of counts associated with the cells were converted to give the amount of virus attached by dividing by the CPM:HAU ratio (see text).

Saturation of the cells with adpFPV/R was not observed. If saturation was occurring then a levelling off in the amount of virus attaching would be observed as the amount of virus in the inoculum increased towards saturating amounts.

I have chosen to express the attachment of virus to cells in terms of HAU per cell because:

- (i) The number of cells used in the assay is accounted for, so that attachment to different cell types can be compared e.g. less than 10^6 organ culture cells were used compared to 10^8 chicken erythrocytes. Also the number of tissue culture cells per confluent monolayer was variable between experiments e.g. $4.71 \pm 1.90 \times 10^6$ BHK cells per 5cm Petri dish.
- (ii) The CPM:HAU ratio was determined within each assay, dividing the number of counts attached to each cell (CPM/cell) by the CPM:HAU ratio converted the measurement of attachment to HAU/cell. This correction was used to account for quality of the preparation of radiolabelled virus being used. The amount of ^{32}P -radiolabel incorporated into each adpFPV/R preparation varied considerably (891 \pm 1593 CPM:HAU). The ratio for each preparation also declined with time as the radiolabel decayed.
- (iii) Previous studies on the attachment of radiolabelled virus to cells have expressed attachment as a proportion (percentage) of the inoculum applied (Taylor & Dismock, 1985a, 1985b). I found that the cell type greatly affected the proportion of inoculated virus that did attach. Also there was considerable variation between experiments within each cell type (Table 8.1.).

Table 8.1. Proportion of adpFPV/R attaching to different cell types.

<u>Cell type:</u>	<u>Proportion of inoculum attaching (%)</u>	<u>Number of experiments</u>	<u>Range of inocula (HAU)</u>
chicken erythrocytes	45.3 (+/- 20.3)	19	1 - 400
organ culture cells	1.7 (+/- 1.3)	21	4 - 4000
BHK cells	23.6 (+/- 10.6)	31	3 - 800
CEF cells	17.0 (+/- 5.5)	6	5 - 450
C3H10T1/2 cells	11.3 (+/- 2.8)	4	7 - 200

Different amounts of ^{32}P adpFPV/R were inoculated onto the various cell types. After incubation at 37°C for 60 minutes the amount of radioactivity attached to the cells was determined and expressed as a percentage of the inoculum applied.

8.2.3. The rate of adpFPV/R attachment.

As well as determining the amount of virus attaching to cells, I compared the rates of attachment of adpFPV/R to tracheal organ culture epithelial cells and to other cell types. The amount of virus attaching following a 60 min. incubation was taken as the 100% value. Figure 8.8. shows that attachment is rapid, irrespective of the cell type studied, the majority (>80%) of the labelled adpFPV/R attaching within 5 min. The amount of virus inoculated (420 HAU) was not sufficient to saturate the cells (section 8.2.2.).

8.2.4. Effect of the proteases on adpFPV/R neutralization.

Before examining the effects of neutralization on the attachment of adpFPV/R to organ culture cells, it was necessary to show that the proteases did not effect this process. For example, protease treatment could affect virus infectivity, or attack the virus/antibody complex, perhaps by altering the ability of antibody to neutralize.

To test the effect of proteases on virus infectivity, adpFPV/R was incubated with either medium alone or medium plus proteases in a manner similar to that used to isolate epithelial cells from tracheal organ cultures. The incubations were carried out at three temperatures. If a reduction in infectivity was caused by enzymatic digestion, such a change might be expected to be temperature dependant. Following incubation the samples were assayed for infectivity (Table 8.2.). Proteases reduced the

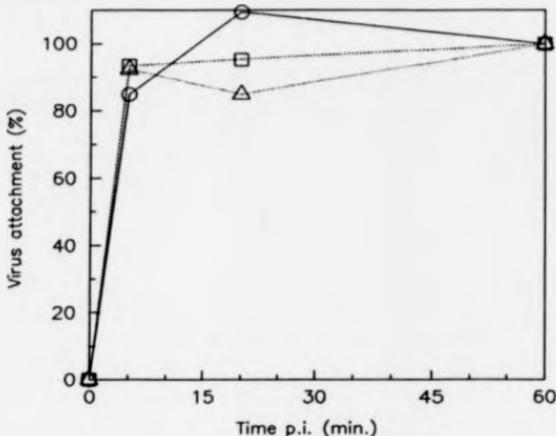


Fig.8.8. Attachment of non-neutralized adpFPV/R to various cell types as a function of time.

^{32}P adpFPV/R (420 HAU) was inoculated onto (i) tracheal organ cultures (\circ), BHK cells (\square) and chicken erythrocytes (\triangle). After incubation at 37°C for various times, the unattached virus was removed by washing with PBS. The cells were then isolated (methods section), resuspended in 1ml PBS, sonicated and the amount of radioactivity determined. The amount of virus attached to the cells at each time interval has been related as a percentage to the value at 60 min. post-inoculation.

Table 8.2.
Effect of protease treatment
on adpFPV/R infectivity.

Temperature of incubation	adpFPV/R* + proteases	adpFPV/R* + medium	$\frac{\text{protease value} \times 100}{\text{medium value} \times 1}$
4°C	6.20×10^7	6.90×10^7	89.9%
20°C	6.50×10^7	7.40×10^7	87.8%
37°C	7.45×10^7	8.45×10^7	88.2%

* infectivities expressed as pfu/ml.

infectivity of the virus by a small amount (10-13%). No temperature dependence was seen indicating that this reduction was not due to an enzymatic protease digestion.

The effect of the proteases on the neutralization process was then examined. Samples of adpFPV/R (1000 HAU/ml) were incubated with an equal volume of either PBS, or the monoclonal antibodies HC2 (1/300) or HC61 (1/1000) for 60 min. at 25°C. To these virus/antibody mixtures was added an equal volume of either (i) PBS, (ii) medium alone, or (iii) medium plus proteases. The samples were incubated for a further 80 min. at 37°C and then assayed for residual infectivity. The results (Table 8.3.) demonstrate that protease treatment did not reduce the neutralizing capacity of the antibodies investigated.

8.2.5. Neutralization of adpFPV/R by HC2 and the effect on virus attachment.

³²P adpFPV/R (1000 HAU/ml) was neutralized with an equal volume of HC2 (1/300) for 60 min. at 25°C, sufficient to cause a 99.2% reduction in infectivity by plaque assay. The amount of neutralized adpFPV/R attaching to different cell types was determined and compared to the respective non-neutralized controls (Figure 8.9.). A significant reduction in virus attachment compared to the controls (100%) was observed on all cell types studied. The lowest level of attachment of neutralized virus was seen on chicken erythrocytes (10.9%), the highest was found on CEM10T1/2 tissue culture cells (68.9%). An intermediate level of attachment by neutralized adpFPV/R was found on cells isolated from mouse tracheal organ cultures (31.0%). However the reduction in attachment caused by neutralization was

Table 8.3.
Effect of protease treatment on the ability of
antibodies to neutralize adpFPV/R infectivity.

Post- neutralization treatment	Control	Neutralization by HC2 (1/300)	Neutralization by HC61 (1/1000)
PBS	7.38 (100%)*	5.62 (1.75%)	6.11 (5.42%)
Medium	7.38 (100%)	5.68 (2.00%)	6.15 (5.94%)
Proteases	7.32 (100%)	5.61 (1.95%)	6.08 (5.71%)

* Log_{10} pfu/ml (% control value)

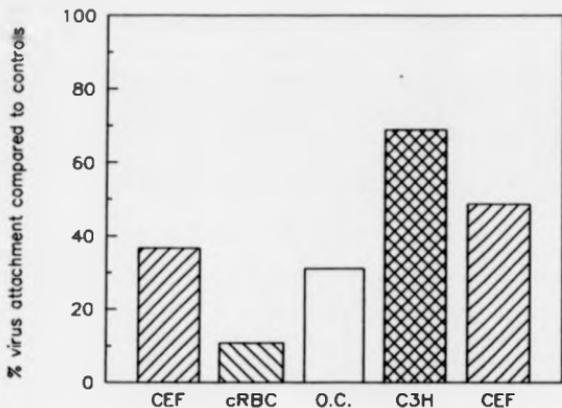


Fig. 8.9. Neutralization of ^{32}P adpFPV/R by HC2 and attachment to various cell types.

^{32}P adpFPV/R (1×10^3 HAU/ml) was incubated at 25°C for 60 min. with $1/300$ dilutions of either HC2 or the anti-H3 monoclonal antibody, 185/1, which is the control. The virus/antibody mixtures were inoculated onto various cell types and incubated at 37°C for 60 min. The excess unattached virus was removed by washing with PBS, before the cells were isolated, sonicated and the amount of cell associated radioactivity determined.

always less than the reduction in infectivity, indicating that failure of attachment was not necessarily the reason why virus had become non-infectious. The differences between my findings and those in previously published works (Possee *et al.*, 1982; Dismock, 1984; Taylor, 1986) will be discussed at the end of this chapter.

8.2.6. Neutralization of adFPV/R by HC61 and the effect on virus attachment.

The finding that IgG (HC2) neutralization of adFPV/R reduced attachment to all cell types, including one of those previously studied by others (section 8.1.), was surprising. To confirm this finding, a similar set of experiments was done using a different monoclonal IgG i.e. HC61.

³²P labelled adFPV/R (4100 HAU/ml) was incubated with a 1/20 dilution of HC61, sufficient to cause greater than a 99.9% reduction in infectivity. A significant reduction in virus attachment was observed on each of the three cell types studied (Figure 8.10.), similar to the situation found using HC2.

8.2.7. Effect of IgG neutralization on the rate of adFPV/R attachment.

It is possible that the reduction in attachment of HC2- and HC61-neutralized adFPV/R resulted from a slower rate of attachment compared to the non-neutralized control. This was studied using BHK cells and chicken erythrocytes. Figures 8.11. and 8.12. show that the

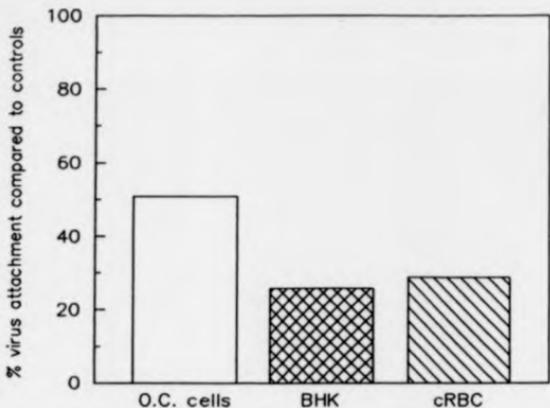


Fig.8.10. Neutralization of ^{32}P adpFPV/R by HC61 and attachment to three types of cells.

^{32}P adpFPV/R (4.1×10^3 HAU/ml) was incubated at 25°C for 60 min with 1/20 dilutions of either HC61 or 185/1. The virus/antibody mixtures were then inoculated onto the different cell types and incubated at 37°C for 60 min. The cells were then washed with PBS to remove the excess unattached virus, before the cells were isolated and the amount of cell associated radioactivity determined.

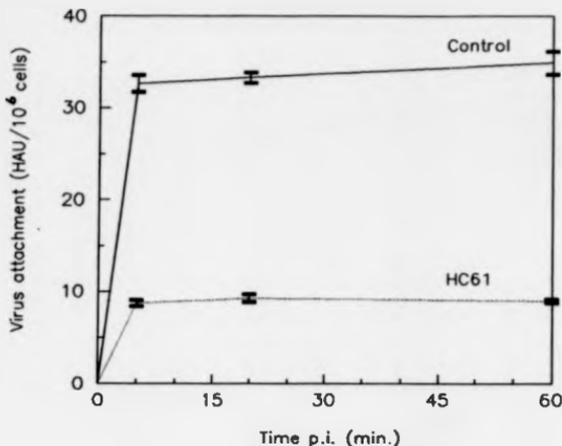


Fig.8.11. Rates of attachment of HC61-neutralized and non-neutralized ³²P adpFPV/R to BHK cells.

³²P adpFPV/R (4.1×10^3 HAU/ml) was incubated at 25°C for 60 min with 1/20 dilutions of either 185/1 (—) or HC61 (---). The virus/antibody mixtures were inoculated onto confluent BHK monolayers (1.62×10^6 cells) and incubated at 37°C for different times. The cells were then washed with PBS to remove the excess unattached virus. The cells were then scraped from the Petri dish, resuspended in 1ml PBS, sonicated and the amount of cell associated radioactivity determined.

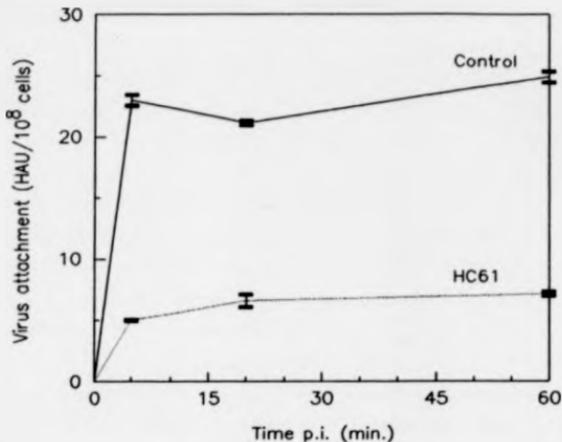


Fig.8.12. Rates of attachment of HC61-neutralized and non-neutralized ³²P adFPV/R to chicken erythrocytes.

³²P adFPV/R (4.1×10^5 HAU/ml) was incubated at 25°C for 60 min with 1/20 dilutions of either 185/1 (—) or HC61 (---). The virus/antibody mixtures were inoculated onto chicken erythrocytes (1×10^8 cells) and incubated at 37°C for different times. The cells were then washed with PBS to remove unattached virus, resuspended in 1ml PBS, sonicated and the amount of cell associated radioactivity determined.

MO61-neutralized adpFPV/R attached at a similar rate to the non-neutralized virus (but again to a reduced level) i.e. the majority of the neutralized virus that is able to attach did so within 5 min. post-inoculation.

8.3. Discussion.

My results demonstrate that tracheal organ cultures can be used to study the attachment of influenza virus to differentiated cells of the respiratory tract. The proportion of the inoculum attaching to the ciliated epithelial cells of organ cultures (Table 8.1.) is low (1.7%), possibly reflecting a restricted access for the inoculum to the ciliated epithelium on the luminal organ culture surface.

An alternative method of assessing virus attachment to whole organ cultures is to determine the infectivity of the inoculum before and after inoculation, the difference representing the amount of virus attached.

Using this technique Kingsman *et al.* (1977b) reported that roughly 10% of A/Moscow/1019/65 (H2N2) attached to ferret nasal turbinate organ cultures after 5, 60 or 240 min. post-infection at 4°C. Schiff (1974) reported that over 90% of the A/PR/8 (H1N1) attached to 2-day old hamster tracheal organ cultures at 120 minutes post-infection (in fact, there was no residual infectivity in the supernatant culture fluid!). A difference in attachment was found using organ cultures from 4-week old hamsters, where only 50% of the inoculum attached. Schiff concluded that influenza virus showed 1st order kinetics of binding and that possibly the older animals had less receptors on the cells. It was noted that the surface area of the organ cultures from animals of different ages could vary and that caution needed to be taken in interpreting the results.

Probably the reason for the difference between my findings and those described above, is the method of assay. I studied attachment of virus to cells which were subsequently removed from organ cultures whereas others studied attachment to whole organ cultures. I found, using radiolabelled virus, that tracheal organ cultures retained a large population of loosely or non-attached virions, which could be recovered by extensive washing. The ciliated nature of the cells, or the ring-shaped structure of the cultures, may be responsible for the retention of the virus. The age and species of the animal used, together with variations in the virus strain may also affect the results, as indicated above.

The proportion of virus attaching to cells did not appear to depend on the amount of virus in the inoculum, but was dependant on the cell type being studied. I found a larger proportion of the inoculum attached to tissue culture cells than attached to epithelial cells of organ cultures. The greatest amount attached to chicken erythrocytes. This may reflect an increase in access for the virus to the target cells. Saturation of the cells with virus did not occur at the highest inocula tested. Taylor & Dismock (1985a, 1985b) using A/FPV/R, found that 20-30% of the inoculum attached to EHK cells, this agrees well with my findings. If the levels of attachment found by Taylor & Dismock (1985a, 1985b) are converted to HAU per cell, using additional data provided, again good agreement is found with my results. Interestingly their data also reveal great variation in the quantity of radiolabel incorporated per virus particle, ranging from 213 CPN:HAU (Taylor & Dismock, 1985b) to 619 and 4255 CPN:HAU (Taylor & Dismock, 1985a). Matlin *et al.* (1981) found that a maximum of between 35% to 55% A/FPV/R attached to MDCK cells. The virus bound to the microvilli

and penetrated the cells at the base of these structures, a situation similar to that seen using tracheal organ cultures (Dourashekin & Tyrrell, 1970). Richman et al. (1986) found only 10% of A/HK/68 (H3N2) attached to MDCK or LLC cells. The observation that only a small proportion of inoculum is able to attach to cells appears to be common to many virus systems e.g. Venezuelan equine encephalomyelitis (VEE) (Roehrig et al., 1968), avian infectious bronchitis virus (IBV) (Cavanagh & Davis, 1986), rabies virus (Dietzschold et al., 1987). It seems that both the virus strain and the cell type under study can affect the amount of virus attaching to cells.

At a constant inoculum (100 HAU), the extent of attachment of adpFPV/R to different cell types can be compared (Table 8.4.). The values calculated in this table should only be considered as approximate. On a per cell basis the greatest number of adpFPV/R virions attach to C3H10T1/2 cells, and the least to chicken erythrocytes. This finding does not account for the relative size and surface areas of different cells. On a unit area basis a different picture emerges, nearly 4-fold more virions attach to BHK cells than to C3H10T1/2 cells. The relatively low number of virions attaching per chicken erythrocyte now falls into line with CEF and C3H10T1/2 cells and does reflect the small surface area of these cells compared to the others studied. The ciliated nature of the organ culture cells greatly complicates any surface area estimations. Edwards et al. (1986), working on ciliated human adenoidal organ cultures, estimated that 2×10^8 cells occupied 3mm^2 . If the cells were not ciliated and this value was applied to my data, there would be 6.67 virions per $10\mu\text{m}^2$ of organ culture cell surface. This is certain to be an over-estimate as no account is taken of the increased surface area due to the cilia. The differences in the amounts of adpFPV/R attaching to different cell types may reflect either (a) the numbers of

Table 8.4.

Comparison of the levels of attachment of ^{32}P labelled adpFPV/R to different cell types.

Cell Type	a Log ₁₀ HAU attached per 10 ⁶ cells	b virions per cell	cell number	c mean cell surface area	virions per 10 μm ²
O.C. cells	+0.4	100	varies	not known	
BHK cells	+0.9	316	5.0 x 10 ⁶	392.7 μm ²	8.05
cRBC's	-0.6	10	1.0 x 10 ⁶	88.9 "	1.12
CEF cells	+0.7	199	2.3 x 10 ⁶	853.7 "	2.33
C3H10T1/2 cells	+1.6	1584	2.6 x 10 ⁵	7551.9 "	2.10

NOTES:-

- The results are taken from figures 8.3 - 8.5, where the inoculum size is 100 HAU.
- For A/FPV/R 1 HAU represents 3.98×10^7 virus particles (Taylor *et al.*, 1987). This is assumed to apply also to adpFPV/R.
- Calculated by dividing the area of a 5cm diameter petri dish by the cell number. Assumes that the cells lie uniformly flat.
- Chicken erythrocytes have an average volume of $79 \mu\text{m}^3$ (Cook *et al.*, 1979). The surface area has been calculated assuming the cells are spherical.

cell receptors for the virus, or (b) the affinity of the virus for the particular cell receptors. If the diameter of adpFPV/R is assumed to be similar to that of A/FPV/R i.e. 119.5nm (Taylor *et al.*, 1967), then over 100 virions could pack into a $10\mu\text{m}^2$ area. Therefore less than 8% of the BHK cell surface area is covered by attached virions.

The rate of attachment of adpFPV/R was rapid irrespective of cell type (Figure 8.8.), the majority of virus attached within 5 min. post-inoculation. As the incubation time was increased the rate of virus attachment slowed considerably, with little additional virus able to attach. Others have found that A/FPV/R attaches more gradually to cultured cells and that the rate slows after 60 to 90 min. post-inoculation (Matlin *et al.*, 1981; Taylor & Dimmock, 1985a, 1985b). Schiff (1974) demonstrated that 70% of the A/HK/68 inoculum attached to whole hamster tracheal organ cultures within 30 min. post-inoculation. These findings are surprising and may reflect a slow rate of diffusion rather than attachment. Attachment of influenza virus to cells is temperature-independent (Taylor & Dimmock, 1985a), probably because the abundance of cell receptors gives a high probability of virus/cell collision (Lonberg-Holm & Philipson, 1980). An abundance of cell receptors would predict a rapid rate of attachment, as I have found. My data do not allow the rate constant to be accurately calculated, considerably shorter time intervals would be required. The use of cell suspensions rather than monolayers would also be advantageous and would increase the "encounter efficiency" of virus-cell collisions (Lonberg-Holm, 1981).

I found that neutralization of adpFPV/R by monoclonal IgG antibodies directed against the haemagglutinin did cause a significant reduction in attachment, the size of which depended on the cell type studied. However, the reduction in attachment never equalled the reduction in infectivity caused by neutralization. My observation that neutralizing monoclonal IgG reduces attachment of influenza virus to chicken erythrocytes confirms the findings of Possee (1981) who used polyclonal IgG to neutralize A/FPV/R. A difference with published data (Possee *et al.*, 1982; Dimmock, 1984; Taylor, 1986) is my finding that there is a reduction in attachment of IgG neutralized virus to CEF and BHK cells. This was not attributable to a slower rate of attachment. The discrepancy between my observations and those of Taylor (1986) was especially worrying because we both used the same BHK cell line, identical monoclonal antibodies (HC2 and HC61), a similar inoculum size and virus strain (A/FPV/R). The major difference between protocols was that Taylor used saturating concentrations of neutralizing antibody whereas I used lower, sub-saturating amounts. At sub-saturating IgG concentrations substantial aggregation of A/FPV/R occurs (Taylor *et al.*, 1987) and this may affect the levels of attachment of neutralized adpFPV/R. It therefore appears that the antibody to virus ratio may be important in interpreting and understanding IgG neutralization of influenza virus. Although my findings clearly show that the reduction in attachment of virus does not fully account for the reduction in infectivity caused by IgG neutralization, they do indicate that the mechanism(s) of neutralization of influenza virus are more complex than previously proposed (Dimmock, 1984; Dimmock, 1987).

RESULTS 3.
Neutralization of influenza
virus by IgG.

2.1. Introduction.

My initial findings (section 8.) showed that contrary to the data of others (Reviewed by Dimmock 1984, 1987) IgG neutralization did cause a significant reduction in the ability of the virus to attach to cells, which may depend on the target cell type, the antibody:virus ratio and the ability of antibody to aggregate virus at sub-saturating antibody concentrations.

The aim of this chapter is to investigate the mechanisms of IgG neutralization of influenza virus. Three neutralizing IgG monoclonal antibodies (HC2, HC10 and HD61) directed against the H7 haemagglutinin were selected. All were of the IgG2a sub-class and each was directed against a different non-overlapping antigenic site (personal communication P.Garner, and Dr.A.Hey). I used three cell types. Tracheal organ culture cells were used because these are the natural target for influenza virus and thus the best model with which to study the natural influenza virus infection. The use of such differentiated cells might possibly reveal differences between the in vitro and in vivo mechanisms of neutralization. BHK cells were included as a model of in vitro neutralization. This cell type has been previously used to study the neutralization of influenza virus (Taylor & Dimmock, 1985a, 1985b; Taylor, 1986). An alternative would have been to use CEF cells as these too had been used in earlier studies (Poesse et al., 1982), but CEF cells are a primary cell culture and hence are heterogeneous. The third choice of cell type was the chicken erythrocyte. Influenza virus attachment to these cells can be visualised with the naked eye (i.e. haemagglutination assay), as can the one of the effects of

antibody binding (i.e. haemagglutination inhibition). Also, attachment of influenza virus to these cells does not lead to appreciable internalization of the virus or to productive infection (Cook *et al.*, 1979).

In order to define the components of the neutralization mixture I have used a constant amount of virus throughout in terms of haemagglutination units (HAU) since overall it is the reaction of antibody with all virus particles having surface haemagglutinin protein which is important as well as the reaction with infectious virus. The choice of the virus concentration was influenced by amount of radioactive virus which attached to the cells, especially to tracheal organ culture cells, and the number of virus particles which were needed for electron microscopy. I used 1000 HAU/ml mixed with an equal volume of neutralizing antibody, only occasionally did the incorporation of radiolabel into the virus prove too low for assays to be performed with tracheal organ cultures.

The concentration of antibody used is presented in terms of one of its biological activities i.e. haemagglutination-inhibition units (HIU). The relative amounts of antibody and virus present in the mixture is defined by the ratio, \log_2 HIU:HAU. This method of expressing the antibody concentration was subsequently adopted by others in our laboratory (Taylor *et al.*, 1987). Previously antibody concentrations were reported as dilutions or a total protein concentration which made it impossible to compare one antibody preparation with another. Use of the \log_2 HIU:HAU ratio allows such comparisons to be made.

2.2. Results.

2.2.1. IgG neutralization of adFPV/R infectivity on organ cultures.

Since my aim was to investigate the role of the target cell in neutralization, plaque reduction on tissue culture cell lines could not be taken as proof that neutralization was also occurring on the epithelial surface of tracheal organ cultures. As plaque reduction could not be performed using organ cultures, neutralization was examined by comparing the ability of neutralized and non-neutralized adFPV/R to multiply on tracheal organ cultures.

The results for the HC2 are shown in Figure 9.1.. Two different antibody concentrations were used, the higher concentration neutralized and coated the virus but caused no virus aggregation whilst the lower antibody concentration neutralized the virus and caused considerable aggregation (personal communication, Dr.S.J.Armstrong). The infectivity of the virus was neutralized by more than 99.5% in both cases.

Multiplication was retarded by neutralizing antibody, the neutralized adFPV/R showed negligible multiplication in the organ cultures during the first 12 hours post-infection and was reduced over the initial 24 to 36 hours post-infection compared to the non-neutralized controls. Infectivity of the virus was not totally abolished as maximum yields of adFPV/R were seen with further incubation resulting from secondary cycles of multiplication. The virus produced could still be neutralized by HC2 and did not result from antibody-escape mutations. Limitations in the amounts

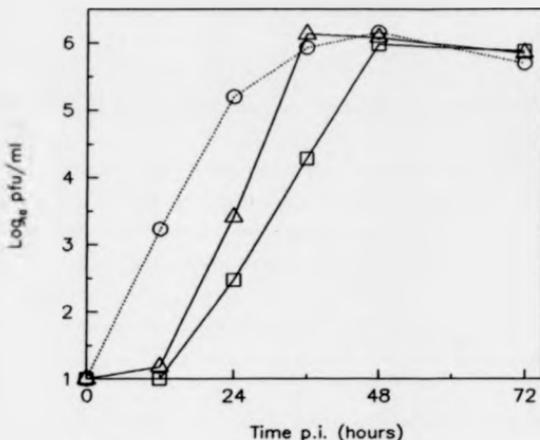


Fig.9.1. Neutralization of adpFPV/R by HC2 and the effect on virus multiplication on tracheal organ cultures.

adpFPV/R was diluted to 1000 HAU/ml before adding an equal volume of non-neutralizing 185/1 (\circ) or HC2 diluted to a Log₁₀ HIU:HAU ratio of +1.0 (\square) or -0.5 (\triangle). The virus plus IgG mixtures were incubated at 25°C for 60 min. 200 μ l of each virus/antibody mixture were added to batches of 18 tracheal organ cultures. After 60 min. at 37°C, the cultures were washed with PBS and seeded as individual cultures into wells of a microtitre tray and 50 μ l of media added. The cultures were incubated at 37°C, and the media was regularly harvested and replaced with fresh media. The infectivity of the pooled media was determined by plaque assay.

of monoclonal antibody available precluded its continued presence in the culture medium. Experiments using HC10 and HC61 (not shown) gave similar results to those for HC2.

9.2.2. Ability of the IgG monoclonal antibodies to neutralize adpFPV/R

In an earlier part of this thesis neutralization of adpFPV/R by HC2, HC10 and HC61 had been determined using a final virus concentration of 1000 pfu/ml (section 7.2.6.). Here a final concentration, after the addition of antibody, of 500 HAU/ml is used, which represents an increase of approximately 10^6 fold in the amount of virus to be neutralized. Therefore the amount of antibody required would also be expected to increase, and this was examined.

The neutralization profiles of each of the monoclones versus the higher adpFPV/R concentration are shown in Figure 9.2.. The amount of antibody used has been expressed as a ratio of antibody to virus, i.e. \log_{10} HIU:HAU, rather than as dilutions, as discussed earlier. The dilutions of antibody required for 50% neutralization are shown in Table 9.1.. These amounts are compared to the amounts required for 50% neutralization of 1000 pfu/ml adpFPV/R (section 7.2.6.). More antibody was required to neutralize 1000 HAU/ml adpFPV/R but the increase in antibody was many orders of magnitude less than the increase in the amount of virus. The efficiency of neutralization was dependent on the amount of virus to be neutralized.

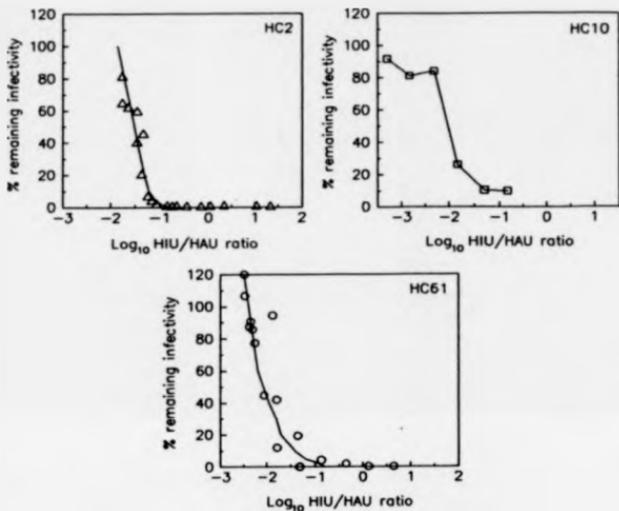


Fig.9.2. Neutralization of adpFPV/R by IgG monoclones.

Dilutions of antibody were added to equal volumes of adpFPV/R (1000 HAU/ml), mixed and incubated for 60 min. at 25°C. The remaining infectivity was determined by plaque assay on CEF cells.

Table 9.1.

The dilutions of IgG required to cause 50% neutralization of two different concentrations of adpFPV/R.

Antibody	versus 1000 HAU/ml		versus 1000 pfu/ml [*]	
	Log ₁₀ HIU:HAU	Dilution (A)	Dilution (B)	(A)/(B)
HC2	-1.5	1/1122	1/12162	10.8
HC10	-2.0	1/1496	1/21878	14.6
HC61	-2.1	1/ 615	1/26303	42.8

* Values previously reported (section 7.2.6.).

9.2.3. Neutralization of adFPV/R by HC2.

The mechanism of neutralization of adFPV/R by HC2 was investigated (section 6.5.4.) using a range of HC2 concentrations. The effects of HC2 on virus aggregation and attachment to the different cell types were examined. The neutralization profile of HC2 has also been included in all the figures to allow a clear comparison of data.

9.2.3.1. Aggregation of adFPV/R caused by HC2.

Dr.S.J.Arstrong kindly performed all the EM procedures on the samples I supplied. The number of virus particles in each of the aggregates was counted and the results are shown in Figure 9.3.

Aggregation of adFPV/R did occur and reached a maximum average aggregate size of 6.3 virus particles at a \log_{10} HIU:HAU ratio of -1.0. The rise in aggregation coincided with the HC2 concentration that caused neutralization. The amount of aggregation fell as the HC2 concentration increased. There was no aggregation (compared to the non-neutralized controls) above a \log_{10} HIU:HAU ratio of +0.5, the virus being monodisperse at this concentration.

9.2.3.2. Reduction in attachment of adFPV/R to organ culture cells caused by HC2.

There was a significant reduction in attachment of adFPV/R to organ culture cells at the same concentration of HC2 required to initiate neutralization (Figure 9.4a). However the size of the reduction in

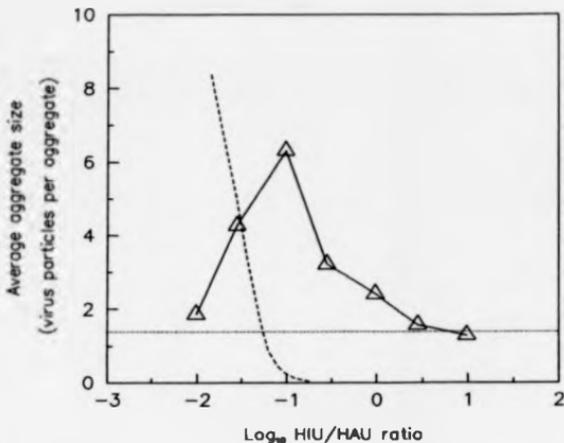


Fig.9.3. Aggregation of adFPV/R due to HC2.

Dilutions of HC2 were added to equal volumes of adFPV/R (1000 HAU/ml), mixed and incubated for 60 min. at 25°C. The samples were then examined by EM and the amount of aggregation determined (---△---) and compared to the non-neutralized adFPV/R controls (-----). Also shown is the Hc2 neutralization profile (.....) from Fig.9.2.

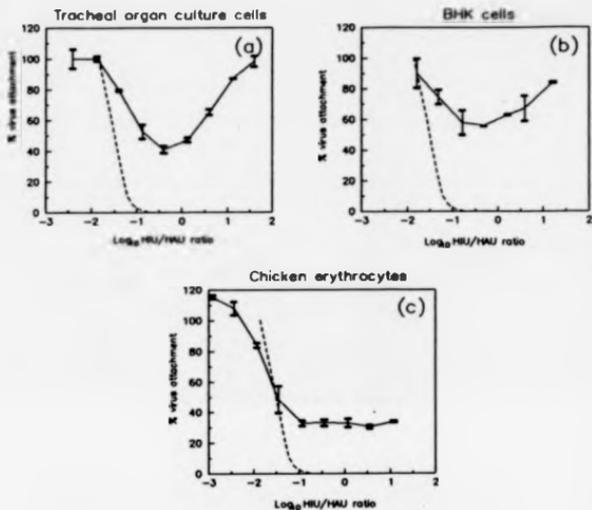


Fig. 9.4. Neutralization of adpFPV/R by HC2 and the effect on attachment to various cells.

Dilutions of HC2 were added to equal volumes of ^{32}P adpFPV/R, mixed and incubated for 60 min. at 25°C . The amounts of virus attaching were determined as previously described (section 6.5.4.). Also shown is the HC2 neutralization profile (-----).

The line is drawn through the average value of two duplicate samples which are shown by the bars. Other attachment data reported in these are presented in a similar manner.

attachment was always less than the reduction in infectivity. The maximum reduction in attachment was 60% whereas infectivity was reduced to 99.9% and this occurred at \log_{10} HIU:HAU ratio of -0.4. As the HC2 concentration was increased the amount of attachment to the organ culture cells also increased so that at a \log_{10} HIU:HAU ratio of +1.0, attachment of adpFPV/R was 100%.

2.2.3.3. Reduction in attachment of adpFPV/R to BHK cells caused by HC2.

Reduction in attachment of HC2-neutralized adpFPV/R to BHK cells was similar to that observed using tracheal organ culture cells, except the magnitude of the reduction was smaller (Figure 9.4b). The maximum reduction in attachment was 45% and occurred at a \log_{10} HIU:HAU ratio of -0.3. Attachment returned to a higher level (82%) at a \log_{10} HIU:HAU ratio of greater than +1.0.

2.2.3.4. Reduction in attachment of adpFPV/R to chicken erythrocytes caused by HC2.

Reduction in attachment of HC2-neutralized adpFPV/R to chicken erythrocytes paralleled the loss of infectivity down to 30% (Figure 9.4c). In contrast to tracheal organ cultures and BHK cells, there was no return to a normal level of attachment even at a \log_{10} HIU:HAU ratio of greater than +1.0, and there was no further decrease in attachment.

9.2.4. Neutralization of adFPV/R by HC10.

The mechanism of neutralization of adFPV/R by HC10 was investigated with the protocol used for HC2. The HC10 neutralization profile is also shown in all relevant figures.

9.2.4.1. Aggregation of adFPV/R caused by HC10.

The maximum average aggregate size was 4.5 virus particles and occurred at \log_{10} HIU:HAU ratio of -2.3 (Figure 9.5.). The rise in aggregation occurred slightly before the HC10 concentration that caused neutralization. The amount of aggregation fell as the HC10 concentration was further increased. Above a \log_{10} HIU:HAU ratio of -1.0 no aggregation of the virus was found.

9.2.4.2. Reduction in attachment of adFPV/R to organ culture cells caused by HC10.

Reduction in attachment occurred at the same concentration of HC10 required to initiate neutralization of the virus (Figure 9.6a). As with HC2, the size of the reduction in attachment was always less than the reduction in infectivity. The maximum inhibition of attachment was 50% and this occurred at a \log_{10} HIU:HAU ratio of -1.2. The amount of HC10-neutralized virus able to attach to the organ culture cells increased when the amount of antibody was also increased. Attachment returned to 95% (compared to the non-neutralized controls) above a \log_{10} HIU:HAU ratio of 0.0.

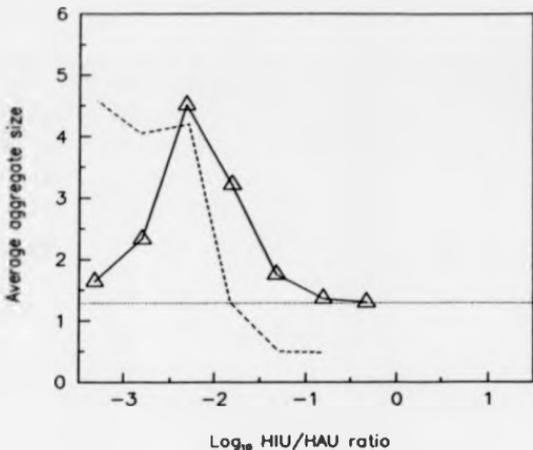


Fig.9.5. Aggregation of adpFPV/R due to HC10.

Dilutions of HC10 were added to equal volumes of adpFPV/R (1000 HAU/ml), mixed and incubated for 60 min. at 25°C. The samples were then examined by EM and the degree of aggregation determined (\triangle) and compared to the non-neutralized adpFPV/R controls (.....). Also shown is the HC10 neutralization profile (-----) from Fig.9.2.

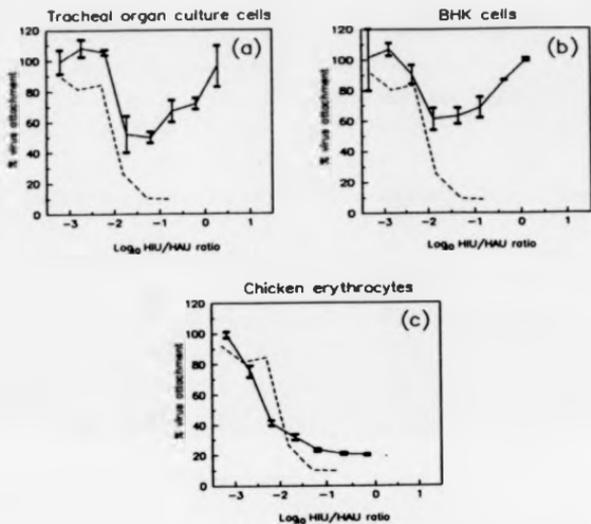


Fig.9.6. Neutralization of adpFPV/R by HC10 and the effect on attachment to various cells.

Dilutions of HC10 were added to equal volumes of ^{32}P adpFPV/R, mixed and incubated for 60 min. at 25°C . The amounts of virus attaching were determined as previously described (section 6.5.4.). Also shown is the HC10 neutralization profile (-----).

2.2.4.3. Reduction in attachment of adpFPV/R to BHK cells caused by HC10.

HC10 reduced attachment of adpFPV/R to BHK cells (Figure 9.6b). Maximum inhibition of attachment was 40% and occurred at a \log_{10} HIU:HAU ratio of -1.5. Neutralized adpFPV/R attached to a similar extent as the non-neutralized controls (i.e. 100%) at a value greater than \log_{10} HIU:HAU -0.5.

2.2.4.4. Reduction in attachment of adpFPV/R to chicken erythrocytes caused by HC10.

A reduction in attachment of HC10-neutralized adpFPV/R to chicken erythrocytes was found (Figure 9.6c). Maximum inhibition was 60% of the non-neutralized controls. As with HC2, no return to normal amounts of attachment was observed at high HC10 concentrations and the amount of attachment remained at 20%.

2.2.5. Neutralization of adpFPV/R by HC61.

The availability of HC61 was limited and three different preparations were used, each had a different undiluted activity, i.e. 4,898; 31,700 and 141,250 HIU/ml. The dilution at which the first preparation (4,898 HIU/ml) caused 50% neutralization is shown in Table 9.1., the other two preparations caused neutralization at proportionally greater dilutions. The use of a relative concentration (\log_{10} HIU:HAU ratio), in preference to a simple dilution, allowed the results from the three preparations to be combined.

The HC61 neutralization profile is in all relevant figures.

9.2.5.1. Aggregation of adpFPV/R caused by HC61.

Figure 9.7. shows that aggregation of adpFPV/R by HC61 did occur. A maximum aggregate size was difficult to deduce because one HC61 preparation caused more aggregation than the other two preparations. The rise in aggregation occurred at a higher antibody concentration than neutralization, and reasons for this are discussed later. The range of HC61 concentrations that caused aggregation of adpFPV/R was greater than that seen for either HC2 or HC10. The amount of aggregation fell gradually as the HC61 concentration was increased. No aggregation was observed at above $\log_{10}\text{HIU:HAU} +1.0$.

9.2.5.2. Reduction in attachment of adpFPV/R to organ culture cells caused by HC61.

Attachment of HC61-neutralized adpFPV/R to organ culture cells was reduced and occurred at exactly the same concentrations required for neutralization (Figure 9.8a). Maximum inhibition of attachment was 70% of the control value and occurred at a $\log_{10}\text{HIU:HAU}$ ratio of -2.0 . Attachment of neutralized adpFPV/R remained low until the $\log_{10}\text{HIU:HAU}$ ratio reached $+0.6$. The amount of neutralized adpFPV/R able to attach increased to 90% as the HC61 concentration increased to $\log_{10}\text{HIU:HAU} +1.5$.

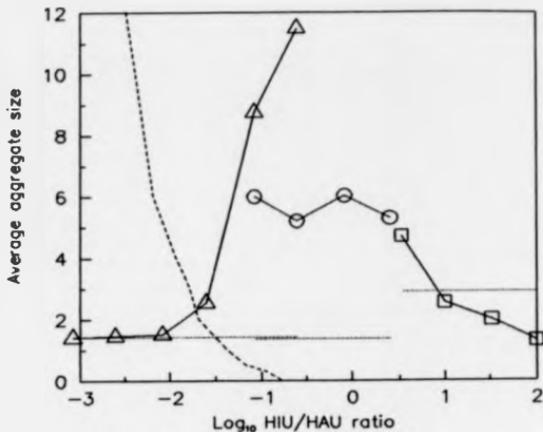


Fig.9.7. Aggregation of adpFPV/R due to HC61.

Dilutions of HC61 were added to equal volumes of adpFPV/R (1000 HAU/ml), mixed and incubated for 60 min. at 25 C. The samples were then examined by EM and the amount of aggregation determined (—) and compared to the non-neutralized adpFPV/R controls (----). Also shown is the HC61 neutralization profile (-----) from Fig.9.2.

The data from 3 separate experiments are shown (○, △, □) each with its respective control.

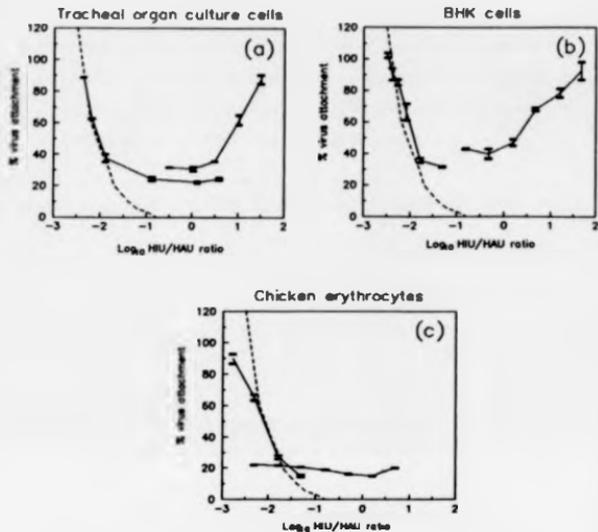


Fig.9.8. Neutralization of adpFPV/R by HC61 and the effect on attachment to various cells.

Dilutions of HC61 were added to equal volumes of ^{32}P adpFPV/R, mixed and incubated for 60 min. at 25°C . The amounts of virus attaching were determined as previously described (section 6.5.4.). Also shown is the HC61 neutralization profile (-----).

9.2.5.3. Reduction in attachment of adFPV/R to BHK cells caused by HC61.

Attachment of HC61-neutralized adFPV/R to BHK cells (Figure 9.8b) was similar to that previously found using organ culture cells. The maximum inhibition of attachment was 65% and occurred at a \log_{10} HIU:HAU ratio of -2.0. Attachment of neutralized virus at a \log_{10} HIU:HAU ratio of greater than +1.5 was 90% compared to the non-neutralized controls.

9.2.5.4. Reduction in attachment of adFPV/R to chicken erythrocytes caused by HC61.

HC61-neutralized adFPV/R attached poorly (80% inhibition) to chicken erythrocytes (Figure 9.8c), and as with HC2 and HC10, there was no increase in attachment at high HC61 HIU:HAU ratios.

9.2.6. Removal of cell receptors for adFPV/R by neuraminidase and the effect on attachment.

The above experiments confirmed that the attachment of IgG-neutralized adFPV/R to BHK and organ culture cells was unaffected at high IgG concentrations. In these situations it is possible that the neutralized virus attached to cells via a different cell receptor from that used by non-neutralized virus, as occurs with vesicular stomatitis virus (Schlegel & Wade, 1983). Antibody bound to neutralized virus may enable the virus to attach to the cells via Fc cell receptors as discussed in section 5.3. To test this the cells were pre-treated with neuraminidase before the addition

of virus in order to remove the sialic acid cell receptors. Attachment of virus via an Fc cell receptor would not be affected by neuraminidase treatment.

The results (Table 9.2.) show that pre-treatment of each of the three cell types with neuraminidase reduced the amount of virus able to attach. The attachment of infectious virus to cells was reduced showing that the neuraminidase did remove the sialic acid cell receptors. The attachment of IgG-neutralized virus was reduced by a similar amount demonstrating that the attachment of IgG-neutralized virus was also via a neuraminidase-sensitive cell receptor.

9.2.7. Internalization of IgG neutralized edoFPV/R by cells.

IgG-neutralized virus is able to attach to cells although the amount attaching is dependant on the HIU:HAU ratio. Internalization of attached neutralized virus was determined by treating the cells with neuraminidase at different times post-inoculation (section 6.5.5.). Virus remaining on the cell surface would remain sensitive to removal by neuraminidase, whereas virus being internalized would become progressively more resistant to removal by neuraminidase.

HC2 and HC61 were used, Table 9.3. shows that there was the same increase in neuraminidase resistance of neutralized and non-neutralized virus between 5 min. and 60 min. post-inoculation on either BHK or organ culture cells.

Table 9.2. Effect of neuraminidase pre-treatment on attachment of adFPV/R.

Inoculum ^a	Cell Type	Amount of ³² P- <i>adFPV/R</i> attached to:		Reduction in attachment due to neuraminidase treatment (%)
		Untreated cells	Neuraminidase treated cells ^b	
185/1 ^d	O.C.	2.30 x 10 ⁻⁶	0.51 x 10 ⁻⁶	78.0
	BHK	4.99 x 10 ⁻⁸	1.83 x 10 ⁻⁸	63.2
	cRBC	9.33 x 10 ⁻⁸	3.66 x 10 ⁻⁸	60.8
HC2 [Log H/H +0.55]	O.C.	1.24 x 10 ⁻⁶	0.54 x 10 ⁻⁶	56.0
	BHK	3.31 x 10 ⁻⁸	1.13 x 10 ⁻⁸	65.8
	cRBC	2.38 x 10 ⁻⁸	0.83 x 10 ⁻⁸	65.0
HC10 [Log H/H +0.17]	O.C.	ND	ND	ND
	BHK	2.92 x 10 ⁻⁶	1.03 x 10 ⁻⁶	64.7
	cRBC	2.41 x 10 ⁻⁸	0.92 x 10 ⁻⁸	61.8
HC61 [Log H/H -0.31]	O.C.	0.57 x 10 ⁻⁶	0.16 x 10 ⁻⁶	72.6
	BHK	2.28 x 10 ⁻⁶	0.86 x 10 ⁻⁶	62.5
	cRBC	1.94 x 10 ⁻⁸	0.74 x 10 ⁻⁸	62.0

a. 1000 HAU/ml³²P-*adFPV/R* was incubated with an equal volume of antibody for 60 minutes at 25°C.

b. Expressed in terms of HAU per cell.

c. Cells pretreated with 0.5U neuraminidase for

60 minutes at 37°C (section 6.5.5).

d. 185/1 is an irrelevant non-neutralizing monoclonal IgG.

ND: Not done.

Table 9.3. Internalization of IgG-neutralized adFPV/R by cells.

Monoclonal	Log ₁₀ HIU/HAU	Cell type	% attachment ^a	Neuraminidase resistance (%)	
				5' p.i.	60' p.i.
185/1 ^d	N/A	O.C.	100	33.3	67.3
HC2	+0.65	O.C.	36	42.9	97.9
185/1	N/A	BHK	100	26.1	86.5
HC2	+0.85	BHK	80	32.0	128.9
HC2	-0.45	BHK	46	29.2	92.3
185/1	N/A	O.C.	100	38.9	68.1
HC61	-0.26	O.C.	50	45.1	79.0
185/1	N/A	BHK	100	35.0	64.0
HC61	+0.70/+0.39 ^e	BHK	62	35.0	99.1
HC61	-0.30/-0.61 ^e	BHK	41	35.0	124.2

a. Compared to the non-neutralized controls at 60 min. p.i.

b. Cells treated with neuraminidase for 60 min. at 37°C (section 6.5.5.).

c. Relative increase in neuraminidase resistance; value at 60 min. p.i. divided by the 5 min. p.i. value. No increase (i.e. value of 1.0)

would indicate that no virus had been internalized.

d. Control samples treated with the irrelevant monoclonal antibody, 185/1.

e. Due to a dilution error the ratio differs between the 5 min. p.i. sample and the 60 min. p.i. sample.

Two different IgG concentrations were used when studying internalization by BHK cells, the higher concentration causing little reduction in the attachment of virus and no aggregation, whilst the lower caused considerable aggregation and a large reduction in the attachment of virus. The results demonstrate that antibody concentration used to neutralize the virus did not affect the internalization of virus that attached to these cells.

A similar set of experiments were performed using chicken erythrocytes (not shown). Less than 10% of the attached neutralized and infectious virus was neuraminidase resistant at either 5 min. or 60 min. post-inoculation, indicating that minimal internalization of the attached virus took place in these cells.

2.3. Discussion.

In this chapter I have investigated three mechanisms by which IgG antibodies could cause neutralization of influenza virus :- aggregation of the virus, inhibition of virus attachment to cells and prevention of virus entry into cells. In this discussion I shall examine the data for each of these stages separately before combining them into a more complete view of the neutralization process.

2.3.1. IgG-mediated aggregation of adnFPV/R.

If it is assumed that each aggregate represents a single infectious virus unit then aggregation of the virus reduces the number of infectious units within a virus population.

The amount of aggregation caused by the binding of IgG molecules to influenza virus haemagglutinin was highly dependent on the antibody concentration:virus concentration ratio and this confirms the findings of Taylor *et al.* (1987). My data are summarized in Table 9.4. For HC2 a similar \log_{10} HIU:HAU ratio of -1.5 caused both the initial loss of infectivity (50% neutralization) and the initial rise in aggregation (half-maximal average aggregate size).

The \log_{10} HIU:HAU ratio for HC61 causing the initial rise in aggregation (-1.3) was higher than that required for neutralization (i.e. \log_{10} HIU:HAU -2.1). The fact that neutralization occurred at a HC61 concentration which did not cause appreciable aggregation of the virus suggests that aggregation contributed little to the overall process of neutralization.

The opposite situation was found with HC10 where the initial rise in aggregation (i.e. \log_{10} HIU:HAU -2.6) preceded neutralization (i.e. \log_{10} HIU:HAU -2.0). This was surprising because the infectivity would be expected to diminish in proportion to the aggregate size. However more aggregation may occur on the EM grid than in solution as has been previously suggested by Taylor *et al.* (1987) or aggregates may be dispersed in solution by pipetting and vortexing. Obviously the specificity of the monoclonal antibody as well as the HIU:HAU ratio is a contributory factor to aggregation. This suggestion is supported by Wrigley *et al.* (1983) who demonstrated that the position of the antigenic site on the haemagglutinin affected the formation of aggregates of bromelain treated haemagglutinin in

Table 9.4. Summary of aggregation of adpFPV/R by IgG monoclonal antibodies.

	Antibody ratio (log ₁₀ IU:HAU)		
	HC2	HC10	HC61
50% neutralization ^a	-1.5	-2.0	-2.1
Half-maximal aggregation ^b	-1.6	-2.6	-1.3
Maximum aggregation ^b	-1.0	-2.3	-0.6
Return to monodispersion ^b	+0.4	-0.8	+1.0
Max. average aggregate size ^b	6.3	4.5	11.3 <-> 6.0
Maximum potential neutralization due to aggregation ^c	79%	71%	89% <-> 78%

a. Data from Table 9.1.

b. Data from Figures 9.3., 9.5. and 9.7. respectively.

c. If it is assumed that each aggregate represents a single infectious unit then aggregation of the virus reduces the number of infectious units within a virus population. The maximum potential neutralization due to aggregation can be calculated from the maximum average aggregate size e.g.

For HC2:

- ∴ infectious (control) virus aggregate size = 1.3 particles/aggregate
- ∴ per 100 virions there are 100/1.3 aggregates
- ∴ theoretical infectivity = 76.92

- For HC2 neutralized virus max. aggregate size = 6.3 particles/aggregate
- ∴ per 100 virions there are 100/6.3 aggregates
- ∴ theoretical infectivity = 15.87

- ∴ remaining infectivity = 15.87/76.92 X 100 = 20.63%
- ∴ neutralization = 79.4%

suspension by monoclonal IgG antibodies. All the monoclonal antibodies used in this chapter were IgG2a and hence we are not considering variations in subclass properties.

All the monoclonal IgG antibodies caused a considerable amount of aggregation over a wide range of antibody concentrations. From my data the maximum potential loss of infectivity due to aggregation is 70-90% (Table 9.4.), but this never fully accounts for the observed neutralization of greater than 99%.

Increasing the antibody concentration beyond that required for maximal aggregation resulted in a decrease in the amount of aggregation until eventually the virus was monodisperse, whilst there was no return in infectivity of the virus. Due to limitations in the availability of the monoclonal antibodies I could not assess the amount of antibody required for saturation of adFPV/R. Taylor *et al.* (1987) demonstrated that saturation of A/FPV/R by either HC2, HC10 or HC61 labelled with ¹²⁵I, occurred at a constant log₁₀ HIU:HAU ratio of +0.9. Since the adFPV/R strain is derived from and is still antigenically similar to the A/FPV/R strain (section 7.2.6.), it is likely that both viruses would be saturated by the same concentration of IgG. On the basis of this assumption, my data show that IgG saturation of the virus did not always coincide with the antibody concentration at which aggregation was no longer apparent (Table 9.4.). There was good agreement with HC61 and reasonable agreement with HC2 but there was a 50-fold difference with HC10. The reasons for this are not clear but again possibly may reflect the differences between the antigenic sites to which the antibodies bind (Wrigley *et al.*, 1983).

9.3.2. IgG-mediated reduction in attachment of adpFPV/R to cells.

A second possible mechanism by which IgG antibodies could neutralize influenza virus is by inhibiting its attachment to cells. A reduction in the capacity of the virus to attach would result in a proportional loss of virus infectivity. The infectivity assays (section 9.2.2.) were performed using CEF cells but parallel assays using BHK cells instead gave identical neutralization profiles (results not shown). The adpFPV/R virus could be neutralized by each of the monoclonals to an equal extent on either cell type. All three IgG monoclonals inhibited attachment of adpFPV/R to each of the cell types studied and the data are summarized in Table 9.5. In each case the initial inhibition of attachment (i.e. half-maximal) occurred at the same antibody concentration as that required for the initial loss of infectivity (i.e. 50% neutralization). This would suggest that at low IgG concentrations, the inhibition of attachment may be responsible for the loss of infectivity except for HC2 where at no point did the magnitude of such inhibition fully account for the amount of neutralization observed.

The magnitude of the reduction in attachment was dependent on the cell type used. For each antibody the largest reduction was found using chicken erythrocytes, intermediate reductions with tracheal organ culture cells and the least with BHK cells. The specificity of the antibody also influenced the size of the reduction: with tracheal organ culture or BHK cells HC61 caused more inhibition of attachment than either HC2 or HC10. All three antibodies caused similar reductions in attachment of neutralized adpFPV/R to chicken erythrocytes.

Table 9.5. Summary of IgG inhibition of oadFPV/R attachment to various cell types.

		Antibody ratio (log ₁₀ HIU:HAU):			
		HC2	HC10	HC61	
50% neutralization		-1.5	-2.0	-2.1	
Tracheal organ culture cells	Initial inhibition of attachment	-1.2	-1.9	-2.1	
	Maximum inhibition of attachment	-0.4 (40%)*	-1.7 (50%)	-0.8 (25%)	
	Return to 80%+ attachment	+1.1	+0.3	+1.5	
BHK cells	Initial inhibition of attachment	-1.4	-2.1	-2.0	
	Maximum inhibition of attachment	-0.3 (55%)	-1.8 (60%)	-1.3 (30%)	
	Return to 80%+ attachment	+1.2	-0.3	+1.7	
Chicken erythrocytes	Initial inhibition of attachment	-1.7	-2.4	-2.2	
	Maximum inhibition of attachment	-0.8 (30%)	-1.2 (20%)	-1.3 (20%)	

* In parenthesis is the percentage of virus which attached compared to the non-neutralized controls.

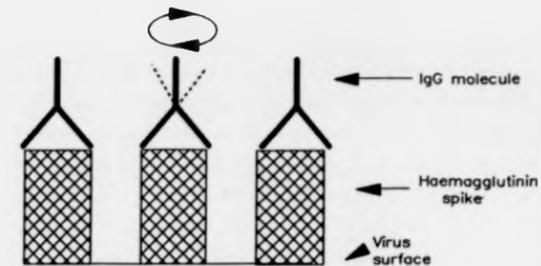
When BHK cells were used, the attachment of virus returned towards 100% as the antibody concentration was increased whilst there was no return of virus infectivity. Earlier work had shown that IgG-neutralized A/FPV/R attached normally to CEF cells (Pomies *et al.*, 1982), mouse L929, human foreskin and BHK cells (Taylor & Dismock, 1985a; Taylor, 1986). None of these previous studies varied the concentration of IgG used to neutralize the virus and only high or saturating concentrations of neutralizing antibody were used. These workers implied on the basis of these results that the attachment of IgG-neutralized influenza virus to cultured cells was unaffected regardless of antibody concentration. My data confirm their findings that with high or saturating IgG concentrations normal amounts of influenza virus attached to BHK cells, but show that with sub-saturating IgG concentrations this is not the case.

The attachment of IgG-neutralized adpFPV/R to tracheal organ culture cells and BHK cells proved to be similar and attachment returned towards 100% as the antibody concentration was increased. It would therefore appear that BHK cells are a good model of the *in vivo* situation but the amount of IgG-neutralized virus attaching to tracheal organ culture cells was always lower (by roughly 20%) than that attaching to BHK cells.

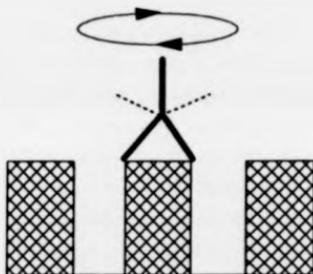
At high IgG concentrations, where maximum amounts of attachment occurred, it was possible that the IgG-neutralized virus was attaching to the cells via an Fc receptor and not via sialic acid as used by infectious virus. My data show that this was not the case because removal of sialic acid from cells using neuraminidase affected equally the attachment of both infectious and neutralized virus (Table 9.2.). Hence IgG antibody does not necessarily block attachment of virus to the receptor.

The question arises as how virus that is coated or saturated with IgG molecules attach in normal amounts to cells?. The receptor binding pocket on the haemagglutinin does not constitute an antigenic site for antibody binding (Wiley *et al.*, 1981; Weis *et al.*, 1988). It was proposed by Dimmock (1987) that the antibody 'footprint' did not obscure the receptor binding pocket and that if the cell receptors are long enough to penetrate the fringe of IgG molecules bound to the virus then normal attachment could occur. On the other-hand, Weis *et al.* (1988) states that the binding of antibody would probably obscure the receptor binding pocket and that neutralization would result from an inhibition of virus attachment to cells. My data clearly demonstrate that this is not the case and support the view of Dimmock.

It is strange that virus with many IgG molecules bound attached to cells better than virus with only a few IgG molecules bound. Attached IgG antibodies are able to undergo axial rotation and flexing (Burton, 1986; Feinstein *et al.*, 1986; Rumphrey, 1986) and this may provide a possible explanation. It is conceivable that when only a few antibody molecules are bound then such molecules will have space to flex and rotate (Figure 9.9.). This movement will impede formation of the haemagglutinin-sialic acid linkage and lead to an overall lower level of virus attachment. When many antibody molecules are bound to the virus then the antibody molecules are more tightly packed and their movement and flexing will be reduced. Virus-cell attachment will not be impeded and greater amounts of neutralized virus will attach to the cells.



Many IgG molecules bound to virus particles



Few IgG molecules bound to virus particles

Fig.9.9.

Diagram of IgG binding to HA spikes of influenza particles and the limitations placed on antibody movement and flexing at high/saturating conditions.

Tracheal organ cultures have been previously used as a model on which to study neutralization. It was shown that neutralization of avian infectious bronchitis virus (IBV) by IgG protected chick embryo tracheal organ cultures from ciliostasis (Cherry & Taylor-Robinson, 1970; Darbyshire *et al.*, 1979). My results (Figure 9.1.) show that IgG neutralization of adpFPV/R occurred on mouse tracheal organ cultures and was apparent as a reduction in the amount of virus multiplication during the initial 24-36 hours post-infection.

Chicken erythrocytes are a convenient but inappropriate cell type on which to study the infectious pathway of influenza virus because the attachment of virus to these cells does not lead to a productive infection although viral proteins are synthesized by the cells in small amounts (Cook *et al.*, 1979; Dimmock *et al.*, 1981). Attachment of neutralized adpFPV/R to chicken erythrocytes differed from that to either BHK or tracheal organ culture cells as the amount attaching remained low even when high antibody concentrations were used. This confirms the preliminary findings of Pomesse (1981) and would further support the view that chicken erythrocytes are a poor model on which to study the infectious route of influenza virus. The sialic acid-bearing cell receptor for influenza virus on chicken erythrocytes is probably glycoporphin. This extends 5nm from the erythrocyte cell surface (Viitala & Jernsfelt, 1985) and Dimmock (1987) proposed that this was not long enough to penetrate the layer of IgG coating the virus particles and therefore the attachment of influenza virus to erythrocytes is sterically blocked by the IgG molecules bound to the virus haemagglutinin.

2.3.3. Internalization of IgG-neutralized virus.

Resistance to removal by neuraminidase (*Clostridium perfringens* V) has been used by others to demonstrate the internalization of influenza virus by cells (Richman *et al.*, 1986; Matlin *et al.*, 1981). The virus that is attached to the external surface of the cell can be removed by neuraminidase whereas internalized virus cannot as it is no longer available to the action of neuraminidase. Therefore internalization is measured by an increase in neuraminidase resistance with respect to time.

Table 9.3. shows that at 5 min. post-infection a mean of 33% of the attached infectious virus was resistant to neuraminidase. Probably some internalization has already occurred because on chicken erythrocytes, where little or no internalization occurs, less than 10% of the virus is resistant to neuraminidase. When the virus was incubated with cells for 60 min. the proportion resistant to neuraminidase increased to over 64% indicating that the attached virus was being internalized.

The effect of IgG-neutralization on the internalization process is also shown in Table 9.3. Both HC2 and HC61 reduced the amount of virus attaching to BHK and tracheal organ culture cells. Neutralization by either HC2 or HC61 did not prevent the internalization of virus that had attached to either cell type. In all cases the increase in neuraminidase resistance observed was at least as large, and frequently greater, than that found using infectious virus. This suggests that neutralization by IgG antibodies does not prevent internalization of the virus that has attached to cells and may, in some cases (e.g. BHK cells), improve internalization. The data

also show that while antibody had the capacity to aggregate virus neither the concentration of antibody nor the state of aggregation affected internalization of the virus that had attached to BHK cells.

IgG-neutralization of A/FPV/R does not affect the amount of virion RNA accumulating in the nucleus nor the rate at which it does so in CEF cells (Pomese et al., 1982) and BHK cells (Taylor & Dismock, 1985a; Taylor, 1986). Therefore by implication, internalization of the neutralized virus must have occurred. Taylor (1986) also demonstrated that neutralization of A/FPV/R with saturating amounts of IgG did not prevent internalization by BHK cells when measured as the increase in resistance to removal of virus by proteinase K. The data in this thesis have advanced this concept by suggesting that (i) IgG-neutralized influenza virus is internalized by tracheal epithelial cells and (ii) IgG-neutralized virus is internalized regardless of whether it is neutralized by high or low concentrations of antibody.

With chicken erythrocytes the majority (more than 90%) of the attached infectious adpFPV/R was removed by neuraminidase treatment at both 5 min. and 60 min. post-infection. Very small amounts of virus appeared to be internalized but too little radioactivity remained after neuraminidase treatment to allow accurate assessment (data not shown). It has been previously shown that small amounts of influenza virus are internalized and viral proteins are synthesised by chicken erythrocytes but no productive infection results (Cook et al., 1979; Dismock et al., 1981) and my findings support this.

2.3.4. How is influenza virus neutralized by IgG?

When high IgG concentrations were used the virus was not aggregated and attachment to BHK or tracheal organ culture cells was not inhibited. The IgG-neutralized virus that attached to cells did so via sialic acid receptors and was successfully internalized by the cells. The infectivity of the virus was neutralized by more than 99.9% at these high IgG concentrations and therefore, under these conditions, the mechanism of IgG neutralization involved neither aggregation, inhibition in the attachment to cells nor inhibition of internalization of attached virus by cells. Neutralization must therefore occur at a later stage of the infectious pathway and this is reviewed in detail in the general introduction to this thesis.

I have shown in this chapter that at low, sub-saturating, IgG concentrations neutralized virus was aggregated and its attachment to cells was impaired. This was seen with each of the monoclonal IgG antibodies, irrespective of the antigenic site to which they are directed, and with both tracheal organ culture and BHK cells.

IgG-mediated aggregation and inhibition of attachment of influenza virus to cells will result in some loss of infectivity but if virus is neutralized when neither of these occurs, then the question arises as to whether aggregation or reduced attachment ever play any major role in neutralization?

I would argue as follows, on the basis of my findings and those of others, that aggregation of the virus and the inhibition of virus attachment, either separately or in combination, are important in the overall process of neutralization.

(i) If the binding of a single IgG molecule to the virus resulted in neutralization then it could be argued that only virus already neutralized was aggregated and failed to attach.

Taylor *et al.* (1987) demonstrated that at the concentration of antibody required for 50% neutralization of A/FPV/R there were approximately 50 IgG molecules bound per virion. Therefore the binding of a single IgG molecule did not automatically result in neutralization. To reconcile this finding with the single (or limited) hit kinetics of neutralization they proposed that there were neutralization 'relevant' and 'irrelevant' haemagglutinin spikes on the virus surface. The binding of antibody to a 'relevant' spike led to a loss of infectivity whereas binding to an 'irrelevant' spike did not. They assumed, on the basis of earlier work, that neutralization resulted solely from an impairment of a post-internalization stage of the infectious pathway, involving a specific communicative interaction between the 'relevant' haemagglutinin spike and the virus core, possibly involving the M2 protein. They concluded that there were 20-40 neutralization 'relevant' haemagglutinin spikes per virion. Unfortunately these workers failed to account for any possible loss of infectivity resulting from either aggregation or inhibition of virus attachment, even though they did show that aggregation did occur at the point of 50% neutralization.

At high IgG concentrations my data would support the proposal that neutralization occurred post-internalization but at lower IgG concentrations both aggregation and inhibition of attachment could greatly enhance neutralization. It is unlikely that aggregation and inhibition of attachment would require a specific interaction between the haemagglutinin and the virus core. Therefore some loss of infectivity would be expected to result from the binding of antibody to the virus irrespective of whether it was bound to a 'relevant' or 'irrelevant' spike. From my data, the loss of adpFPV/R infectivity due to aggregation and inhibition of attachment together could be calculated (Figure 9.10.). For HC61 the calculated loss of infectivity very closely paralleled the actual loss of infectivity down to 95% neutralization. For HC2 the calculated loss paralleled the actual loss of infectivity to approximately 85% neutralization although there was a small difference between the antibody ratios. The problems with the aggregation data for HC10 (discussed above) resulted in a substantial difference between the calculated and actual losses of infectivity. As previously discussed, increasing the IgG concentration eventually resulted in a decrease in the amount of both aggregation and inhibition of attachment. At these higher concentrations more antibody molecules are bound per virion and therefore the chances of an antibody binding to a neutralization 'relevant' spike is increased, the post-internalization mechanism of neutralization then becomes more probable and more significant.

- (ii) If neutralization resulted solely from the binding of IgG to 'relevant' haemagglutinin spikes, then the efficiency of neutralization (i.e. the amount of antibody required to neutralize a given amount of virus) should be largely independent of virus concentration. Increasing the

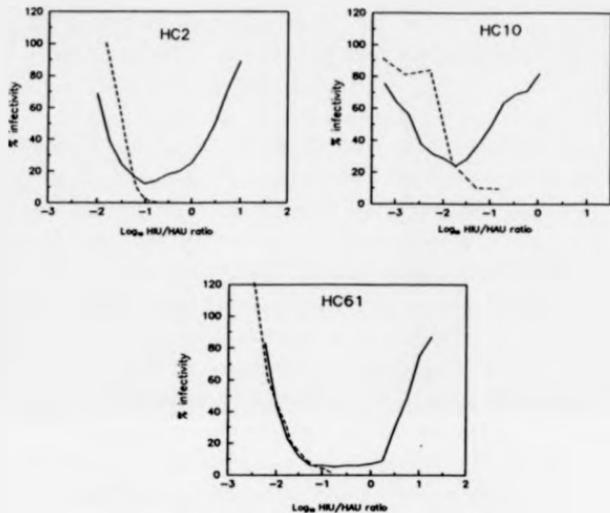


Fig.9.10. Calculated loss of adpFPV/R infectivity due to the combined effects of IgG-mediated aggregation and inhibition of attachment to tracheal organ culture cells. Data from:
 HC2; Fig.9.3. and Fig.9.4a.
 HC10; Fig.9.5. and Fig.9.6a.
 HC61; Fig.9.7. and Fig.9.8a.
 (—) calculated loss of infectivity
 (---) actual loss of infectivity

concentration of virus would not increase the proportion of neutralization 'relevant' spikes per virion for a given virus preparation. At 50% neutralization only 50 of the 1000 haemagglutinin spikes (or 3000 antigen sites) have antibody bound (Taylor *et al.*, 1987) and therefore, as the antigen is in vast excess, an increase in virus concentration will have little effect on the antibody binding.

Table 9.1. demonstrates that the efficiency of IgG neutralization, in terms of the number virions neutralized per molecules of IgG, of adpFPV/R was highly dependent on the concentration of virus to be neutralized. An increase of more than 100,000 fold in the virus concentration required only an increase of 10-50 fold in the antibody concentration to effect the same degree of neutralization. The dependence of neutralization on the virus concentration could be partly explained by introducing the concept of virus aggregation as one of the causes of neutralization. An increase in virus concentration would facilitate aggregation of the virus by reducing the average distance between virions in solution. Aggregation would also reduce the average number of IgG molecules required for neutralization because antibodies will be able to bind and cross-link two virions per IgG molecule. This cross-linking of two virions by a single IgG molecule would improve the probability of an IgG binding for to a neutralization 'relevant' haemagglutinin spike.

The dependence of the efficiency of neutralization on virus concentration means that comparisons of data carried out on widely disparate virus concentrations should be treated with caution e.g. Taylor *et al.* (1987) used 13,000 pfu (less than 0.1 HAU) when studying the neutralization of A/FPV/R but used 20 HAU when studying the attachment of antibody to the

virus and 200 HAU for virus aggregation. All my data on the neutralization, attachment and internalization of adpFPV/R has been obtained using a constant virus concentration i.e. a final inoculum concentration of 500 HAU/ml.

(iii) The contribution of additional mechanisms of neutralization at sub-saturating IgG concentrations can be seen in the data of Taylor et al. (1987). They found the neutralization of A/FPV/R by HClO was more efficient at low, sub-saturating IgG concentrations, less than 200 IgG molecules bound per virion, than at high IgG concentrations, more than 300 IgG molecules bound per virion (data reproduced in Figure 9.11.). The binding of the first 200 IgG molecules to virions reduced the infectivity by over 60-fold whereas the binding of a further 200 IgG molecules only reduced the infectivity by less than 3-fold.

I would argue that the improved efficiency of neutralization at sub-saturating IgG concentrations was the result of enhancement by aggregation and inhibition of attachment. Although aggregation and inhibition of attachment may be important factors under experimental conditions used it is difficult to assess their importance in vivo as the relative concentrations of antibody and virus are not known.

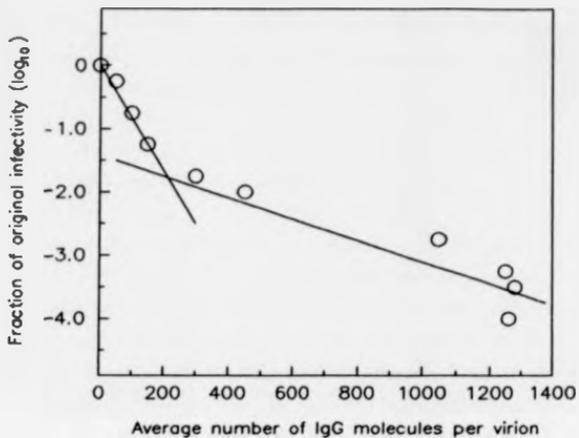


Fig.9.11. Reproduced from Taylor *et al.*, 1987.

Reduction in the infectivity of 2×10^7 pfu A/FPV/R by HC10 IgG correlated with the number of IgG molecules binding per A/FPV/R particle.

9.3.5. Summary.

The results presented in this chapter are based on the use of constant concentration of virus which allows clear comparison of neutralization by IgG and the effects on virus aggregation, attachment and internalization. The relative concentration of antibody to virus was shown to be crucially important in both the aggregation and inhibition of attachment. Markedly different situations were found between high, saturating IgG concentrations and lower, sub-saturating concentrations.

At high IgG concentrations the virus was monodisperse, attached to BHK and tracheal organ culture cells in similar amounts to infectious virus and was internalized. At lower IgG concentrations each of the IgG monoclones aggregated the virus and inhibited its attachment to cells but did not affect the internalization of the virus that attached to BHK or tracheal organ culture cells. The specificity of the antibody influenced the magnitude of these effects, HC61 caused the greatest aggregation and inhibition of attachment whereas HC10 caused the least. The cell type also affected the attachment of neutralized virus and the probable in vivo situation, modelled using tracheal organ culture cells, was similar to that found using cultured BHK cells rather than chicken erythrocytes.

RESULTS 4.
Neutralization of influenza
virus by IgM.

10.1. Introduction.

Taylor & Dimmock (1965b) previously demonstrated that neutralization of A/FPV/R by rabbit polyclonal IgM reduced virus attachment to BHK cells by 50% at 37°C. Attachment was temperature dependent and little or no virus attached when the temperature was lowered to 4°C. The IgM-neutralized virus that had attached was not internalized by the cell and could be largely removed using proteinase K; no virion RNA was found in the cell nucleus. These workers proposed that IgM neutralized influenza virus by sterically hindering virus attachment and internalization. One limitation of the Taylor & Dimmock work described above was that they used only BHK cells and a single, high concentration of IgM which produced 99% neutralization of the virus. In this chapter I aim to extend these findings by investigating the IgM-neutralization process by using a range of IgM concentrations and examining aggregation of virus, attachment to cells and subsequent internalization of attached virus by cells. The same three cell types will be used as previously for IgG-neutralization, including chicken erythrocytes because of their importance in the assay of influenza virus (i.e. haemagglutination) and antibody (i.e. haemagglutination-inhibition). It was also of interest to see if the interaction of IgM-neutralized virus and chicken erythrocytes was as different from other cell types as we had found previously using IgG.

10.2. Results.

10.2.1. Purification of IgM from mouse serum.

As a monoclonal IgM was not available, polyclonal IgM neutralizing for adpFPV/R was prepared by inoculating C3H mice with A/FPV/D (H7N7) which has a haemagglutinin closely related to the A/FPV/R (H7N1) and an unrelated neuraminidase (section 6.4.1.2.). The C3H mice were the same strain from which tracheal organ cultures were derived. The antiserum was specific for the H7 haemagglutinin glycoprotein and had a haemagglutination inhibition titre of 1/2560 and an anti-neuraminidase titre of less than 1/200 (section 6.4.4.).

IgM antibodies were purified using a Sephacryl S-300 column (section 6.4.2.). Initially IgM was precipitated using polyethylene glycol (PEG). This was only partly satisfactory as it precipitated a significant (but variable) proportion of antibody from the serum, but was better than ammonium sulphate which precipitated only small amounts of antibody (data not shown).

5ml aliquots of PEG-precipitated serum were passed through the column (Figure 10.1.). The IgM eluted in the first protein peak with a good separation (60 min.) between the IgM and the other serum proteins. There was little HI activity eluting in the position expected of IgG. Increasing the amount of sample overloaded the column and resulted in a poor separation of antibody classes. Many aliquots of serum were run on the S-300 column and the fractions containing HI activity were pooled and concentrated using Centricon-10 tubes (section 6.4.2.) and stored at 4°C.

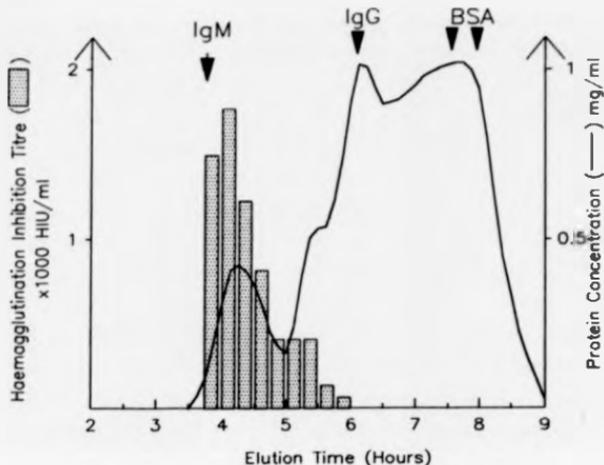


Fig.10.1. Isolation of H7-specific IgM from PEG-precipitated mouse serum on a Sephacryl S-300 column.

5ml of PEG-precipitated C3H mouse serum was applied to the Sephacryl S-300 column and the proteins eluted in TBS. The fractions were collected at 15 min. intervals and assayed for haemagglutination inhibition (section 6.4.5.) and protein concentration (section 6.4.8.).

10.2.2. Verification that the purified antibody was IgM.

The antibody was confirmed as IgM by Ouchterlony analysis (section 6.4.5.). There was immunoprecipitation with the goat anti-mouse (GAM) IgM but not with GAM IgG or GAM IgA (Figure 10.2.).

The purified antibody was further characterized by SDS-PAGE (section 6.4.6.). Under non-reducing conditions (Figure 10.3a) a strong band was seen which hardly entered the gel. Increasing the running time of the gel or reducing the polyacrylamide concentration did not significantly improve the mobility of the band. The high molecular weight of this protein was thus consistent with the expected large size of pentameric IgM, molecular weight (Mr) of 900,000. The contaminating smaller proteins were probably IgM-breakdown products as they increased when the antibody was stored over prolonged periods. Under reducing conditions (Figure 10.3b) a strong band with a molecular weight of 75-77,000 was seen and this agrees well with the expected Mr of mouse μ heavy chain (Mr 75,000). No light chains (Mr roughly 25,000) were seen, nor was the J chain (Mr 15,000). The IgG gave a band at 50-54,000 which corresponded with the expected mouse γ heavy chain (Mr 50,000), and again there was no evidence of light chains, even though the antibody was monoclonal and should have given better definition. The Mr of bovine serum albumin (BSA) was calculated as 69,000 which agreed well with the expected value (67,000).

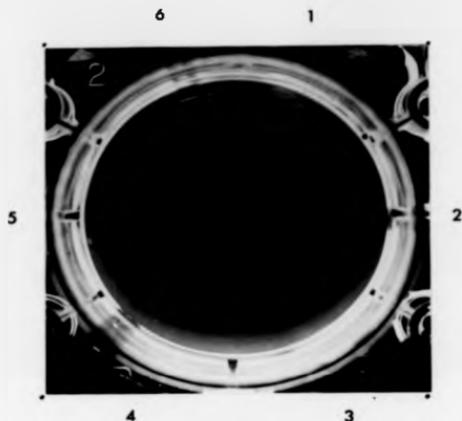


Fig.10.2.
Immunoprecipitation of purified pClgM.

10ul of pClgM was placed in the centre well and wells numbered 1 to 6 were loaded with 10ul of:
GAM IgM 1/10; 1/30; 1/100; 1/300 (wells 1-4)
GAM IgG 1/10; GAM IgA 1/10 (wells 5-6).

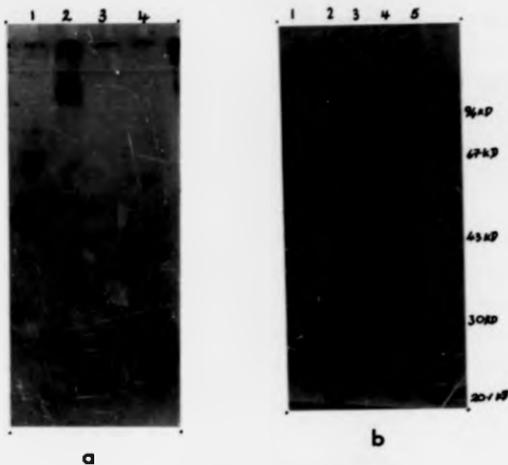


Fig.10.3. SDS-PAGE analysis of purified pcIgM.
 Method detailed in section 6.4.6.

- a) Non-reduced samples run on 10-30% gel.
 track #1 monoclonal IgG
 track #2 pcIgM
 track #3 empty
 track #4 BSA
- b) BME-reduced samples on 20% gel.
 track #1 BSA
 track #2 molecular weight markers
 track #3 BSA
 track #4 monoclonal IgG
 track #5 pcIgM

10.2.3. Neutralization of adpFPV/R by IgM by plaque reduction on CEF cells.

Neutralization of adpFPV/R by IgM was determined by plaque assay using CEF cells only and parallel assays using BHK cells were not performed. I had previously found with IgG that changing the cell type from CEF to BHK did not alter the neutralization profile (section 9.3.) and I assume this also to be true for IgM. The decline in infectivity with increasing concentration of IgM (Figure 10.4.) was more gradual than that seen for IgG (section 9.2.2.) and 50% neutralization occurred at a \log_{10} HIU:HAU ratio of -0.5 . It should be noted that the amount of neutralization found at a \log_{10} HIU:HAU ratio of above 0.0 was variable (80-99%) and it was often found that maximum neutralization of only 90% could be achieved at the highest IgM concentration.

With IgG, I found neutralization to be highly dependant on the concentration of virus to be neutralized, the neutralization of a large amounts of virus could be brought about by a relatively small increase in antibody concentration (section 9.2.2.). This was similarly investigated using IgM (Table 10.1.).

The IgM had a haemagglutination inhibition (HI) titre of 1/10,540. This is measured using 4 HAU of virus, which is equivalent to 16 HAU/ml (section 6.4.3.). In comparison, experiments studying the attachment of virus to cells used 500 HAU/ml (virus plus antibody) inocula. These inocula were assayed for remaining haemagglutination activity (section 6.5.2.). It was found that 50% haemagglutination activity remained when a 1/100 dilution of IgM was used to neutralize the virus. The ability of IgM to neutralize two widely different concentrations of adpFPV/R was determined by plaque

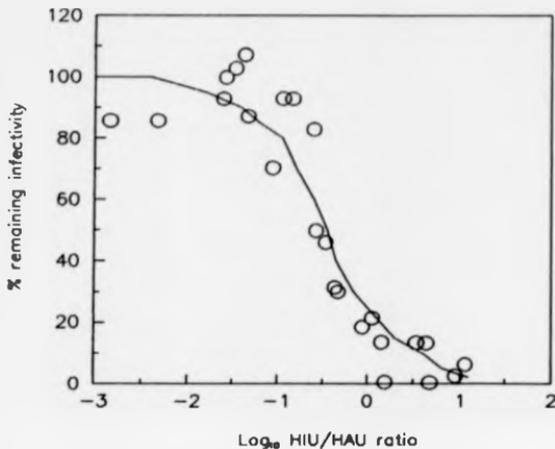


Fig.10.4. Neutralization of adpFPV/R infectivity by pClgM.

Dilutions of pClgM were added to equal volumes of adpFPV/R (1000 HAU/ml), mixed and incubated for 60 min. at 25°C. The remaining infectivity was determined by plaque assay using CEF cells.

Table 10.1. The dilutions of IgM required to cause 50% inhibition in haemagglutination or infectivity of different adFPV/R concentrations.

	50% end-point (dilution)
Haemagglutination inhibition assay using 16 HAU/ml	1/10540 (section 6.4.3.)
Assay of remaining haemagglutination using 500 HAU/ml	1/100 (section 6.5.2.)
Plaque assay using 1000pfu/ml or 0.014 HAU/ml	1/200 (section 6.5.1.)
Plaque assay using 500 HAU/ml	1/110 (section 6.5.1.)

reduction. When the concentration of virus was increased by 70,000 fold (from 0.014 HAU/ml to 1000 HAU/ml) then only twice the amount of IgM was required to cause 50% neutralization (1/200 to 1/110 dilution).

Therefore at high virus concentrations (500 HAU/ml) the same amount of IgM (1/100 dilution) was required to inhibit both infectivity and haemagglutination by 50%. At lower virus concentrations more than 50-fold less IgM was required to inhibit the haemagglutination of 16 HAU/ml by 50% (1/10,540 dilution) than was required to neutralize 0.02 HAU/ml by 50% (1/200 dilution). It would appear that at lower virus concentrations the IgM inhibited haemagglutination far more readily than infectivity. Reasons for this are discussed later.

10.2.4 Neutralization of adFPV/R by IgM on tracheal organ cultures.

Neutralization found by plaque reduction on cultured cells was not taken as evidence that neutralization was necessarily occurring on the epithelial surface of tracheal organ cultures. The ability of the IgM to reduce adFPV/R multiplication on tracheal organ cultures was investigated as before (section 9.2.1.).

Neutralization of the inoculum by IgM reduced the multiplication by 99% during the initial 12 hours incubation (Figure 10.5.), demonstrating that neutralization had occurred. Two concentrations of IgM were used, the higher (\log_{10} HIU:HAU ratio of +0.5) caused 75% neutralization by plaque assay and the lower (\log_{10} HIU:HAU ratio of -0.4) only 54% neutralization. Nevertheless there was little difference in their effectiveness in inhibiting multiplication on organ cultures. At the higher concentration,

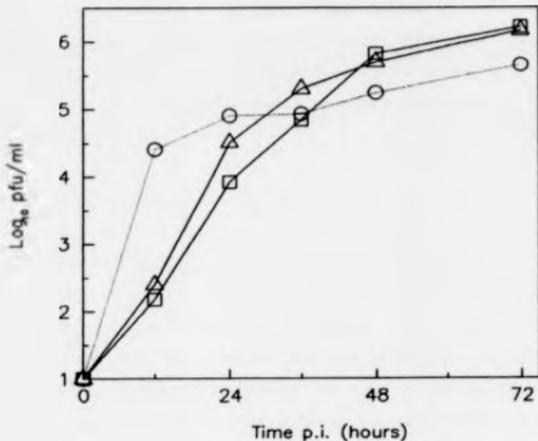


Fig. 10.5. Neutralization of adpFPV/R by pClgM and the effect on virus multiplication on tracheal organ cultures.

adpFPV/R was diluted to 1000 HAU/ml before adding an equal volume of either (i) 1/10 non-neutralizing 185/1 (○), (ii) purified polyclonal IgM (pClgM) diluted to either \log_2 HIU:HAU +0.5 (△) or -0.4 (□). The virus plus antibody mixtures were incubated at 25°C for 60 min. 200ul of each mixture were added to batches of 18 tracheal organ cultures. After 60 min. at 37°C, the cultures were washed with PBS and seeded as individual cultures into wells of a microtitre tray and 50ul of medium added. The cultures were incubated at 37°C, the medium was regularly harvested and replaced with fresh medium. The infectivity of the pooled media was determined by plaque assay.

by EM, the virus particles appeared completely coated with antibody and there was little aggregation (personal communication, Dr.S.J.Armstrong), whereas at the lower concentration the virus was considerably aggregated. Note that IgM was not present in the culture medium and maximal levels of virus multiplication were seen when cells were infected with non-neutralized progeny virus.

10.2.5. Aggregation of adFPV/R by IgM.

IgM aggregated virus (Figure 10.6.) to a similar degree to that found with IgG (section 9.). The initial rise in aggregation (half-maximal) occurred at a \log_{10} HIU:HAU ratio of -1.4 and preceded the initial fall in infectivity (i.e. 50% neutralization) which occurred at a \log_{10} HIU:HAU ratio of -0.5. This suggests that either the EM technique enhanced aggregation or that the aggregates were dispersed by physical agitation (as previously discussed). A maximum average aggregate size of 4.6 occurred at a \log_{10} HIU:HAU ratio of -0.9. Increasing the \log_{10} HIU:HAU ratio to 0.0 lessened the amount of aggregation and the virus was essentially monodisperse at a concentration of above \log_{10} HIU:HAU +0.5.

10.2.6. Attachment of adFPV/R to tracheal organ culture cells and the effect of neutralization by IgM.

IgM reduced attachment of adFPV/R to organ culture cells (Figure 10.7a). The initial fall in attachment (half maximal) occurred at an IgM \log_{10} HIU:HAU ratio of -0.3 which was close to that required for 50% neutralization i.e. \log_{10} HIU:HAU ratio of -0.5. The maximum inhibition of attachment (70%) was not as great as the maximum neutralization (greater

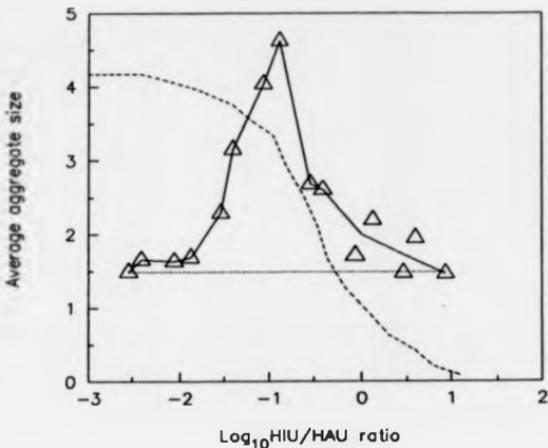


Fig.10.6. Aggregation of adpFPV/R by pcIgM.

Dilutions of pcIgM were added to equal volumes of adpFPV/R (1000 HAU/ml), mixed and incubated for 60 min. at 25°C. The samples were then examined by EM and the amount of aggregation determined (\triangle) and compared to the non-neutralized adpFPV/R controls (-----). Also shown is the pcIgM neutralization profile (.....) from Fig.10.4.

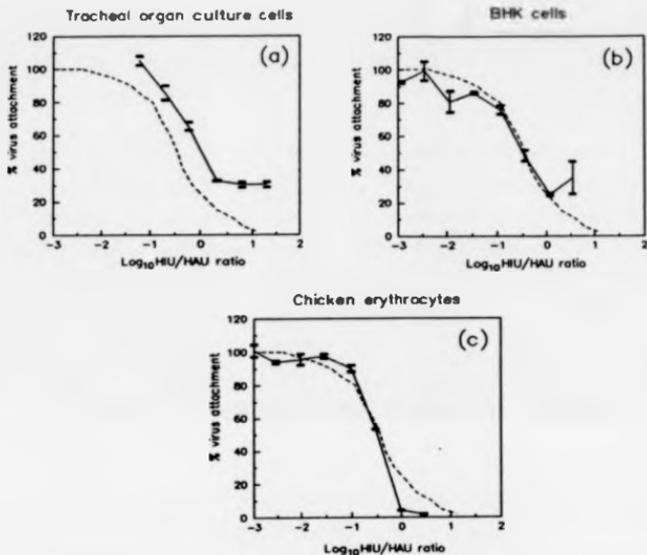


Fig.10.7. Neutralization of adpFPV/R by pClgM and the effect on attachment to various cells.

Dilutions of pClgM were added to equal volumes of ^{32}P adpFPV/R, mixed and incubated for 60 min. at 25°C . The amounts of virus attaching were determined as previously described (section 6.5.4.). Also shown is the pClgM neutralization profile (-----).

than 90%). Above a \log_{10} HIU:HAU ratio of 0.0, where the virus was coated with antibody and was not aggregated (section 9.2.5.). There was no accompanying increase in attachment as seen with IgG.

10.2.7. Attachment of adFPV/R to BHK cells and the effect of neutralization by IgM.

A reduction in attachment of IgM-neutralized adFPV/R to BHK cells was found and occurred at the same IgM concentration as that required for neutralization (Figure 10.7b). The maximum inhibition of attachment was 70% and occurred at a \log_{10} HIU:HAU ratio of 0.0. The slight improvement in attachment at the highest IgM concentration was not significant and subsequent experiments using high IgM ratios (not shown) confirmed that no increase in virus attachment occurred.

10.2.8. Attachment of adFPV/R to chicken erythrocytes and the effect of neutralization by IgM.

Inhibition of attachment of IgM-neutralized adFPV/R to chicken erythrocytes was more than 95% (Figure 10.7c) and the fall again paralleled the loss of infectivity.

10.2.9. Effect of neutralization by IgM on the internalization of adFPV/R.

The capacity of cells to internalize IgM-neutralized adFPV/R was determined (Table 10.2.) by their acquisition of resistance to release by neuraminidase.

Table 10.2. Internalization of pc1gM-neutralized adpFPV/R by cells.

Antibody	Log ₁₀ HIU/HAU	Cell type	Neuraminidase resistance (%)	
			5' p.i.	60' p.i.
185/1 ^a	N/A	O.C.	100	75.3
pc1gM	+0.81	O.C.	2.3	48.8
185/1	N/A	BHK	100	71.1
pc1gM	+0.30	BHK	34	44.8
185/1	N/A	BHK	100	89.9
pc1gM	-0.43	BHK	42	61.5

a. Compared to the non-neutralized controls at 60 min. p.i.

b. Cells treated with neuraminidase for 60 min. at 37°C (section 6.5.5).

c. Relative increase in neuraminidase resistance; value at 60 min. p.i. divided by the 5 min. p.i. value. No increase (i.e. value of 1.0)

would indicate that no virus had been internalized.

d. Control samples treated with the irrelevant monoclonal antibody, 185/1.

The neuraminidase resistance of infectious virus increased with time using either BHK or tracheal organ culture cells and this, as discussed previously (section 9.2.7.), was taken as a demonstration that internalization was occurring.

In agreement with the results above, neutralization by IgM reduced the amounts of virus attaching to cells (Table 10.2., column 4). The IgM-neutralized adFPV/R that attached did not become resistant to the action of neuraminidase and hence was not internalized by the cells. Even when the IgM concentration was reduced to a \log_{10} HIU:HAU ratio of -0.43 (Table 10.2., rows 5 and 6), internalization by BHK cells was still impaired to the same extent.

10.3. Discussion.

10.3.1. Aggregation of adFPV/R by IgM.

IgM antibodies might be expected to cause more aggregation than IgG because of their increased antigen binding capacity (i.e. valency of 10 compared with 2), but both caused comparable amounts of aggregation. In theory aggregation caused by IgM could have reduced infectivity by up to 70% (Table 10.3.). This calculated loss of infectivity has to be treated with caution as aggregation preceded neutralization, as discussed above (section 10.2.5.). Increasing the IgM concentration abolished aggregation whilst the infectivity remained low, suggesting that aggregation was not the sole mechanism of neutralization. A similar situation was found by Thomas et al. (1986) who investigated the neutralization of poliovirus. They found the amount of IgM-mediated aggregation of poliovirus decreased under conditions of antibody excess.

Table 10.3. Summary of data on the aggregation of adFPV/R by purified IgM.

	Antibody ratio (log ₁₀ HIU:HAU)
50% neutralization ^a	-0.5
Half-maximal aggregation ^b	-1.4
Maximum aggregation ^b	-0.9
Return to monodispersion ^b	+0.5
Max. average aggregate size ^b	4.6
Maximum potential neutralization due to aggregation ^c	67.4%

a. Data from Fig.10.4.

b. Data from Fig.10.6.

c. Calculated from the maximum average aggregate size e.g.

Infectious (control) virus aggregate size = 1.5 particles /aggregate

∴ per 100 virions there are 100/1.5 aggregates

∴ theoretical infectivity = 66.67

For pIgM neutralized virus max. aggregate size = 4.6 particles/aggregate

∴ per 100 virions there are 100/4.6 aggregates

∴ theoretical infectivity = 21.74

∴ remaining infectivity = 21.74/66.67 x 100 = 32.61%

∴ neutralization = 67.4%

I unsuccessfully attempted to saturate adpFPV/R with [125 I]-IgM but failed to find a concentration where antibody no longer continued to attach to the virus. However at a \log_{10} HIU:HAU ratio of +1.5 the binding of IgM began to tail-off, indicating that saturation was being approached (data not shown). Taylor (1986) also failed to find a satisfactory sharp saturation end-point for polyclonal IgM.

10.3.2. Attachment of adpFPV/R to cells and the effect of IgM.

Taylor & Dismock (1985b) demonstrated that neutralization of A/FPV/R by a high IgM concentration resulted in more than a 50% reduction in attachment of virus to BHK cells. They also showed that levels of attachment were lower if cells were inoculated and incubated at temperature of 25°C, while at 4°C attachment was negligible. They concluded that the IgM, because of its large size, was sterically hindering the attachment of virus to cells and that "at higher temperatures the increased thermal agitation could be responsible for bringing receptor and target molecules into the correct juxtaposition".

My attachment data are summarized in Table 10.4.. Like Taylor & Dismock (1985b) I found that IgM-neutralization reduced the attachment of adpFPV/R to BHK cells. IgM also reduced the attachment of virus to tracheal organ culture cells to a similar level which suggests that BHK cells are a suitable cell type on which to investigate neutralization. For both cell types the inhibition of attachment paralleled loss of infectivity and occurred at approximately the same \log_{10} HIU:HAU ratio, suggesting that the

Table 10.4. Summary of data on IgM inhibition of odpFPV/R attachment to cells.

CEF cells	50% neutralization	Antibody ratio (log ₁₀ HIU:HAU)
		-0.5
Tracheal organ culture cells	Initial inhibition of attachment Maximum inhibition of attachment	-0.3 +0.3 (30%)*
BHK cells	Initial inhibition of attachment Maximum inhibition of attachment	-0.5 +0.1 (30%)
Chicken erythrocytes	Initial inhibition of attachment Maximum inhibition of attachment	-0.5 0.0 (5%)

* In parenthesis is the percentage of virus which attached compared to the non-neutralized controls.

inhibition of attachment played an important role in the neutralization process. Unlike IgG, increasing the \log_{10} HIU:HAU ratio to +0.5 did not restore attachment.

Neutralization by IgM resulted in a concomitant reduction in attachment of adFPV/R to chicken erythrocytes and was over 5-fold more than found using BHK or tracheal organ culture cells. The magnitude of the reduction was greater than with IgG (section 9.) and this may be due to the greater size of IgM compared to IgG which would increase the steric hindrance. The amount of IgM-neutralized virus attached to the chicken erythrocytes remained low when the IgM was increased to a \log_{10} HIU:HAU ratio of +0.5.

Limitations in the availability of IgM prevented examination of attachment above a \log_{10} HIU:HAU ratio of +0.5. However EM and aggregation data showed that above a \log_{10} HIU:HAU ratio of 0.0 virus was coated with antibody and was monodisperse. It therefore seems likely that saturation of the virus with IgM was being approached at a \log_{10} HIU:HAU ratio greater than +0.5 and that the amount of virus attachment found at this point would not have significantly altered if the IgM concentration could have been increased.

10.3.3. Internalization of IgM-neutralized adFPV/R by cells.

Taylor & Dismock (1985b) reported that more than 80% of IgM-neutralized virus that managed to attach to BHK cells remained susceptible to removal by proteinase K following 2 hours incubation at 25° or 37°C and that none of the virion RNA was found in the cell nucleus. They concluded that the

majority of the attached IgM-neutralized virus remained on the outer surface of the cells and that the IgM interfered with the endocytotic event responsible for internalization.

Using neuraminidase and various concentrations of IgM, I also found that neutralization of adpFPV/R by IgM not only reduced the attachment of virus to cells but also impaired the internalization of the virus that had attached. The large size of the IgM molecule may possibly be preventing the internalization process, which is not affected by the smaller IgG (section 9.). The amount of neutralization caused by IgM tended to be more variable than that found for IgG and frequently as much as 10% original infectivity could remain even when the highest antibody concentration was used. Reasons for this are discussed later.

10.3.4. Mechanism of neutralization of influenza virus by IgM.

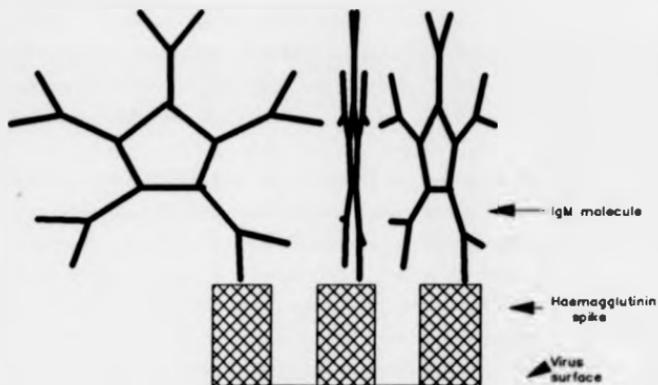
IgM-mediated aggregation of the virus presumably results in the loss of some infectivity. However aggregation cannot be the sole cause of neutralization because increasing the antibody concentration decreased aggregation without any return of infectivity. Aggregation probably contributes to neutralization by enhancing the neutralizing activity of the antibody shifting the neutralization profile to the left (i.e. to a lower antibody concentration). At low relative IgM concentrations aggregation could be an important cause of neutralization as there may be insufficient antibody to neutralize the virus by other means.

On data based on a single concentration of IgM, Taylor & Dismock (1985b) proposed that the large size of the IgM molecules bound to the virus particles probably sterically hindered virus attachment and blocked internalization. I have confirmed this and shown that the fall in virus attachment coincided with the loss of infectivity. The amount of IgM-neutralized virus attached to BHK and tracheal organ culture cells remained low at high log HIU:HAU ratios and could account for most of the observed neutralization (70% out of a total 90-99%). Therefore inhibition of virus attachment appears to be a major mechanism by which IgM antibodies neutralize influenza virus. Although IgM greatly reduced the attachment of virus to cells, approximately 30% of the virus did attach. Thus inhibition of attachment does not fully account for neutralization but impairment of internalization of this attached IgM-neutralized virus may explain the additional loss of infectivity.

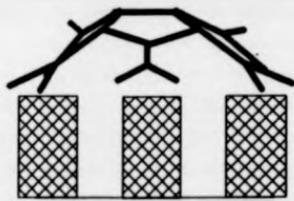
Results here suggest a different explanation of the findings of Cascino et al. (1986). They produced a human IgM monoclonal (UT 17.9) which bound to the H3 haemagglutinin, caused haemagglutination inhibition (HI) but did not neutralize the virus. They proposed that "the epitope for neutralization overlaps with, but is smaller than, the epitope for haemagglutination, or that it only partially overlaps with the latter". They argued that simple steric hindrance which caused HI differed from neutralization and that neutralization must in some way be related to the antigenic site against which the antibody was directed. However erythrocytes are a poor model for the study of neutralization (discussed in section 9.) and therefore the conclusions of Cascino et al. (1986) about neutralization, which are based on the use of erythrocytes, need to be treated with caution. Also their monoclonal IgM, UT 17.9, had a very low haemagglutination inhibition (HI)

titre (1/32) and it is possible that they failed to detect neutralization because the HI activity was too low. My data (Table 10.1.) would support this interpretation and showed that HI was a much more sensitive test than neutralization when low virus concentrations are used.

By EM examination Dr. S.J. Armstrong (personal communication) has detected bound IgM using anti-IgM linked to colloidal gold. The amount of antibody used shows that neutralization does not result simply from the binding of one IgM molecule to the influenza virus particle but was dependant on the number of IgM molecules bound. Maximum aggregation and 50% neutralization required 31 and 172 molecules of IgM per virion respectively, using CEF cells for the latter. The lowest concentrations of IgM bind to the virus particles but fail to neutralize or reduce the attachment to CEF cells and only caused a small amount of aggregation. It was also found that the distance between the viral envelopes of aggregated virus particles was the same for virus with a few IgM molecules bound as for infectious virus and therefore it did not appear that IgM would sterically hinder virus attachment. In contrast when higher concentrations of IgM were used, the attachment of virus to CEF cells was reduced and neutralization resulted. A significant increase in distance between the viral envelopes within aggregates was also found, this would suggest that the IgM molecules are sterically hindering virus attachment at these higher, neutralizing IgM concentrations. Dr. Armstrong proposes that the conformation the bound IgM adopts affects the attachment and neutralization of the virus (illustrated in Figure 10.8.). At low, non-neutralizing concentrations the IgM binds to the virus in the 'staple' conformation (Feinstein *et al.*, 1986) and this conformation does not aggregate the virus or reduce either the attachment, internalization or infectivity. At high concentrations more IgM molecules



Many IgM molecules bound to virus particles
with the IgM adopting 'planar' conformation.



Few IgM molecules bound to virus particles
with the IgM adopting the 'staple' conformation.

Fig.10.8.

Diagram of IgM binding to HA spikes of influenza particles and the limitations placed on antibody conformation at differing antibody concentrations.

bind to the virus particles. They are unable to adopt the 'staple' conformation and instead adopt the 'planar' conformation. Dr. Armstrong proposes that the 'planar' conformation aggregates the virus particles, sterically hinders the attachment and blocks the internalization process of any virus which does attach.

The conformation adopted by the bound IgM molecules may explain my observation that haemagglutination can be more readily inhibited than infectivity. The depth of antibody surrounding the virus particles is relatively small when IgM is bound in the 'staple' conformation compared to the 'planar' conformation. Both the 'planar' and 'staple' conformations may sterically hinder the interaction of the virus haemagglutinin with the short erythrocytes receptor (Viitala & Jarnefelt, 1985; Dimmock, 1987). I previously found that the smaller IgG molecule was capable of blocking this interaction and reduced the attachment of virus to chicken erythrocytes (discussed in section 9.). In comparison, it would seem that the length of the receptor on BHK and organ culture cells is sufficiently long to penetrate the antibody layer surrounding the virus when either IgG molecules are bound or when IgM is bound in the 'staple' conformation. When more IgM molecules bind to the virus, and the 'planar' conformation is adopted, the depth of the antibody layer becomes sufficiently deep to block the attachment of virus to BHK and organ culture cells, resulting in neutralization.

RESULTS 5.
Neutralization of influenza
virus by IgA.

11.1. Introduction.

The neutralization of influenza virus by IgA was investigated in the same manner as previously reported for IgG (section 9.) and IgM (section 10.). Secretory IgA (sIgA) is the predominant antibody class found in external secretions and is a principal mediator of humoral immunity against infections of the respiratory tract (reviewed by Underdown & Schiff, 1986; Mestecky, 1987). Therefore the mechanisms by which this immunoglobulin class neutralizes respiratory tract pathogens are of considerable interest. My use of epithelial cells from mouse tracheal organ cultures in conjunction with murine IgA was designed to create a model system within one species of animal with which to study influenza virus infection. As it used natural differentiated target cells, the system was also capable of determining if there were significant differences between in vivo mechanisms of neutralization and the in vitro mechanisms of neutralization which have been previously reported.

Taylor & Dismock (1985a) found that secretory IgA (sIgA), prepared from rat bile, reduced the attachment of A/FPV/R to BHK cells at 37°C by roughly 60%. The inhibition of attachment was shown to be temperature dependant, with negligible amounts of neutralized virus attaching at 4°C. The majority of virus that attached remained susceptible to proteinase K removal and the RNA of any virus remaining attached to the cells did not migrate to the cell nucleus. It was also found that IgA monomers, prepared from the same sIgA by differential reduction, neutralized but did not inhibit virus attachment or its internalization by the cells. Taylor & Dismock concluded that the mechanism of neutralization of influenza virus was dependant on the character of the antibody used. Monomeric antibodies, IgG and monomeric

IgA₁ did not block virus attachment or internalization by cells and therefore must neutralize infectivity at a intracellular stage of infection. On the other hand the polymeric antibodies sIgA and later IgM (Taylor & Dimmock, 1985b) inhibited both virus attachment and internalization.

11.2. Results.

11.2.1. Purification of IgA from culture medium.

Taylor (1986) prepared polyclonal sIgA by directly inoculating the Peyer's patches of Wistar rats with EPL-treated A/FPV/D and at 7-9 days post-inoculation cannulating the bile ducts and collecting bile. Bile salts were removed by dialysis and the sIgA purified by either affinity chromatography or gel filtration. I unsuccessfully attempted to adapt this method for use with mice: EPL-treated A/FPV/D was administered to C3H mice by either intranasal, intragastric or intravenous inoculation and the bile collected from mice sacrificed 7 days post-inoculation. The methodology and results are not presented because in all cases the haemagglutination-inhibition titre of the harvested bile was low (less than 16 HIU/ml) and the small amount of bile per mouse (less than 20ul) made this an impractical source of sIgA.

Dr. V. Gerhard (Philadelphia, USA) kindly supplied a hybridoma cell-line (#37-66-1) that produced monoclonal IgA directed against the haemagglutinin of A/PR/8 (H1N1). The culture medium harvested from these cells (tcf#37-66-1) had a high haemagglutination-inhibition titre against A/PR/8

(more than 28,000 HIU/ml), and did not cross-react with other haemagglutinin sub-types or with the HI haemagglutinin of A/WSN (results not shown).

The antibody in tcf#37-66-1 failed to bind to protein-A beads which had previously been reported to bind human IgA2 in addition to IgG antibodies (Archibald *et al.*, 1987). This may reflect the fact that murine immunoglobulin A has only one subclass, whereas man has two subclasses (IgA1 and IgA2) and rabbits possibly three subclasses (Underdown & Schiff, 1986).

I initially attempted to purify the IgA from tcf#37-66-1 using Jacalin, a lectin isolated from the jackfruit *Artocarpus integrifolia*. Jacalin has an IgA-binding specificity and has been previously used to purify human serum and secretory IgA (Rogue-Barreira & Campos-Neto, 1985) and hamster IgA (Ray *et al.*, 1988). Jacalin failed to bind any of the HI activity in tcf#37-66-1.

Immune precipitation followed by gel filtration has been used to purify IgA from human serum (Meestecky *et al.*, 1971). I loaded 5ml batches of tcf#37-66-1 culture medium directly on to a Sephacryl S-300 column and eluted with TBS (section 6.4.2.). I did not include an immune precipitation step because I was working with hybridoma cell culture medium which would not contain any other immunoglobulins specific for influenza virus. PEG precipitation was not used as it failed to precipitate significant amounts of antibody and as culture medium was used instead of serum it was found that the column did not become blocked. A typical elution profile is shown in Figure 11.1. HI antibody eluted with the first peak and good separation

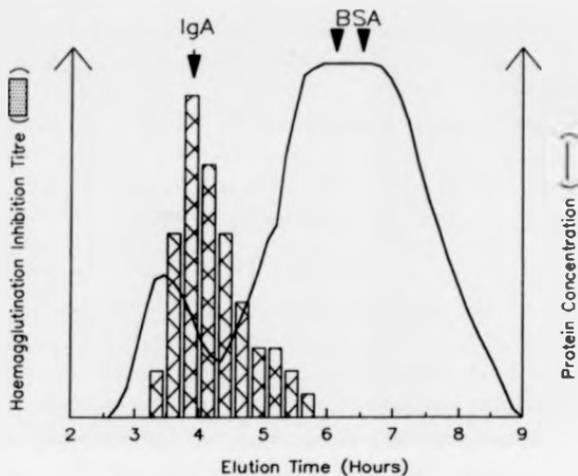


Fig.11.1. Isolation of presumptive IgA from the culture medium of the hybridoma #37-66-1.

5ml of undiluted tcf#37-66-1 was applied to the S-300 column and the IgA eluted with TBS (section 6.4.2.). Fractions were collected at 15 min. intervals and assayed for haemagglutination inhibition against A/PR/B and protein concentration (section 6.4.3. and 6.4.8.).

was found between this and the other major proteins of the culture medium. The fact that the HI antibody eluted in the first peak and at a time similar to that previously found for IgM rather than IgG (section 10.2.1.) suggested that the IgA was in a polymeric rather than a monomeric form.

Repeated attempts were made, without much success, to prepare monomeric IgA using the differential reduction method of Taylor & Dismock (1965a). I found that if 0.12M iodoacetamine was added as a blocking agent following the reduction by beta-mercaptoethanol, then small amounts of monomer were obtained.

11.2.2. Verification that the purified antibody was IgA.

The fractions obtained from the S-300 column were characterized by SDS-PAGE using non-reducing conditions (Figure 11.2a). A band was found (tracks 3, 4, 5) with a higher molecular weight than IgG (track 11) and was only observed in the fractions which contained HI activity, suggesting that this was the putative IgA. Fractions from the second large protein peak of the S-300 purification (tracks 6 to 10) appeared to be mainly composed of albumin. Determination of the molecular weight (M_r) of the putative IgA antibody using this gel system proved difficult, so the PAA gel system was used instead and is reported below. The putative IgA antibody was also run on SDS-PAGE gels using reducing conditions (Figure 11.2b). The M_r of the IgA heavy chain (track 3) was 67,000; heavier than found for IgG (track 4) (M_r 60,000) but less than IgM (track 1) (M_r 76,000). A second minor band can be seen in this IgA preparation which had a slightly lower M_r (58,000). The IgA had been stored at 4°C for approximately 1 week and possibly the IgA antibody undergoes some degradation upon storage similar to that found

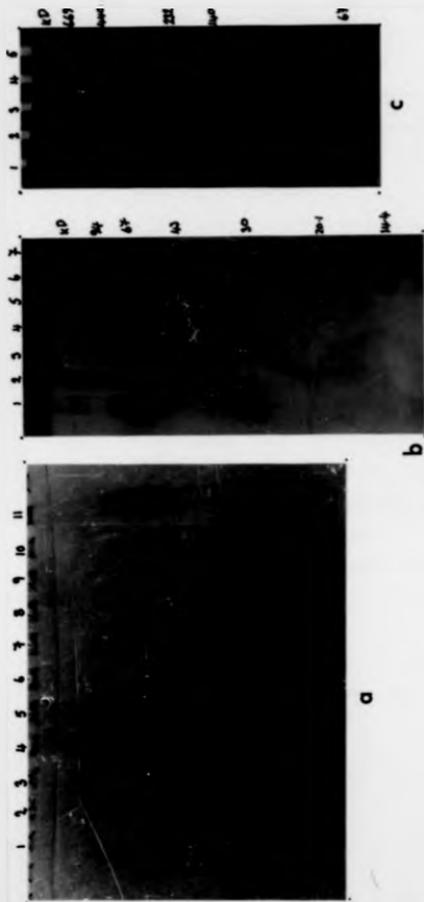


Fig.11.2. Gel electrophoresis of putative IgA antibody.

a) Non-reduced samples from S-300 column run on 10-30% SDS-PAGE gel.

track #1 BSA
 tracks #2-#10 half-hourly fractions from S-300 column commencing at 3 hours.
 track #11 monoclonal IgG

b) Reduced samples on 20% SDS-PAGE gel.

track #1 polyclonal IgM
 track #2 purified IgA preparation
 track #3 purified IgA preparation
 track #4 monoclonal IgG
 track #5 empty
 track #6 molecular weight markers
 track #7 BSA

c) Samples run on 4-30% PAA gel.

track #1 polyclonal IgM
 track #2 monoclonal IgG
 track #3 purified IgA preparation
 track #4 molecular weight markers
 track #5 BSA

for IgM (section 10.2.2.). With a different IgA preparation (track 2), bands were observed with higher Mr values and these possibly result from incomplete reduction of the antibody. The J chain was not visible for either IgA preparation. Light chains can be seen with Mr of 27,000 (IgG), 28,000 (IgM) and 29,000 (IgA).

The IgA was further characterised on PAA gels using non-reducing, non-denaturing conditions (Figure 11.2c). The IgG (track 2) had a Mr of 210,000, which was higher than the expected value of approximately 160,000. The reasons for this are not clear. The IgM (track 1) had been stored for more than 2 months and gave a series of bands with high Mr values. These probably represent IgM degradation products. The IgA (track 3) gave 4 visible bands. The smallest band had a Mr of 470,000 which agrees with that expected for a dimeric structure. The Mr values of the other IgA bands were greater than thyroglobulin (Mr 669,000), the largest molecular weight marker used. These bands probably represent larger polymers of IgA i.e. trimers, tetramers and pentamers. Underdown & Schiff (1986) have reported the presence of large polymers of human IgA in preparations of secretory IgA.

The antibody was confirmed as IgA by Ouchterlony analysis (Figure 11.3.). immunoprecipitation was found against the GAM anti-IgA and not against GAM anti-IgG or GAM anti-IgM.

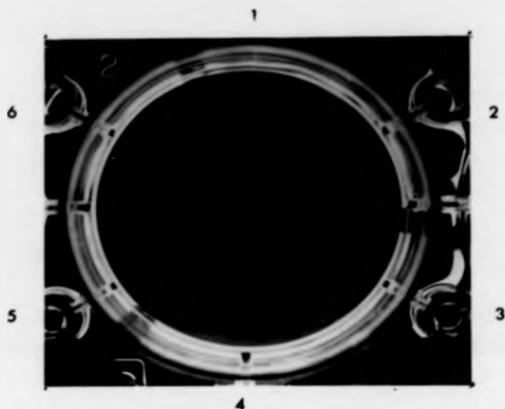


Fig.11.3. Immunoprecipitation of purified IgA.

10 μ l of purified IgA was placed in the centre well and wells numbered 1 to 6 were loaded with 10 μ l of:
GAM IgA 1/10; 1/30; 1/100; 1/300; GAM IgG 1/10;
GAM IgM 1/10. Method described in section 6.4.5.

11.2.3. Neutralization of A/PR/8 with purified IgA by plaque reduction.

The specificity of the IgA antibody isolated from tcf#37-66-1 meant that I had to change from using the adpFPV/R strain of influenza virus to the A/PR/8 strain, which also multiplied well on mouse tracheal organ cultures (section 7.2.3.).

The neutralization profile of the purified IgA versus A/PR/8 determined by plaque reduction on MDCK cells is shown in Figure 11.4. A 50% loss of infectivity was found at a \log_{10} HIU:HAU ratio of -1.5. Increasing the antibody ratio to above -1.0 caused greater than 99.9% neutralization.

11.2.4. Neutralization of A/PR/8 on tracheal organ cultures.

The ability of the IgA to reduce A/PR/8 multiplication on tracheal organ cultures was investigated as described previously for IgG and IgM antibodies. The multiplication of IgA-neutralized A/PR/8 was reduced during the initial 24 hours post-inoculation (Figure 11.5.) again demonstrating that neutralization had occurred. Further incubation of the cultures, in the absence of antibody, resulted in normal amounts of virus multiplication, the progeny virus could still be neutralized by the IgA and did not result from neutralization-escape mutations. I used two different IgA concentrations to neutralize the virus and found that both reduced the multiplication of virus to a similar degree.

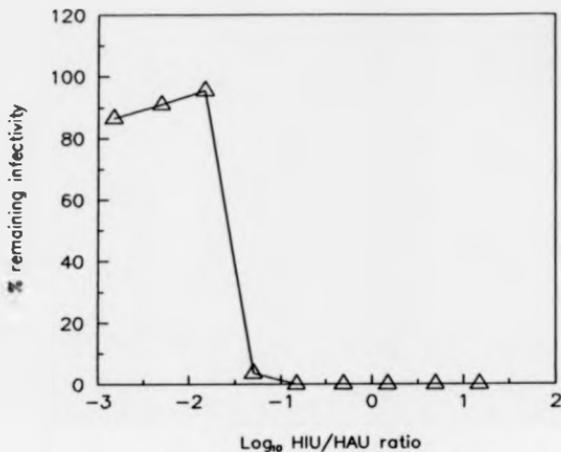


Fig.11.4. Neutralization of A/PR/8 infectivity by IgA.

Dilutions of IgA were added to equal volumes of A/PR/8 (1000 HAU/ml), mixed and incubated for 60 min. at 25°C. The remaining infectivity was determined by plaque assay using MDCK cells.

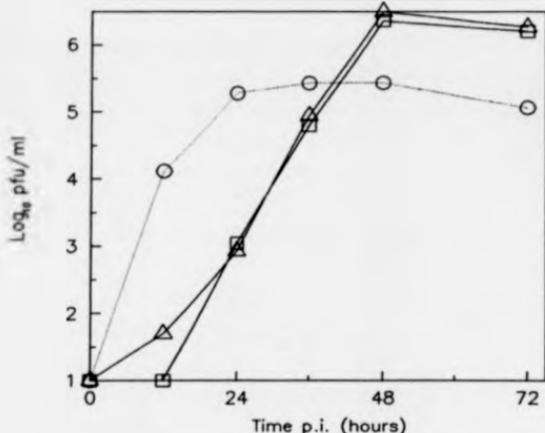


Fig.11.5. Neutralization of A/PR/8 by IgA and the effect on virus multiplication on tracheal organ cultures.

A/PR/8 was diluted to 1000 HAU/ml before adding an equal volume of either (i) 1/10 non-neutralizing 185/1 (○), or IgA: (ii) logH/H + 1.28 (△), or logH/H + 0.28 (□). The virus/antibody mixtures were incubated at 25°C for 60 min. 200 μ l of each virus/antibody mixture were added to batches of 18 tracheal organ cultures. After 60 min. at 37°C, the cultures were washed with PBS and seeded as individual cultures into wells of a microtitre tray and 50 μ l of medium added. The cultures were incubated at 37°C, and the medium was regularly harvested and replaced with fresh medium. The infectivity of the pooled medium was determined by plaque assay.

11.2.5. Aggregation of A/PR/8 by IgA.

A/PR/8 was aggregated by IgA (Figure 11.6.) with the initial rise in aggregation (half-maximal) occurring at a \log_{10} HIU:HAU ratio of -1.5. This coincided with the initial fall in infectivity. The maximum average aggregate size of IgA-neutralized virus was 3.4 and occurred at a \log_{10} HIU:HAU ratio of -1.2. Increasing the IgA concentration beyond a \log_{10} HIU:HAU ratio of -1.2 reduced the amount of aggregation and the virus was essentially monodisperse at a \log_{10} HIU:HAU ratio of above -0.7.

The maximum potential loss of infectivity due to IgA aggregation was 41% whereas virus was neutralized by more than 99%.

11.2.6. Attachment of A/PR/8 to tracheal organ culture cells and the effect of neutralization by IgA.

The results of two separate experiments are shown in Figure 11.7. In both cases a significant reduction in attachment of A/PR/8 to organ culture cells was found and the initial fall in attachment (half maximal) occurred at an IgA \log_{10} HIU:HAU ratio of -1.2 which is close to that required for 50% neutralization and the same as for maximum aggregation i.e. \log_{10} HIU:HAU ratio of -1.5. In both cases the maximum reduction in virus attachment was between 80-90% and occurred at an IgA \log_{10} HIU:HAU ratio of approximately -0.8. Variations were seen between the two separate experiments: in one (solid line) the rise in attachment became apparent at a \log_{10} HIU:HAU ratio of +0.4 and at a ratio of +1.1 attachment had returned to 100%, whereas in

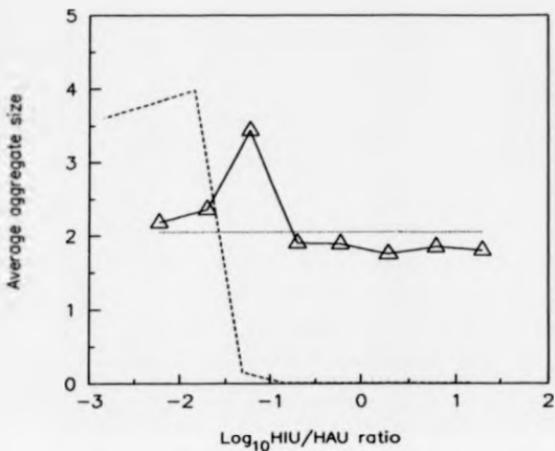


Fig.11.6. Aggregation of A/PR/8 by IgA.

Dilutions of IgA were added to equal volumes of A/PR/8 (1000 HAU/ml), mixed and incubated for 60 min. at 25°C. The samples were then examined by EM and the amount of aggregation determined (\triangle) and compared to the non-neutralized A/PR/8 controls (-----). Also shown is the IgA neutralization profile (.....) from Fig.11.5.

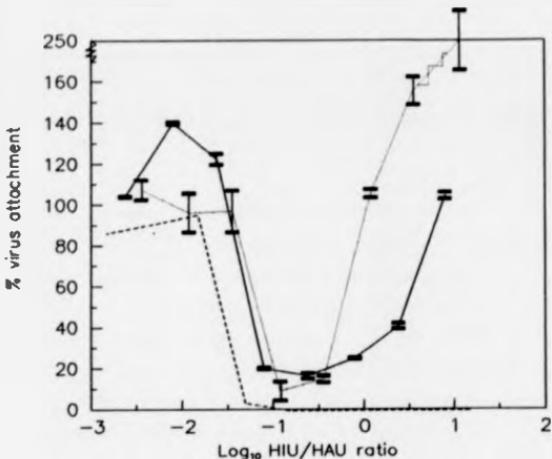


Fig.11.7. Neutralization of A/PR/B by IgA and the effect on attachment to epithelial cells of mouse tracheal organ cultures.

Dilutions of IgA were added to equal volumes of ³²P A/PR/B (1122 HAU/ml, CPM:HAU 82/516), mixed and incubated for 60 min. at 25°C. Batches of 26/40 organ cultures were inoculated with 400ul virus/antibody. The levels of virus attachment were determined as previously described (section 6.5.4.). Each organ culture batch yielded 2.11/10.0 X 10⁵ cells. Also shown is the IgA neutralization profile (-----) from Fig.11.4.

the other (dotted line) the rise in attachment occurred at a \log_{10} HIU:HAU ratio of -0.3 and rose to 250% at a ratio of +1.2. The reasons for these differences will be discussed later.

11.2.7. Attachment of A/PR/8 to BHK cells and the effect of neutralization by IgA.

A reduction in attachment of IgA-neutralized A/PR/8 to BHK cells was found (Figure 11.8.). The fall in attachment paralleled the loss in infectivity and the maximum inhibition of attachment was 70% and occurred at \log_{10} HIU:HAU ratio of -1.5. In contrast to my findings using tracheal organ culture cells, increasing the IgA concentration did not increase attachment which remained low even at a \log_{10} HIU:HAU ratio of +1.0. These data agree with Taylor & Dismock (1985a) even though they used sIgA whilst I used polymeric IgA lacking secretory component (discussed later).

11.2.8. Attachment of A/PR/8 to chicken erythrocytes and the effect of neutralization by IgA.

Neutralization of A/PR/8 by IgA reduced the attachment of the virus to chicken erythrocytes by more than 90% (Figure 11.9.). The fall in attachment paralleled the loss of infectivity and remained low at a \log_{10} HIU:HAU ratio of greater than +1.0.

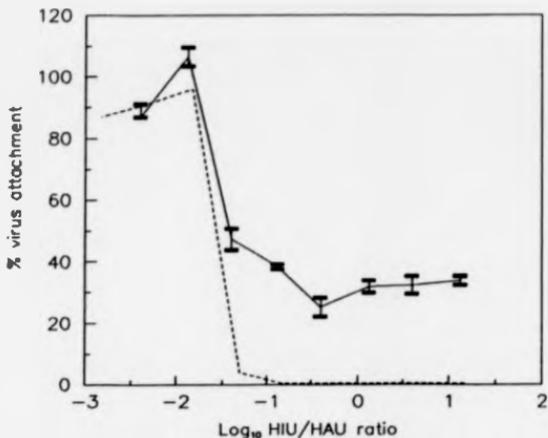


Fig.11.8. Neutralization of A/PR/B by IgA and the effect on attachment to BHK cells.

Dilutions of IgA were added to equal volumes of ³²P A/PR/B (1125 HAU/ml, CPM:HAU 79), mixed and incubated for 60 min. at 25°C. 100ul of virus/antibody was inoculated onto each confluent BHK monolayer (1.5 X 10⁶ cells). The levels of virus attachment were determined as previously described (section 6.5.4.). Also shown is the IgA neutralization profile (-----).

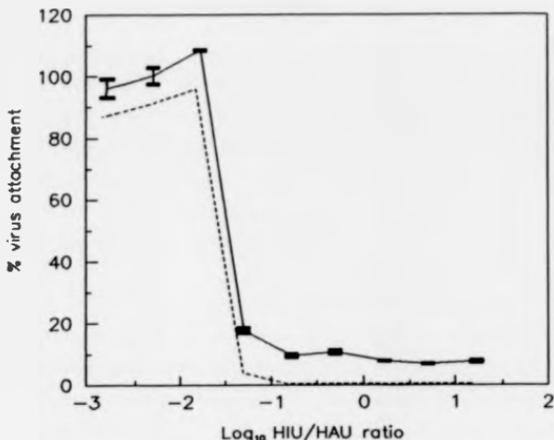


Fig.11.9. Neutralization of A/PR/8 by IgA and the effect on attachment to chicken erythrocytes.

Dilutions of IgA were added to equal volumes of ³²P A/PR/8 (1124 HAU/ml, CPM:HAU 93), mixed and incubated for 60 min. at 25°C. 100ul of virus/antibody was inoculated into suspensions of chicken erythrocytes (1 X 10⁸ cells). The levels of virus attachment were determined as previously described (section 6.5.4.). Also shown is the IgA neutralization profile (-----).

11.2.9. Pre-treatment of cells with neuraminidase and effect on A/PR/8 attachment.

The enhanced attachment of IgA-neutralized virus to organ culture cells may result from attachment to a different cell receptor, such as an IgA Fc cell receptor (discussed later). To test this, cells were pre-treated with neuraminidase before the addition of virus. Table 11.1. shows that pre-treatment of both organ culture and BHK cells with neuraminidase reduced the attachment of infectious virus.

Pre-treatment with neuraminidase reduced the attachment of IgA-neutralized A/PR/8 and infectious virus to BHK cells to a similar extent. In contrast the attachment of IgA-neutralized virus to organ culture cells was not greatly affected by neuraminidase pre-treatment. Apparently IgA-neutralized virus could attach to organ culture cells via a neuraminidase-resistant cell receptor which is clearly different from the sialic acid receptor used by infectious virus. This will be discussed below.

11.2.10. Effect of neutralization by IgA on the internalization of A/PR/8.

It was of interest to investigate whether the virus that had attached to the cells remained external to the cell (i.e. sensitive to removal by neuraminidase) or was internalized (i.e. resistant to removal by neuraminidase).

Table 11.2. shows that the resistance of attached infectious virus to neuraminidase, using either BHK or tracheal organ culture cells, increased with time and internalization of virus had occurred.

Table 11.1. Effect of pre-treating cells with neuraminidase on the attachment of A/PR/8.

Cell type	Inoculum ^a	Amount of ³² P-A/PR/8 attached to:		Reduction in attachment due to neuraminidase treatment (%)
		Untreated cells	Neuraminidase treated cells ^b	
Tracheal organ culture cells	Infectious virus	11.17 x 10 ⁻⁶	4.83 x 10 ⁻⁶	56.7
	IgA(log HIU/HAU +0.02) ^d	13.96 x 10 ⁻⁶	11.19 x 10 ⁻⁶	19.9
	IgA(log HIU/HAU -0.96) ^d	4.24 x 10 ⁻⁶	4.12 x 10 ⁻⁶	2.8
	IgA(log HIU/HAU -1.96) ^d	9.31 x 10 ⁻⁶	8.43 x 10 ⁻⁶	9.5
BHK cells	Infectious virus	1.98 x 10 ⁻⁶	0.64 x 10 ⁻⁶	67.4
	IgA(log HIU/HAU +0.06) ^d	0.91 x 10 ⁻⁶	0.42 x 10 ⁻⁶	53.4
BHK cells	Infectious virus ^e	1.72 x 10 ⁻⁶	0.69 x 10 ⁻⁶	60.1
	IgA(log HIU/HAU -0.13) ^e	0.53 x 10 ⁻⁶	0.23 x 10 ⁻⁶	56.0

a. 1000 HAU/ml ³²P-A/PR/8 was incubated with an equal volume of antibody for 60 minutes at 25°C.

b. Expressed in terms of HAU per cell.

c. Cells pretreated with 0.5U neuraminidase for 60 minutes at 37°C. (section 6.5.5).

d. Ratio of HIU:HAU A/PR/8 in parenthesis.

Table 11.2. Internalization of IgA-neutralized A/PR/8 by cells.

IgA	Log ₁₀ HIU/HAU	Cell type	Neuraminidase resistance (%)		Increase ^c
			% attachment ^a	60' p.i.	
-	N/A	O.C.	100	40.1	97.0
+	+0.45	O.C.	595	29.3	61.9
-	N/A	BHK	100	40.3	71.3
+	+0.06	BHK	46	40.8	44.3
-	N/A	BHK	100	49.4	77.6
+	-0.13	BHK	31	44.5	43.0

a. Compared to the non-neutralized controls at 60 min. p.i.

b. Cells treated with neuraminidase for 60 min. at 37°C. (methods 5.5.)

c. Relative increase in neuraminidase resistance; value at 60 min. p.i. divided by the 5 min. p.i. value. No increase (i.e. value of 1.0) would indicate that no virus had been internalized.

Neutralization of the virus by IgA reduced the amounts of virus attaching to BHK cells as expected. Neuraminidase resistance of attached IgA-neutralized A/PR/8 did not increase with time, unlike infectious virus, thus neutralization by IgA impairs the internalization of the virus. It was also found that the two different IgA concentrations used to neutralize the virus both affected internalization on these cells.

The internalization of IgA-neutralized A/PR/8 by organ culture cells was less clear. A high IgA concentration used to neutralize the virus resulted in enhanced attachment (59%) and confirmed data in Figure 11.7. Not surprisingly at 60 min. p.i. the majority of IgA-neutralized virus was not detached by neuraminidase, either it was still attached by a neuraminidase-resistant receptor or had been internalized. However after 5 min. p.i. the IgA-neutralized virus was detached by neuraminidase. This was surprising as I had earlier demonstrated that the pre-treatment of organ culture cells with neuraminidase did not affect the attachment of IgA-neutralized virus (Table 11.1.). The possible reasons for this are discussed later (section 11.3.4.).

11.2.11. Neutralization of A/PR/8 by monomeric IgA and the effect on attachment to BHK cells.

The differential reduction of the IgA to monomeric IgA was too inefficient to allow a full range of experiments to be performed. Therefore only a preliminary investigation into neutralization and the effect on attachment of virus to BHK cells was carried out (Figure 11.10.).

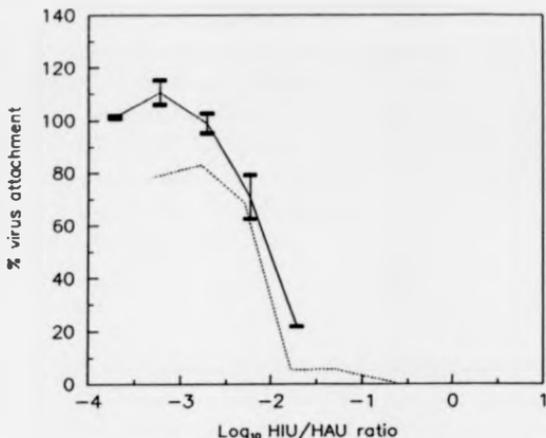


Fig.11.10. Neutralization of A/PR/8 by monomeric IgA and the effect on attachment to BHK cells.

Dilutions of mIgA were added to equal volumes of ³²P-A/PR/8 (942 HAU/ml, CPM:HAU 89), mixed and incubated for 60 min. at 25°C. 100ul of virus/antibody was inoculated onto each confluent BHK monolayer (5.8×10^6 cells). The levels of virus attachment were determined as previously described (section 6.5.4.). Also shown is the mIgA neutralization profile determined by plaque reduction using MDCK cells (-----).

The monomeric IgA efficiently neutralized A/PR/8 by plaque reduction; 50% neutralization occurred at a \log_{10} HIU:HAU ratio of -2.1 and more than 95% neutralization was achieved at the highest \log_{10} HIU:HAU ratio. The attachment of virus neutralized by monomeric IgA to BHK cells was reduced and the fall in attachment paralleled the loss of infectivity. At the highest \log_{10} HIU:HAU ratio (-1.6) the attachment of virus was low (80%) but the lack of available monomeric IgA prevented me from determining if attachment increased at high concentrations as described by Taylor & Dismock (1985a) using rat monomeric IgA.

11.3. Discussion.

My data are summarized in Table 11.3.. Direct comparisons between the data presented in this chapter with those presented earlier in the thesis need to be treated with caution because two different influenza virus strains were used.

11.3.1. Aggregation of A/PR/8 by IgA.

Infectious A/PR/8 was slightly more aggregated (average 2.0 virions/aggregate) compared to adpFPV/R (average <1.5 virions/aggregate). The rise in IgA-mediated aggregation of A/PR/8 coincided with neutralization but IgA caused less aggregation of A/PR/8 than previously found when examining IgG- or IgM-mediated aggregation of adpFPV/R. This was surprising, polymeric IgA would have been expected to cause more aggregation than IgG because of its greater valency. I can offer no reason as to why the IgA failed to substantially aggregate the virus.

Table 11.3. Summary of IgA inhibition of A/PR/8 attachment to various cell types.

	Antibody ratio (log ₁₀ HIU:HAU)
50% neutralization	-1.5
Tracheal organ culture cells	Initial inhibition of attachment Maximum inhibition of attachment Return to 80%+ attachment
BHK cells	Initial inhibition of attachment Maximum inhibition of attachment
Chicken erythrocytes	Initial inhibition of attachment Maximum inhibition of attachment

* In parenthesis is the percentage of virus which attached compared to the non-neutralized controls.

11.3.2. Attachment of A/PR/8 to chicken erythrocytes and the effect of IgA.

IgA reduced attachment of A/PR/8 to chicken erythrocytes by 90% and the fall in attachment occurred at the same \log_{10} HIU:HAU ratio required for neutralization. The amount of virus attaching remained low when the IgA concentration was increased to a \log_{10} HIU:HAU ratio of more than +1.0. The magnitude of the reduction was similar to that found using adFPV/R and IgM (section 10.), and greater than seen using adFPV/R and IgG (section 9.). The size of the polymeric IgA antibody molecules (dimers, trimers, tetramers etc.) are larger than IgG molecules and would result in more steric hindrance of attachment.

11.3.3. Attachment of A/PR/8 to EHK cells and the effect of IgA.

Taylor & Dimock (1985a) found that polymeric rat sIgA, when used at a concentration sufficient to neutralize the virus by 96.5%, reduced attachment of A/FPV/R to EHK cells by 60% at 37°C. They also found that the virus that did attach to the cells was not internalized and remained susceptible to removal using proteinase K. They concluded that the polymeric sIgA sterically hindered both the attachment and the internalization stages of infection and that neutralization resulted from a combination of these two effects.

My data confirm the findings of Taylor & Dimock (1985a) and extend them by using a range of antibody:virus ratios. I found the initial inhibition of attachment of A/PR/8 to EHK cells by the polymeric IgA occurred at the same \log_{10} HIU:HAU ratio (-1.5) as required for neutralization (Table 11.3.). This strengthens the proposition that the inhibition of attachment is largely

responsible for the loss of infectivity. The reduction in attachment cannot be the sole mechanism of neutralization because attachment was inhibited by a maximum of 70% whereas the virus was neutralized by 99.9%. The additional loss of infectivity probably results from impairment of the internalization process. IgA-neutralized virus that did attach to BHK cells was not internalized but remained on the cell surface and was susceptible to removal using neuraminidase (Table 11.2.). Therefore polymeric IgA neutralized influenza virus on BHK cells by a similar mechanism to IgM; i.e. by inhibiting virus attachment and impairing internalization.

Taylor & Dismock (1965a) found that monomeric rat IgA failed to inhibit the attachment of A/FPV/R to BHK cells and they proposed that monomeric IgA behaved in a manner similar to monomeric IgG which also failed to reduce virus attachment. I have previously demonstrated that IgG used at high log HIU:HAU ratios, failed to inhibit the attachment of adpFPV/R to BHK cells but at lower ratios the attachment of virus to BHK cells was reduced (section 9.). If monomeric IgA and IgG act in a similar manner then one would expect an inhibition in the attachment of virus to BHK cells to occur at low, sub-saturating log₁₀ HIU:HAU ratios. I found that low concentrations of monomeric IgA reduced the attachment of A/PR/8 to BHK cells by 80% and the fall in attachment coincided with neutralization. If influenza virus is neutralized by monomeric IgA via the same mechanisms as IgG one would predict that (i) increasing the log₁₀ HIU:HAU ratio would result in virus attachment returning towards 100% and (ii) monomeric IgA-neutralized virus that had attached would be internalized by BHK cells. Unfortunately these experiments could not be performed because of limitations in availability of monomeric IgA.

Taylor & Dismock (1985a) found that 96 μ g of rat sIgA yielded 47 μ g of monomeric IgA by differential reduction and there was negligible loss of HI activity. In contrast, using the same technique, I found no differential reduction of mouse polymeric IgA to monomeric IgA units, and only by adding iodoacetamine could any reduction be achieved which even then was inefficient (only 15% of the HI activity was recovered). The reasons for this are not clear; rat polyclonal sIgA seems inherently more susceptible to reduction by BME than mouse monoclonal IgA, and possibly the lack of a secretory component on the latter may somehow confer increased resistance to BME reduction.

11.3.4. Attachment of A/PR/8 to tracheal organ culture cells and the effect of IgA.

The attachment of IgA-neutralized virus to differentiated tracheal organ culture cells was very different to that found using the relatively dedifferentiated BHK cells. When low \log_{10} HIU:HAU ratios of IgA were used, the attachment of A/PR/8 to tracheal organ culture cells was reduced by 80%. The fall in attachment paralleled the loss of infectivity and both occurred at a similar \log_{10} HIU:HAU ratio, again suggesting cause and effect. In contrast to my findings with BHK cells, increasing the concentration of IgA resulted in increased attachment of virus to tracheal organ culture cells and at the highest \log_{10} HIU:HAU ratios attachment was enhanced compared to infectious virus. As there was no concomitant return in infectivity, the inhibition of attachment cannot account for the neutralization observed at these high IgA concentrations and therefore additional mechanisms must apply.

Others have demonstrated that IgA enhanced the attachment of virus to cells. Nguyen *et al.* (1986) used sIgA purified from porcine milk to neutralize transmissible gastroenteritis virus (TGEV) and found enhanced attachment of virus to both swine testis and pig kidney cells. The enhancement of attachment by sIgA was concentration dependant: there was no inhibition of attachment at low (0.2ug/ml) concentrations but attachment increased three fold at high (11ug/ml) concentrations. Incubation of the cells with sIgA prior to addition of virus did not enhance virus attachment. They argued on the basis of this finding that the increased attachment was not due to sIgA-neutralized virus binding via an Fc receptor but instead resulted from aggregation, such that the attachment of one virus particle carried along with it many more virions. I feel this particular conclusion needs to be treated with caution as they merely titrated the residual infectivity of the supernatants instead of measuring radioactive counts attaching, which would have been the more logical and sensitive assay. The sIgA-neutralized TGEV that had attached to the cells remained susceptible to proteinase K removal following incubation at 37 C for 60 min. and therefore was not internalized.

I found the removal of the sialic acid by treating the cells with neuraminidase before inoculation failed to reduce the attachment of IgA neutralized virus (Table 11.1.), suggesting that the virus was binding via a neuraminidase resistant receptor. Possible explanations are that: (i) Treatment of the cells with neuraminidase altered the charge on the cells which resulted in spurious interactions between the IgA-neutralized virus and the cells. This explanation is unlikely because a non-specific charge effect would be expected to apply to other cell types and no such effect was noted with BHK cells. (ii) The IgA-neutralized virus may have attached

to the poly-Ig receptor present on epithelial cells which selectively transports polymeric IgA to the mucosal lumen (reviewed by Underdown & Schiff, 1986). This explanation is unlikely because the poly-Ig receptor is cleaved during the course of transport, although the cellular location of cleavage is not known. The IgA transport pathway is constitutive and SC component is found to be secreted in a free form even if IgA had not bound to the poly-Ig receptor. Therefore, if receptor cleavage occurred intracellularly then IgA-neutralized virus would not attach to the cells. If receptor cleavage occurred at the apical surface of cells then IgA-neutralized virus would attach but would probably be released immediately by protease action together with SC component. (iii) The IgA-neutralized virus may have attached to the cells via a Fc receptor. The incidence and functions of Fc receptors for IgA are poorly understood compared to receptors specific for IgG (reviewed Unkeles *et al.*, 1988). IgA Fc receptors have been found on cells of the immune system (reviewed by Kilian *et al.*, 1988; Underdown & Schiff, 1986). Albrechtsen *et al.* (1988) found a receptor (Mr 60,000) on human blood PMN cells that bound monomeric serum IgA1. Gorter *et al.* (1988) demonstrated that the binding of serum IgA to rat peritoneal macrophages was isotype specific and could not be blocked using either IgG or IgM antibodies. Briere *et al.* (1988) showed that human sIgA bound to and induced expression of IgA-specific receptors on T cells (both CD4+ and CD8+) isolated from the tonsils but not from peripheral blood. The induction of receptors did not occur if serum IgA was used, suggesting that the polymeric structure of IgA or presence of the SC component was an important factor. The presence of an IgA Fc receptor on cells prepared from tracheal organ cultures could also explain the variations I found both in the amount of enhanced attachment achieved (105-250%), and in the concentration of IgA required for increased

attachment (\log_{10} HIU:HAU ratio of -0.3 to $+0.4$) (Figure 11.7.). The variation may be due to the number of IgA receptors present on different tracheal organ culture cell preparations because in both experiments the same virus and antibody were used. Unfortunately I lacked the time to investigate the possible Fc-mediated mechanism of attachment. This mechanism could have been investigated using the techniques employed by Peiris *et al.* (1982) to examine ADE of flavivirus infection of macrophages: i.e. by blocking Fc-mediated attachment using anti-Fc receptor antibodies and by papain treating the IgA to give monovalent Fab' fragments, which are unable to interact with Fc-receptors. Another approach would have been to examine whether radiolabelled IgA bound to tracheal organ culture cells.

I attempted to determine if the IgA-neutralized virus that had attached to the organ culture cells was internalized. If the IgA-neutralized virus attached to a different (neuraminidase-resistant) receptor, it would have been expected that treatment with neuraminidase of cells to which IgA-neutralized virus had already attached would be ineffective. I found this not to be the case, both infectious and IgA-neutralized A/PR/8 that had attached to organ culture cells were susceptible to removal by neuraminidase (Table 11.2.). If the IgA-neutralized virus had attached to a sialic acid cell receptor, then removal of virus by neuraminidase would only result in re-attachment via the neuraminidase resistant cell receptor. Possible explanations for my findings include: (i) release by neuraminidase into the medium of a population of IgA-neutralized virus that had bound to sialic acid receptors. The released virus may have reattached to neuraminidase-resistant receptors but the rate of reattachment was reduced because the addition of the neuraminidase solution diluted the medium, or (ii) the receptor to which the IgA-neutralized virus attached was sensitive

to the action of neuraminidase only after virus had bound and not before. This could be possible because in man the IgA Fc receptor is heavily glycosylated (Albrechtsen *et al.*, 1988). If the situation in the mouse is similar then this explanation could be tested by attaching radiolabelled IgA antibodies to the cells prior to the treatment with neuraminidase. If the above explanation is correct, then the bound IgA should be eluted from the cell surface into the medium following neuraminidase treatment. It should be noted that the hinge region of the human IgA molecule (Gorter *et al.*, 1988) and the SC component (Kilian *et al.*, 1988) are also heavy glycosylated and therefore may be the targets for neuraminidase action.

The fact that the neuraminidase resistance of attached IgA-neutralized virus increased by two-fold with time indicated that the virus was internalized. This suggestion should be treated with caution until further investigations into receptor-mediated IgA-neutralized virus attachment have been carried out.

11.3.5. Mechanism of neutralization of influenza virus by IgA.

Polymeric IgA caused little aggregation of A/PR/8 (compared with adpFPV/R and IgG or IgM sections 9.3.1. and 10.3.1.) suggesting that this plays only a minor role in neutralization. My data show that at all ratios of antibody:virus the mechanism of neutralization of influenza virus by polymeric IgA on BHK cells involves both the inhibition of attachment of virus to cells and its subsequent internalization and support the more limited data of Taylor & Dimmock (1985a). The large molecular sizes of the polymeric IgA molecules (which range from dimeric to larger polymeric units) probably hinder the attachment and internalization of A/PR/8

sterically in manner similar to that found using adpFPV/R and IgM (section 10.3.4.). Problems in its preparation prevented any conclusions concerning monomeric IgA from being made and further work needs to be done in this area.

Reduction in attachment of A/PR/8 to tracheal organ culture cells and neutralization occurred at the same IgA concentration suggesting that the inhibition of virus attachment is a mechanism of IgA neutralization. Increasing the amount of antibody resulted in an enhancement of attachment but no return of infectivity. It was possible that this was mediated through Fc receptors. If so then the binding of sub-neutralizing amounts of antibody to the virus should enhance virus attachment and infectivity (ADE), as with other viruses (section 5.3.). This was not found; sub-neutralizing amounts of IgA did not enhance A/PR/8 attachment and sub-saturating amounts of IgA neutralized the virus and substantially reduced attachment. The reasons why enhanced attachment was only apparent using high IgA:virus ratios are not clear. Further work, as described above, has to be done to examine the mechanism by which IgA-neutralized virus attaches to tracheal organ culture cells, and to determine whether or not it is mediated by an IgA-specific Fc receptor. To my knowledge this would be the first report of an IgA-specific Fc receptor on a cell which does not belong to the immune system. Until this work is done no firm conclusions can be drawn relating to internalization of IgA-neutralized virus by tracheal organ culture cells or the mechanism of neutralization operating at high IgA:virus ratios.

REFERENCES.

- Abou-Donia H., Jennings R. & Potter C.W. (1980). Growth of influenza A viruses in hamsters. *Arch.Virol.*, **65**, 99-107.
- Air G.M., Richie L.R., Laver W.G. & Colman P.M. (1985). Gene and protein sequence of an influenza neuraminidase with haemagglutinin activity. *Virol.*, **143**, 117-122.
- Air G.M., Webster R.G., Colman P.M. & Laver W.G. (1987). Distribution of sequence differences in influenza NS neuraminidase of tern and whale viruses and crystallization of the whale neuraminidase complexed with antibodies. *Virol.*, **160**, 346-354.
- Albrecht M., Yeaman G.R. & Kerr M.A. (1988). Characterisation of the IgA receptor from human polymorphonuclear leucocytes. *Immunol.*, **64**, 201-205.
- Ali M., Maassab H.F., Jennings R. & Potter C.W. (1982a). Infant rat model of attenuation for recombinant influenza viruses prepared from cold-adapted attenuated A/Ann Arbor/6/60. *Infect. & Immunity*, **38**, 610-619.
- Ali M.J., Teh C.Z., Jennings R. & Potter C.W. (1982b). Transmissibility of influenza viruses in hamsters. *Arch.Virol.*, **72**, 187-197.
- Alseide J.D. & Tyrrell D.A.J. (1967). The morphology of three previously uncharacterized human respiratory viruses that grow in organ culture. *J.Gen.Virol.*, **1**, 175-178.
- Amit A.G., Mariuzza R.A., Phillips S.E.V. & Poljak R.J. (1985). Three-dimensional structure of an antigen-antibody complex at 6A resolution. *Nature (London)*, **313**, 156-158.
- Anderson K.B. (1987). Cleavage fragments of the retrovirus surface glycoprotein gp70 during virus entry. *J.Gen.Virol.*, **68**, 2193-2202.
- Anderson R.G.W., Brown M.S. & Goldstein J.L. (1977). Role of the coated endocytotic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell*, **10**, 351-364.
- Anderson L.J., Bingham P. & Hierholzer J.C. (1988). Neutralization of respiratory syncytial virus by individual and mixtures of F and G protein monoclonal antibodies. *J.Virol.*, **62**, 4232-4238.
- Andes E.M., Katz J.M., Jackson D.C. & White D.O. (1981). *In vitro* antibody responses to influenza virus: II. Specificity of helper T cells recognizing haemagglutinin. *J.Immunol.*, **127**, 669-672.
- Archibald D.W., Barr C.E., Tarosian J.P., McLane M.F. & Essex M. (1987). Secretory IgA antibodies to human immunodeficiency virus in parotid saliva of patients with AIDS and AIDS-related complex. *J.Inf.Dis.*, **155**, 793-796.
- Austin F.J. & Webster R.G. (1986). Antigenic mapping of an avian H1 influenza virus haemagglutinin and interrelationships of H1 viruses from humans, pigs and birds. *J.Gen.Virol.*, **67**, 983-992.
- Barrett T., Wolstenholme A.J. & Mahy B.W.J. (1979). Transcription and replication of influenza virus RNA. *Virol.*, **98**, 211-225.

- Bartles J.R. & Hubbard A.L. (1988). Plasma membrane protein sorting in epithelial cells: do secretory pathways hold the key? *TIBS*, **13**, 181-184.
- Basak S., Compans R.G. & Oldstone M.B.A. (1984). Restricted mobility of influenza haemagglutinin on HeLa cell plasma membranes. pp361-364 "segmented negative strand viruses". Eds. Compans R.W. & Bishop D.H.L.. Publ. Academic Press, New York.
- Bean Jr. W.J. & Simpson R.W. (1973). Primary transcription of the influenza virus genome in permissive cells. *Virology*, **56**, 646-651.
- Bean Jr W.J., Cox N.J. & Kendal A.P. (1980). Recombination of human influenza A viruses in nature. *Nature (London)*, **284**, 638-640.
- Beaton A.R. & Krug R.M. (1984). Synthesis of the templates for influenza virion RNA replication *in vitro*. *Proc.Natl.Acad.Sci.(USA)*, **81**, 4682-4686.
- Beaton A.R. & Krug R.M. (1986). Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5' capped end. *Proc.Natl.Acad.Sci.(USA)*, **83**, 6282-6286.
- Becht H., Hammerling U. Rott R. (1971). Undisturbed release of influenza virus in the presence of univalent antineuraminidase antibodies. *Virology*, **46**, 337-343.
- Beebe D.P., Schreiber R.D & Cooper N.R. (1983). Neutralization of influenza virus by normal human sera: mechanisms involving antibody and complement. *J.Immunol.*, **130**, 1317-1322.
- Belsho R.B., Smith M.H., Hall C.B., Bretts R. & Hay A.J. (1988). Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. *J.Virology*, **62**, 1508-1512.
- Beyer W.E.P., Ruzgrok R.W.H., van Driel H. & Masural N. (1986). Influenza virus strains with a fusion threshold of pH 5.5 or lower are inhibited by amantadine. *Arch.Virology*, **90**, 173-181.
- Bjorkman P.J., Saper M.A., Sarracini B., Bennett W.S., Strominger J.L. & Wiley D.C. (1987a). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature (London)*, **329**, 506-512.
- Bjorkman P.J., Saper M.A., Sarracini B., Bennett W.S., Strominger J.L. & Wiley D.C. (1987b). The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature (London)*, **329**, 512-518.
- Blaas D., Fetzelt E. & Kuechler E. (1982). Identification of the cap binding protein of influenza virus. *Nucleic Acid Res.*, **10**, 4803-4812.
- Boech F.X., Orlich M., Klenk H-D. & Rott R. (1979). The structure of the haemagglutinin, a determinant for the pathogenicity of influenza viruses. *Virology*, **113**, 197-207.

- Blaskovic P., Rhodes A.J., Doane F.W. & Labzoffsky N.A. (1972a). Infection of chick embryo tracheal organ cultures with influenza A2 (Hong Kong) virus. II. Electron microscopy. *Arch.Virol.*, **36**, 250-266.
- Blaskovic P., Rhodes A.J. & Labzoffsky N.A. (1972b). Combined cell and organ culture for isolation of influenza A2 viruses. *Arch.Virol.*, **39**, 299-302.
- Blaskovic P., Rhodes A.J. & Labzoffsky N.A. (1972c). Infection of chick embryo tracheal organ cultures with influenza A2 (Hong Kong) virus. I. Cytopathology, histopathology, immunofluorescence, hemadsorption and titration of the released infectious progeny virus. *Arch.Virol.*, **37**, 104-113.
- Blaskovic P., Bohman K., Rhodes A.J. & Labzoffsky N.A. (1973). The effect of different media on maintenance of chick embryo tracheal organ cultures and replication of influenza virus. *Arch.Virol.*, **42**, 210-213.
- Boudresault A. (1979). Live influenza vaccine: Screening of attenuated virus strains by a 50R ciliary activity inhibition test in organ cultures of ferret trachea. *Can.J.Microbiol.*, **25**, 279-284.
- Bouloy M., Plotch S.J. & Krug R.M. (1978). Globin mRNAs are primers for the transcription of influenza virus RNA *in vitro*. *Proc.Natl.Acad.Sci.(USA)*, **75**, 4886-4890.
- Bouloy M., Plotch S.J. & Krug R.M. (1980). Both the 7-methyl and the 2'-O-methyl groups in the cap of mRNA strongly influence its ability to act as primer for influenza virus RNA transcription. *Proc.Natl.Acad.Sci.(USA)*, **77**, 3952-3956.
- Braas J., Ulanen I. & Krug R.M. (1983). Molecular model of a eucaryotic transcription complex: Functions and movements of influenza P proteins during capped RNA-primed transcription. *Cell*, **34**, 609-618.
- Braas-Markson J., Jaudon C. & Krug R.M. (1985). Expression of a functional influenza viral cap-recognizing protein by using a bovine papilloma virus vector. *Proc.Natl.Acad.Sci.(USA)*, **82**, 4326-4330.
- Braciale T.J., Braciale V.L., Henkel T.J., Sasmbrook J. & Gething M-J. (1984). Cytotoxic T lymphocyte recognition of the influenza hemagglutinin gene product expressed by DNA-mediated gene transfer. *J.Exp.Med.*, **159**, 341-354.
- Braciale T.J., Morrison L.A., Sweetser M.T., Sasmbrook J., Gething M-J. & Braciale V.L. (1987a). Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. *Immunol.Rev.*, **99**, 95-114.
- Braciale T.J., Braciale V.L., Winkler M., Stroynowski I., Hood L., Sasmbrook J. & Gething M-J. (1987b). On the role of the transmembrane anchor sequence of influenza hemagglutinin in target cell recognition by class I MHC-restricted, hemagglutinin-specific cytolytic T lymphocytes. *J.Exp.Med.*, **166**, 678-692.

- Breschkin A.M., Ahern J. & White D.O. (1981). Antigenic determinants of influenza virus haemagglutinin. VIII. Topography of the antigenic regions of influenza virus haemagglutinin determined by competitive radioimmunoassay with monoclonal antibodies. *Virology*, **113**, 130-140.
- Briçon P., Rombault B. & Boeys A. (1985a). Hit-and-run neutralization of poliovirus. *J.Gen.Virology*, **66**, 2495-2499.
- Briçon P., Thomas A.A.M. & Boeys A. (1985b). Lack of quantitative correlation between the neutralization of poliovirus and the antibody-mediated pH shift of the virions. *J.Gen.Virology*, **66**, 609-613.
- Briere F., Pallard X. & De Vries J.E. (1988). Induction of the receptor for the Fc portion of IgA by secretory IgA on human T cell lines and T cell clones. *Eur.J.Immunol.*, **18**, 445-451.
- Brown L.E., French R.A., Gawler J.M., Jackson D.C., Dyal-Smith M.L., Anders E.M., Tregear G.W., Duncan L., Underwood P.A. & White D.O. (1988). Distinct epitopes recognized by I-Ad-restricted T-cell clones within the antigenic site E on influenza virus haemagglutinin. *J.Virology*, **62**, 305-312.
- Bukrinskaya A.G., Vorkunova N.K. & Narnanbetova R.A. (1980). Rimantadine hydrochloride blocks the second step of influenza virus uncoating. *Arch.Virology*, **66**, 275-282.
- Bukrinskaya A.G., Vorkunova N.K., Kornilayeva G.V., Narnanbetova R.A. & Vorkunova G.K. (1982a). Influenza virus uncoating in infected cells and effect of rimantadine. *J.Gen.Virology*, **60**, 49-59.
- Bukrinskaya A.G., Vorkunova N.K. & Puhkarskaya N.L. (1982b). Uncoating of a rimantadine-resistant variant of influenza virus in the presence of rimantadine. *J.Gen.Virology*, **60**, 61-66.
- Burton R.D. (1986). Is IgM-like dislocation a common feature of antibody function?. *Immunol.Today*, **7**, 165-167.
- Cambridge G., MacKenzie J.S. & Keast D. (1976). Cell-mediated immune responses to influenza virus infections in mice. *Infect. & Immunity*, **132**, 36-43.
- Campbell D., Sweet C. & Smith H. (1979). Comparisons of virulence of influenza virus recombinants in ferrets in relation to their behaviour in man and their genetic constitution. *J.Gen.Virology*, **44**, 37-44.
- Casali P., Rice G.P.A. & Oldstone M.B.A. (1984). Viruses disrupt functions of human lymphocytes. Effects of measles virus and influenza virus on lymphocyte-mediated killing and antibody production. *J.Exp.Med.*, **159**, 1322-1337.
- Casino I., Benedetto A., Mazzilli M.C., Amici C., Caspariando M.P., Testa L., Zaniratti S., Chessa L. & Gandini E. (1986). A nonneutralizing human IgM monoclonal antibody inhibiting haemagglutination of H3N2 influenza A strains. *Hybridoma*, **5**, 307-318.

- Caton A.J., Brownlee G.G., Yewdell J.W. & Gerhard W. (1982). The antigenic structure of the influenza virus A/PR/8/34 haemagglutinin (H1 subtype). *Cell*, **31**, 417-427.
- Cavanagh D. & Davis P.J. (1986). Coronavirus IBV: Removal of spike glycopolypeptide S1 by urea abolishes infectivity and haemagglutination but not attachment to cells. *J.Gen.Virol.*, **67**, 1443-1448.
- Cavanagh D., Mitkis F., Sweet C., Collie M.H. & Smith H. (1979). The localization of influenza virus in the respiratory tract of ferrets: Susceptible nasal mucosa cells produce and release more virus than susceptible lung cells. *J.Gen.Virol.*, **44**, 505-514.
- Cavanagh D., Davis P.J., Darbyshire J.H. & Peters R.W. (1986). Coronavirus IBV: Virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. *J.Gen.Virol.*, **67**, 1435-1442.
- Calada F., Schumaker V.N. & Sercarz E.E. (1983). The changes in protein antigen conformation induced by antibody, pp211-212 "Protein conformation as an immunological signal". Eds. Calada F., Schumaker V.N. & Sercarz E.E. Publ. Plenum, New York.
- Chanas A.C., Gould E.A., Clegg J.C.S. & Varas M.G.R. (1982). Monoclonal antibodies to Sindbis virus glycoprotein E1 can neutralize, enhance infectivity, and independently inhibit haemagglutination of haemolysis. *J.Gen.Virol.*, **59**, 37-46.
- Chen K-S. & Quinnan Jr G.V. (1988). Induction, persistence and strain specificity of haemagglutinin-specific secretory antibodies in lungs of mice after intragastric administration of inactivated influenza virus vaccines. *J.Gen.Virol.*, **69**, 2779-2784.
- Chen K-S., Burlington D.B. & Quinnan Jr G.V. (1987). Active synthesis of haemagglutinin-specific immunoglobulin A by lung cells of mice that were immunized intragastrically with inactivated influenza virus vaccine. *J.Virol.*, **61**, 2150-2154.
- Cherry J.D. & Taylor-Robinson D. (1970). Large-quantity production of chick embryo tracheal organ cultures and use in virus and mycoplasma studies. *App.Microbiol.*, **12**, 658-662.
- Choppin P.W. & Pons M.W. (1970). The RNAs of infective and incomplete influenza viruses grown in MDCK and HeLa cells. *Virology*, **42**, 603-610.
- Clegg J.C.S., Chanas A.C. & Gould E.A. (1983). Conformational changes in Sindbis virus E1 glycoprotein induced by monoclonal antibody binding. *J.Gen.Virol.*, **64**, 1121-1126.
- Cogliano R.C. & Saell Jr P.A. (1978). Specific immunity to influenza virus in ferret organ cultures. *B.J.Exp.Pathol.*, **59**, 21-31.
- Colman P.M., Varghese J.N. & Laver W.G. (1983). Structure of the catalytic and antigenic sites in influenza virus neuraminidase. *Nature (London)*, **303**, 41-44.

- Colman P.M., Lever W.G., Varghese J.M., Baker A.T., Tulloch P.A., Air G.M. & Webster R.G. (1987). Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. *Nature* (London), **326**, 358-363.
- Colonna R.J., Callahan P.L., Leippe D.M., Ruckert R.R. & Tomassini J.E. (1989). Inhibition of rhinovirus attachment by neutralizing monoclonal antibodies and their Fab fragments. *J.Virol.*, **63**, 36-42.
- Compans R.W. & Dimmock N.J. (1969). An electronmicroscopic study of single-cycle infection of chick embryo fibroblasts by influenza virus. *Virology*, **29**, 499-515.
- Compans R.W., Dimmock N.J. & Meier-Ewert H. (1969). Effect of antibody to neuraminidase on the maturation and haemagglutinating activity of influenza A2 virus. *J.Virology*, **3**, 528-534.
- Compans R.W., Klentz H.-D., Caligiuri L.A. & Choppin P.W. (1970). Influenza virus proteins. I. Analysis of polypeptides of the virion and identification of spike glycoproteins. *Virology*, **42**, 880-889.
- Cook R.F., Avery R.J. & Dimmock N.J. (1979). Infection of chicken erythrocytes with influenza and other viruses. *Infect. & Immunity*, **22**, 396-402.
- Couch R.B. & Kasel J.A. (1983). Immunity to influenza in man. *Ann.Rev.Microbiol.*, **37**, 529-549.
- Couch R.B., Webster R.G., Kasel J.A. & Cate T.R. (1979). Efficacy of purified influenza subunit vaccines and the relation to the major antigenic determinants on the haemagglutinin molecule. *J.Inf.Dis.*, **140**, 553-559.
- Coutelier J-P. & van Snick J. (1988). Neutralization and sensitization of lactate dehydrogenase-elevating virus with monoclonal antibodies. *J.Gen.Virology*, **69**, 2097-2100.
- Coutelier J-P., van der Logt J.T.M., Heessen F.W.A., Warnier G. & van Snick J. (1987). IgG2a restriction of murine antibodies elicited by viral infections. *J.Exp.Med.*, **165**, 64-69.
- Cox N.J., Black R.A. & Kendal A.P. (1989). Pathways of evolution of influenza A (H1N1) viruses from 1977 to 1986 as determined by oligonucleotide mapping and sequencing studies. *J.Gen.Virology*, **70**, 299-313.
- Cretescu L., Beare A.S. & Schild G.C. (1978). Formation of antibody to matrix protein in experimental human influenza A virus infections. *Inf. Immun.*, **22**, 322-327.
- Crowell R.L. (1987). Cellular receptors in virus infections. *ASM News*, **53**, 422-425.
- Dahlgren U.I.H., Ahlstedt S. & Hanson L.A. (1987). The localization of the antibody response in milk or bile depends on the nature of the antigen. *J.Immunol.*, **138**, 1397-1402.

- Daniels R.S., Douglas A.R., Skehel J.J. & Wiley D.C. (1983). Analyses of the antigenicity of influenza haemagglutinin at the pH optimum for virus-mediated membrane fusion. *J.Gen.Virol.*, **64**, 1657-1662.
- Daniels R.S., Jefferies S., Yates P., Schild G.C., Rogers G.M., Paulson J.C., Wharton S.A., Douglas A.R., Skehel J.J. & Wiley D.C. (1987). The receptor-binding and fusion properties of influenza virus variants selected using anti-haemagglutinin monoclonal antibodies. *EMBO J.*, **6**, 1459-1465.
- Darbyshire J.H., Rowell J.G., Cook J.K.A. & Peters R.W. (1979). Taxonomic studies of avian infectious bronchitis virus using neutralization tests in tracheal organ cultures. *Arch.Virol.*, **61**, 227-238.
- Davey J., Colman A. & Dismock N.J. (1985). Location of influenza virus M₁ NP and M₂ proteins in microinjected cells. *J.Gen.Virol.*, **66**, 2319-2334.
- Davey J., Hurlley S.M. & Warren G. (1985). Reconstitution of an endocytic fusion event in a cell-free system. *Cell.*, **43**, 643-652.
- Davies D.R. & Metzger H. (1983). Structural basis of antibody function. *Ann.Rev.Immunol.*, **1**, 87-117.
- Davis A.C. & Schulman M.J. (1989). IgM - Molecular requirements for its assembly and function. *Immunol.Today*, **10**, 118-128.
- Davis J.F. & Bucher D.J. (1981). Interaction of neuraminidase with M-protein in liposomes. pp203-207. "The replication of negative strand viruses" Ed. Bishop D.H.L. & Compans R.W. Publ. Elsevier, North Holland.
- Della-Porta A.J. & Westaway E.G. (1977). A multi-hit model for the neutralization of animal. *J.Gen.Virol.*, **38**, 1-19.
- Desselberger U. & Palese P. (1978). Molecular weights of RNA segments of influenza A and B viruses. *Virol.*, **88**, 394-399.
- Detjen B.M., St. Angelo C., Katze M.G. & Krug R.M. (1987). The three influenza virus polymerase (P) proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. *J.Virol.*, **61**, 16-22.
- Dhar R. & Ogra P.L. (1985). Local immune responses. *Br.Med.Bull.*, **41**, 28-33.
- Diaz-Rodríguez P. & Boudreaux A. (1982). Inhibition of ciliary activity in organ cultures of ferret trachea in reference to genetic and biological characters of influenza virus strains. *Can.J.Microbiol.*, **28**, 809-813.
- Dietzschold B., Tollis M., Lafon M., Munner W.H. & Koprowski H. (1987). Mechanisms of rabies virus neutralization by glycoprotein-specific monoclonal antibodies. *Virol.*, **161**, 29-36.
- Dismock N.J. (1982). Initial stages in infection with animal viruses. *J.Gen.Virol.*, **52**, 1-22.

- Dismock N.J. (1984). Mechanisms of neutralization of animal viruses. *J.Gen.Virol.*, **65**, 1015-1022.
- Dismock N.J. (1987). Multiple mechanisms of neutralization of animal viruses. *TIBS*, **12**, 70-75.
- Dismock N.J. & Watson D.H. (1969). Proteins specified by influenza virus in infected cells: Analysis by polyacrylamide gel electrophoresis of antigens not present in the virus particle. *J.Gen.Virol.*, **3**, 499-509.
- Dismock N.J., Cook R.F., Bean W.J. & Wignall J.M. (1981). Cellular and viral control processes affect the expression of matrix protein during influenza virus infection of avian erythrocytes. pp345-351 "The replication of negative strand viruses". Ed. Bishop D.H.L. & Compans R.W., Elsevier, North Holland.
- Doherty P.C., Effros R.B. & Bannink J. (1977). Heterogeneity of the cytotoxic response of thymus-derived lymphocytes after immunization with influenza viruses. *Proc.Natl.Acad.Sci.(USA)*, **74**, 1209-1213.
- Doms R.W. & Helenius A. (1986). Quaternary structure of influenza virus hemagglutinin after acid treatment. *J.Virol.*, **60**, 833-839.
- Doms R.W., Helenius A. & White J. (1985). Membrane fusion activity of the influenza virus hemagglutinin. *J.Biol.Chem.*, **260**, 2973-2981.
- Doms R.W., Gething M-J., Hennesberry J., White J. & Helenius A. (1986). Variant influenza virus hemagglutinin that induces fusion at elevated pH. *J.Virol.*, **57**, 603-613.
- Dourasashkin R.R. & Tyrrell D.A.J. (1970). Attachment of two myxoviruses to ciliated epithelial cells. *J.Gen.Virol.*, **2**, 77-88.
- Dowdle W.R., Downie J.C. & Lever W.G. (1974). Inhibition of virus release by antibodies to surface antigens of influenza viruses. *J.Virol.*, **13**, 269-275.
- Drescher J., Verhagen W., Flik J. & Stachan R. (1987). Cooperative investigation of monoclonal and polyclonal antibodies directed against strain-specific and common antigenic sites on influenza H1N1 virus hemagglutinin. *Intervirology*, **27**, 205-217.
- Edwards K.M., Snyder P.N., Stephens D.S. & Wright P.F. (1986). Human adenoid organ culture: A model to study the interaction of influenza A with human nasopharyngeal mucosa. *J.Inf.Dis.*, **153**, 41-47.
- Eisenlohr L.C., Gerhard W. & Hockett C.J. (1987). Role of receptor-binding activity of the viral hemagglutinin molecule in the presentation of influenza virus antigens to helper T cells. *J.Virol.*, **61**, 1375-1383.
- Eisni E.A., Kao S-Y., Lewis A.J., Crainic R. & Wimmer E. (1983a). Functional basis of poliovirus neutralization determined with monospecific neutralizing antibodies. *J.Virol.*, **46**, 466-474.

- Ennis E.A., Ostapchuk P., Wismer E. (1983b). Bivalent attachment of antibody onto poliovirus leads to conformational alteration and neutralization. *J.Virol.*, **49**, 547-550.
- Ennis A. (1982). Some newly recognized aspects of resistance against and recovery from influenza. *Arch.Virol.*, **73**, 207-217.
- Ennis F.A., Yi-Hua Q. & Schild G.C. (1982). Antibody and cytotoxic T lymphocyte responses of humans to live and inactivated influenza vaccines. *J.Gen.Virol.*, **58**, 273-281.
- Ennis F.A., Rook A.H., Yi-Hua Q., Schild G.C., Riley D., Pratt R. & Potter C.W. (1981a). HLA-restricted virus-specific cytotoxic T-lymphocyte responses to live and inactivated influenza vaccines. *Lancet*, **2**, 887-891.
- Ennis F.A., Meager A., Beare A.S., Yi-Hua Q., Riley D., Schwartz G., Schild G.C. & Rook A.H. (1981b). Interferon induction and increased natural killer-cell activity in influenza infections in man. *Lancet*, **2**, 891-893.
- Fanger M.W., Shen L., Graziano R.F. & Guyre P.W. (1989). Cytotoxicity mediated by human Fc receptors for IgG. *Immunol.Today*, **10**, 92-99.
- Feinstein A. & Mann E.A. (1969). Conformation of the free and antigen-bound IgM antibody molecule. *Nature (London)*, **224**, 1307-1309.
- Fenton R.J., Jennings R. & Potter C.W. (1977). Differential response of ferrets to infection with virulent and avirulent influenza viruses: A possible marker of virus attenuation. *Arch.Virol.*, **55**, 55-66.
- Feinstein A., Richardson N. & Tausig M.J. (1986). Immunoglobulin flexibility in complement activation. *Immunol.Today*, **7**, 169-174.
- Frankova V. (1975). Inhalatory infection of mice with influenza A₀/PR8 virus. I. The site of primary virus replication and its spread in the respiratory tract. *Acta Virol.*, **19**, 29-34.
- Frankova V. & Rychterova V. (1975). Inhalatory infection of mice with influenza A₀/PR8 virus. II. Detection of the virus in the blood and extrapulmonary organs. *Acta Virol.*, **19**, 35-40.
- Fuller A.O. & Spear P.G. (1985). Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack of inhibition by potent neutralizing antibodies. *J.Virol.*, **55**, 475-482.
- Gentry S.E., Culp D.J., Roberts Jr. N.J., Marin M.G., Simons R.L. & Latchney L.R. (1988). Influenza virus infection of tracheal gland cells in culture. *J.Virol.*, **62**, 1524-1529.
- Gerhard W. & Webster R.G. (1978). Antigenic drift in influenza A virus. I. Selection and characterization of antigenic variants of A/PR/8/34 (H₁N₁) influenza virus with monoclonal antibodies. *J.Exp.Med.*, **148**, 363.

- Gerhard W., Yevdell J.W., Frankel M.E. & Webster R.G. (1981). Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. *Nature (London)*, **290**, 713-717.
- Germain R.N. (1986). The ins and outs of antigen processing and presentation. *Nature (London)*, **322**, 687-698.
- Gerna G., Revello M.G., Davis M., Petruzzelli E., Achilli G., Percivelle E. & Torsellini M. (1987). Synergistic neutralization of rubella virus by monoclonal antibodies to viral haemagglutinin. *J.Gen.Virol.*, **68**, 2007-2012.
- Gething M-J., Dome R.W., York D. & White J. (1986). Studies on the mechanism of membrane fusion: Site-specific autagenesis of the haemagglutinin of influenza virus. *J.Cell Biol.*, **102**, 11-23.
- Gething M-J., McCasmon K. & Sambrook J. (1986). Expression of wild-type and mutant forms of influenza haemagglutinin: The role of folding in intracellular transport. *Cell*, **46**, 939-950.
- Gienez H.B., Kair H.M. & Cash P. (1989). *In vitro* enhancement of respiratory syncytial virus infection of U937 cells by human sera. *J.Gen.Virol.*, **70**, 89-96.
- Gitelman A.K., Kaverin N.V., Kharitononkov I.G., Rudneva I.A., Sklyanskaya E.L. & Zhdanov V.M. (1986). Association of the haemagglutination inhibition and the infectivity neutralization in the reactions in influenza A/USSR/90/77 (H1N1) virus variants with monoclonal antibodies. *J.Gen.Virol.*, **67**, 2247-2251.
- Glezen W.P., Decker M., Joseph S.W. & Mercreeedy jr. R.G. (1987). Acute respiratory disease associated with influenza epidemics in Houston, 1981-1983. *J.Inf.Dis.*, **155**, 1119-1126.
- Gollins S.W. & Porterfield J.S. (1984). Flavivirus infection enhancement in macrophages: radioactive and biological studies on the effect of antibody on viral fate. *J.Gen.Virol.*, **65**, 1261-1272.
- Gollins S.W. & Porterfield J.S. (1986). A new mechanism for the neutralization of enveloped viruses by antiviral antibody. *Nature (London)*, **321**, 244-246.
- Gorev N.E. (1975). The effect of species and organ-specific features of tissue systems on the reactogenic and immunogenic properties of influenza virus. *Acta Virol.*, **19**, 41-46.
- Gorter A., Hiesstra P.S., van der Voort E.A.M., van Es L.A. & Daha M.R. (1988). Binding of human IgA1 to rat peritoneal macrophages. *Immunol.*, **64**, 207-212.
- Gotch F., Rothbard J., Howland K., Townsend A. & McMichael A. (1987). Cytotoxic T lymphocytes recognise a fragment of influenza virus matrix protein in association with HLA-A2. *Nature (London)*, **326**, 881-882.
- Gottschalk A. (1957). The specific enzyme of influenza virus and *Vibrio cholerae*. *Biochim.Biophys.Acta*, **23**, 645-646.

- Gould K.G., Sootney H., Townsend A.R.M., Bastin J. & Brownlee G.G. (1987).
cause H-2k-restricted cytotoxic T cells recognise antigenic determinants in
both the HA1 and HA2 subunits of the influenza A/PR/8/34 haemagglutinin.
J.Exp.Med., **166**, 693-701.
- Grady L.J. & Kinch W. (1985). Two monoclonal antibodies against La Crosse
virus show host-dependent neutralizing activity. *J.Gen.Virol.*, **66**,
2773-2776.
- Greenberg S.B., Criswell B.S., Six H.R. & Couch R.B. (1977). Lymphocyte
cytotoxicity to influenza virus-infected cells: II. Requirement for
antibody and non-T lymphocytes. *J.Immunol.*, **119**, 2100-2106.
- Greenberg S.B., Criswell B.S., Six H.R. & Couch R.B. (1978). Lymphocyte
cytotoxicity to influenza virus-infected cells: Response to vaccination and
virus infection. *Infect. & Immunity*, **20**, 640-645.
- Gregoriades A. (1981). Interaction of influenza M protein with lipid.
pp195-201. "The replication of negative strand viruses" Ed. Bishop D.H.L. &
Compans R.W.. Publ. Elsevier, North Holland.
- Hackett C.J., Dietzschold B., Gerhard W., Ghrist B., Knorr R., Gilleseen D.
& Melchers F. (1983). Influenza virus site recognized by a murine helper T
cell specific for HI strains. *J.Exp.Med.*, **158**, 294-302.
- Hackett C.J., Harwitz J.L., Dietzschold B. & Gerhard W. (1985). A synthetic
peptide of influenza virus haemagglutinin elicits helper T cells with the
same fine recognition specificities as occur in response to whole virus.
J.Immunol., **135**, 1391-1394.
- Heller O., Arrheiter H., Lindenmann J. & Gesser I. (1979). Genetically
determined interferon dependent resistance to influenza virus in mice.
J.Exp.Med., **149**, 601-612.
- Hara K., Beare A.S. & Tyrrell D.A.J. (1974). Growth and pathogenicity of
influenza viruses in organ cultures of ciliated epithelium. *Arch.Virol.*,
44, 227-236.
- Harnett G.B. & Hooper W.L. (1968). Test-tube organ cultures of ciliated
epithelium for the isolation of respiratory viruses. *Lancet*, **i**, 339-340.
- Hashimoto G., Wright P.F. & Karzon D.T. (1983). Antibody-dependent
cell-mediated cytotoxicity against influenza virus-infected cells.
J.Inf.Dis., **148**, 785-794.
- Hay A.J., Lomniczi B., Bellamy A.R. & Skehel J.J. (1977). Transcription of
the influenza virus genome. *Virology*, **83**, 337-355.
- Hay A.J., Kennedy N.C.T., Skehel J.J. & Appleyard G. (1979). The matrix
protein gene determines amantadine-sensitivity of influenza viruses.
J.Gen.Virol., **42**, 189-191.

- Hay A.J., Wolstenholme A.J., Skehel J.J. & Smith M.H. (1985). The molecular basis of the specific anti-influenza action of amantadine. *EMBO Journal*, **4**, 3021-3024.
- Haywood A.M. & Boyer B.P. (1985). Fusion of influenza virus membranes with liposomes at pH 7.5. *Proc.Natl.Acad.Sci. (USA)*, **82**, 4611-4615.
- Haywood A.M. & Boyer B.P. (1986). Time and temperature dependence of influenza virus membrane fusion at neutral pH. *J.Gen.Virol.*, **67**, 2813-2817.
- Heath A.W., Addison C., Ali M., Teele D. & Potter C.W. (1983). *In vivo* and *in vitro* hamster models in the assessment of virulence of recombinant influenza viruses. *Antiviral Research*, **3**, 241-252.
- Heath A.W., Maassab H.F., Odagiri T., DeBorde D.C. & Potter C.W. (1986). Cold-adapted reassortants of influenza A virus: pathogenicity of A/Ann Arbor/6/60 x A/Alaska/6/77 reassortant viruses *in vivo* and *in vitro*. *Arch.Virol.*, **91**, 53-60.
- Heinz F.X., Mandl C., Berger R., Tuma W. & Kunz C. (1984). Antibody-induced conformational changes result in enhanced avidity of antibodies to different antigenic sites on the tick-borne encephalitis virus glycoprotein. *Virology*, **133**, 25-34.
- Helenius A. & Sjaoms K. (1980). Mechanisms of animal virus entry into cells. pp41-54 "The molecular basis of microbial pathogenicity" Ed. Smith H., Skehel J.J. & Turner M.J., Publ. Springer-Verlag, New York.
- Herbst-Laier R. (1970a). Organ cultures in evaluation of antiviral drugs. I. Virus infection in trachea explants as potential model system for evaluation of antiviral drugs against respiratory viruses. *Arch.Virol.*, **30**, 379-396.
- Herbst-Laier R. (1970b). A novel technique for *in situ* demonstration of cell viability and cell injury on the respiratory surface of tracheal explants. *Arch.Virol.*, **30**, 248-251.
- Herz C., Stavnezer E., Krug R.M. & Gurney jr. T. (1981). Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. *Cell*, **26**, 391-400.
- Highlander S.L., Sutherland S.L., Gage P.J., Johnson D.C., Levine M. & Glorioso J.C. (1987). Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. *J.Virol.*, **61**, 3356-3364.
- Hocart M.J., MacKenzie J.S. & Stewart G.A. (1988). The IgG subclass responses induced by wild-type, cold-adapted and purified haemagglutinin from influenza virus A/Queensland/6/72 in CBA/CAH mice. *J.Gen.Virol.*, **69**, 1873-1882.
- Holt P.G. & Schon-Hegrad N.A. (1987). Localization of T cells, macrophages and dendritic cells in rat respiratory tract tissue: Implications for immune function studies. *Immunol.*, **62**, 349-356.

- Hoorn B. (1966). Organ cultures of ciliated epithelium for the study of respiratory viruses. *Acta Pathol. et Microbiol. Scand.*, **66**, 1-37.
- Hoorn B. & Tyrrell D.A.J. (1965). On the growth of certain "newer" respiratory viruses in organ cultures. *B.J. Exp. Pathol.*, **46**, 109-118.
- Hoorn B. & Tyrrell D.A.J. (1966a). Effects of some viruses on ciliated epithelium. *Amer. Rev. Resp. Dis.*, **93**, 153-161.
- Hoorn B. & Tyrrell D.A.J. (1966b). A new virus cultivated only in organ cultures of human ciliated epithelium. *Arch. Virol.*, **18**, 210-225.
- Horne R.W., Waterson A.P., Wildy P. & Farrhas A.E. (1960). The structure and composition of the myxoviruses. I. Electron microscope studies of myxovirus particles by negative staining. *Virology*, **11**, 79-89.
- Hu P.C., Collier A.M. & Baerman J.B. (1975). Alterations in the metabolism of hamster trachea in organ culture after infection by virulent Mycoplasma pneumoniae. *Infect. & Immunity*, **11**, 704-710.
- Huang R.T.C. (1974). Adsorption of influenza virus to charged groups on natural and artificial surfaces. *Med. Micro. Immunol.*, **159**, 129-135.
- Huang R.T.C., Rott R., Wahn K., Klenk H-D. & Kohase T. (1980). The function of the neuraminidase in membrane fusion induced by myxoviruses. *Virology*, **107**, 313-319.
- Huang R.T.C., Rott R. & Klenk H-D. (1981). Influenza viruses cause hemolysis and fusion of cells. *Virology*, **110**, 243-247.
- Huang R.T.C., Dietsch E. & Rott R. (1985). Further studies on the role of neuraminidase and the mechanism of low pH dependence in influenza virus-induced membrane fusion. *J. Gen. Virol.*, **66**, 295-301.
- Hudson J.B., Flavith J. & Dismock N.J. (1978). Early events in influenza virus infection. III. The formation of a nucleoplasmic ribonucleoprotein complex from the input virion. *Virology*, **86**, 167-176.
- Hughes P.G., Compans R.W., Zebedes S.L. & Lamb R.A. (1988). Localization of M2 protein in influenza virus-infected MDCK cells. *J. Cell. Biochem.*, suppl. **12C**, 43.
- Husseini R.H., Sweet C., Bird R.A., Collie M.H. & Smith H. (1983). Distribution of viral antigen within the lower respiratory tract of ferrets infected with a virulent influenza virus: Production and release of virus from corresponding organ cultures. *J. Gen. Virol.*, **64**, 589-598.
- Ioanoglu J., Shivan H., Duke G., Gilbert S., Rueckert R. & Anderegg J. (1983). Neutralization of poliovirus by a monoclonal antibody; kinetics and stoichiometry. *Virology*, **127**, 412-425.
- Iorio R.M. & Brett W.A. (1984). Neutralization of Newcastle disease virus by monoclonal antibodies to the hemagglutinin-neuraminidase glycoprotein: requirement for antibodies to four sites for complete neutralization. *J. Virology*, **51**, 445-451.

- Jackson D.C. & Webster R.G. (1982). A topographic map of the enzyme active center and antigenic sites on the neuraminidase of influenza virus A/Tokyo/3/67 (H2N2). *Virology*, **123**, 69-77.
- Jackson D.A., Caton A.J., McCready S.J. & Cook P.R. (1982). Influenza virus RNA is synthesized at fixed sites in the nucleus. *Nature (London)*, **296**, 366-368.
- Jackson R.L., Segrest J.P., Kahane I. & Marchesi V.T. (1973). Studies on the major sialoglycoprotein of the human red cell membrane. *Biochemistry*, **12**, 3131-3138.
- Janda J.M., Davis A.R., Nayak D.P. & de Barun K. (1979). Diversity and generation of defective interfering influenza virus particles. *Virology*, **95**, 48-58.
- Joassin L., Vincenzotto C., Cloes J.-M., Bouachet M. & Reginster M. (1987). Monoclonal antibodies detect M-protein epitopes on the surface of influenza virions. *Arch. Virol.*, **95**, 183-195.
- Johansson B.E., Moran T.M., Bona C.A., Popple S.W. & Kilbourne E.D. (1987a). Immunologic response to influenza virus neuraminidase is influenced by prior experience with the associated viral hemagglutinin. II Sequential infection of mice simulates human experience. *J. Immunol.*, **139**, 2010-2014.
- Johansson B.E., Moran T.M., Bona C.A. & Kilbourne E.D. (1987b). Immunologic response to influenza virus neuraminidase is influenced by prior experience with the associated viral hemagglutinin. III Reduced generation of neuraminidase-specific helper T cells in hemagglutinin-primed mice. *J. Immunol.*, **139**, 2015-2019.
- Johansson B.E., Bucher D.J. & Kilbourne E.D. (1989). Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection. *J. Virol.*, **63**, 1239-1246.
- Johnson P.R., Feldman S., Thompson J.M., Mahony J.D. & Wright P.F. (1986). Immunity to influenza A virus infection in young children: A comparison of natural infection, live cold-adapted vaccine and inactivated vaccine. *J. Inf. Dis.*, **154**, 121-127.
- Joklik W.K. (1964). The intracellular fate of rabbitpox virus rendered noninfectious by various reagents. *Virology*, **22**, 620-633.
- Jones P.D. and Ada G.L. (1986). Influenza virus-specific antibody-secreting cells in the murine lung during primary influenza virus infection. *J. Virol.*, **60**, 614-619.
- Kappler J.W. & Marrick P.C. (1976). Helper T cells recognise antigen and macrophage surface components simultaneously. *Nature (London)*, **262**, 797-799.

- Kaplan D.R., Griffith R., Braciale V.L. & Braciale T.J. (1984). Influenza virus-specific human cytotoxic T cell clones: Heterogeneity in antigen specificity and restriction by class II MHC products. *Cell Immunol.* **80**, 193-206.
- Karadaghi S., Zakomirdin J.A., Shimene C., Bucher D.J., Tverdiakov V.A. & Kharitonov I.G. (1984). Interaction of influenza virus proteins with planar bilayer lipid membranes. (I) Characterization of their adsorption and incorporation into lipid bilayers. *Biochim.Biophys.Acta.* **778**, 269-275.
- Kates M., Allison A.C., Tyrrell D.A.J. & James A.T. (1961). Lipids of influenza virus and their relation to those of the host cell. *Biochim.Biophys.Acta.* **52**, 455-466.
- Kato N. & Eggers H.J. (1969). Inhibition of uncoating of Fowl Plague Virus by 1-amantadine hydrochloride. *Virology*, **37**, 632-641.
- Katz J.M., Neeve C.W. & Webster R.G. (1987). Host cell-mediated variation in H3N2 influenza viruses. *Virology*, **156**, 386-395.
- Kawakami K., Mizumoto K., Ishihama A., Shinozaki-Yamaguchi K. & Miura K-I. (1985). Activation of influenza virus-associated RNA polymerase. *J.Biochem.* **97**, 655-661.
- Kawaoka Y., Nestorowicz A., Alexander D.J. & Webster R.G. (1987). Molecular analyses of the haemagglutinin genes of H5 influenza viruses: Origin of a virulent turkey strain. *Virology*, **158**, 218-227.
- Kelly D.C. & Dimmock N.J. (1974). Fowl Plague Virus in mammalian cell-avian heterokaryons: Studies concerning the actinomycin D and ultra-violet light sensitive phase in influenza virus replication. *Virology*, **61**, 210-222.
- Kelly D.C., Avery R.J. & Dimmock N.J. (1974). Failure of an influenza virus to initiate infection in enucleate BHK cells. *J.Virology*, **13**, 1155-1161.
- Kennedy-Stoskopf S. & Narayan O. (1986). Neutralizing antibodies to vimsa lentivirus: mechanism of action and possible role in virus persistence. *J.Virology*, **52**, 37-44.
- Kida H., Brown L.E. & Webster R.G. (1982). Biological activities of monoclonal antibodies to operationally defined antigenic regions on the haemagglutinin molecule of A/Seal/Massachusetts/1/80 (H7N7) influenza virus. *Virology*, **122**, 36-47.
- Kida H., Yoden S., Kuwabara M. & Yanagawa R. (1985). Interference with a conformational change in the haemagglutinin molecule of influenza virus by antibodies as a possible neutralization mechanism. *Vaccine*, **3** (suppl), 219-222.
- Kida H., Kawaoka Y., Neeve C.W. & Webster R.G. (1987). Antigenic and genetic conservation of H3 influenza virus in wild ducks. *Virology*, **159**, 109-119.
- Kilbourne E.D. (1974). Virus research. *Science* **184**, 410-411.

- Kilbourne E.D. (1987). Influenza in man. pp157-218 "Influenza". Plenum Medical Book Company, New York & London.
- Kilbourne E.D., Laver W.G., Schulman J.L. & Webster R.G. (1968). Antiviral activity of antiserum specific for an influenza virus neuraminidase. *J.Virol.*, 2, 281-288.
- Kilian M., Mestecky J. & Russell M.W. (1988). Defense mechanisms involving Fc-dependent functions of immunoglobulin A and their subversion by bacterial immunoglobulin A proteases. *Microbiol.Rev.*, 52, 296-303.
- Kingsford L., Ishizawa L.D. & Hill D.W. (1983). Biological activities of monoclonal antibodies reactive with antigenic sites mapped on the G1 glycoprotein of La Crosse virus. *Virology*, 129, 443-455.
- Kingsman S.M., Tom G.L. & Smith H. (1977a). The localization of influenza virus in the respiratory tract of ferrets: The different susceptibilities of fresh and maintained organ cultures. *J.Gen.Virol.*, 37, 249-258.
- Kingsman S.M., Tom G.L. & Smith H. (1977b). The localization of influenza virus in the respiratory tract of ferrets: The importance of early replication events in determining tissue specificity. *J.Gen.Virol.*, 37, 259-270.
- Kjellen L. (1985). A hypothesis accounting for the effect of the host cell on neutralization-resistant virus. *J.Gen.Virol.*, 66, 2279-2283.
- Kjellen L. & von Zisapel G. (1984). Influence of host cells on the infectivity and neutralizability of echovirus type 4 (Pescsek). *Intervirology*, 22, 32-40.
- Klenk H-D. & Choppin P.W. (1969). Lipids of plasma membranes of monkey and hamster kidney cells and of parainfluenza viruses grown in the cells. *Virology*, 38, 255-268.
- Klenk H-D. & Schwarz R.T. (1987). Variations in glycosylation of the influenza virus haemagglutinin of subtype H7. *Arch.Virol.*, 97, 359-363.
- Klenk H-D., Compans R.W. & Choppin P.W. (1970). An electromicroscopic study of the presence or absence of neuraminic acid in enveloped viruses. *Virology*, 43, 1158-1162.
- Kostolansky F., Russ G., Mucha V. & Styk B. (1988). Changes in the influenza virus haemagglutinin at acid pH detected by monoclonal antibodies to glycopeptides HA1 and HA2. *Arch.Virol.*, 101, 13-24.
- Krug R.M. (1983). Transcription and replication of influenza viruses. Chapter 3 pp70-99 "Genetics of influenza viruses". Eds. Palese P. & Kingsbury D.W.. Publ. Springer-Verlag, New York.
- Krug R.M., Broni B.A. & Bouloy M. (1979). Are the 5' ends of influenza viral mRNAs synthesized in vivo donated by host mRNAs?. *Cell*, 18, 329-334.

- Krug R.M., Bouloy M. & Plotch S.J. (1980). RNA primers and the role of host nuclear RNA polymerase II in influenza viral RNA transcription. *Phil. Trans. R. Soc. Lond.*, **B288**, 359-370.
- Krug R.M., Shaw M., Broni B., Shapiro G. & Heller O. (1985). Inhibition of influenza viral mRNA synthesis in cells expressing the interferon-induced Mx gene product. *J. Virol.*, **56**, 201-206.
- Lachmann P.J. (1985). Antibody and complement in viral infections. *Br. Med. Bull.*, **41**, 3-6.
- Laab R.A. (1983). The influenza virus RNA segments and their encoded proteins. "Genetics of influenza viruses" pp21-69, Ed. Palese P. & Kingsbury D.W., Publ. Springer-Verlag, New York.
- Laab R.A. & Choppin P.W. (1977). Synthesis of influenza virus polypeptides in cells resistant to alpha-amantadine: evidence for the involvement of cellular RNA polymerase II in virus replication. *J. Virol.*, **23**, 816-819.
- Laab R.A. & Choppin P.W. (1981). Identification of a second protein (M2) encoded by RNA segment 7 of influenza virus. *Virology*, **112**, 729-757.
- Laab R.A. & Choppin P.W. (1983). The gene structure and replication of influenza virus. *Ann. Rev. Biochem.*, **52**, 467-506.
- Laab R.A. & Green N. (1983). Analysis of the antigen specificity of influenza haemagglutinin-immune human T lymphocyte clones: Identification of an immunodominant region for T cells. *Immunol.*, **50**, 659-666.
- Laab R.A., Zebadee S.L. & Richardson C.D. (1985). Influenza virus M2 protein is an integral membrane protein expressed on the infected cell surface. *Cell*, **40**, 627-633.
- Leemali U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, **227**, 680-685.
- Laver W.G. & Kilbourne E.D. (1966). Identification in a recombinant influenza virus of structural proteins derived from both parents. *Virology*, **30**, 493-501.
- Laver W.G. & Valentine R.C. (1969). Morphology of the isolated haemagglutinin and neuraminidase subunits of influenza virus. *Virology*, **38**, 105-119.
- Laver W.G. & Webster R.G. (1968). Selection of antigenic mutants of influenza viruses. Isolation and peptide mapping of their haemagglutinin proteins. *Virology*, **34**, 193-202.
- Laver W.G. & Webster R.G. (1973). Studies on the origin of pandemic influenza. I. Evidence implicating duck and equine influenza viruses as possible progenitors of the Hong Kong strain of human influenza. *Virology*, **51**, 383-391.
- Laver W.G. & Webster R.G. (1979). Ecology of influenza virus in lower mammals and birds. *Br. Med. Bull.*, **35**, 29-33.

- Laver W.G., Air G.M., Webster R.G., Gerhard W., Ward C.W. & Dopheide T.A.A. (1979). Antigenic drift in type A influenza virus: Sequence differences in the haemagglutinin of Hong Kong (H3N2) variants selected with monoclonal hybridoma antibodies. *Virology*, **98**, 226-237.
- Laver W.G., Air G.M., Dopheide T.A. & Ward C.W. (1980). Amino acid sequence changes in the haemagglutinin of A/Hong Kong (H3N2) influenza virus during the period 1968-77. *Nature (London)*, **283**, 454-457.
- Laver W.G., Webster R.G. & Colman P.M. (1987). Crystals of antibodies complexed with influenza virus neuraminidase show cooperative binding of antibody to wild-type and variant antigens. *Virology*, **156**, 181-184.
- LeCompte J. & Oxford J.S. (1981). Detection of antigenic variation of influenza A matrix protein by a competitive radioimmunoassay. *J.Gen.Virology*, **57**, 403-406.
- Lee P.W.K., Hayes E.C. & Joklik W.K. (1981). Protein of the reovirus cell attachment protein. *Virology*, **108**, 156-163.
- LeFrancois L. & Lyles D.S. (1982). The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. I. Analysis of neutralizing epitopes with monoclonal antibodies. *Virology*, **121**, 157-167.
- Lenard J. & Miller D.K. (1983). Entry of enveloped viruses into cells. pp121-138 "Receptors and recognition", volume 15. Eds. Oestreicher P. & Roth T.F.. Publ. Chapman & Hall, London.
- Lenard J., Wong C.Y. & Compans R.W. (1974). Association of the internal membrane proteins with the lipid bilayer in influenza virus. *Biochim.Biophys.Acta*, **332**, 341.
- Liew F.Y. & Russell S.M. (1980). Delayed-type hypersensitivity to influenza virus. Induction of antigen-specific suppressor T cells for delayed-type hypersensitivity to haemagglutinin during influenza virus infection in mice. *J.Exp.Med.*, **151**, 799.
- Liew F.Y. & Russell S.M. (1983). Inhibition of pathogenic effect of effector T cells by specific suppressor T cells during influenza virus infection in mice. *Nature (London)*, **304**, 541-543.
- Liew F.Y., Russell S.M. & Brand C.M. (1979). Induction and characterization of delayed-type hypersensitivity to influenza virus in mice. *Eur.J.Immunol.*, **9**, 783-790.
- Liew F.Y., Russell S.M., Appleyard G., Brand C.M. & Beale J. (1984). Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. *Eur.J.Immunol.*, **14**, 350-356.
- Lightman S., Cobbold S., Waldmann H. & Askonas B.A. (1987). Do L3T4+ T cells act as effector cells in protection against influenza virus infection. *Immunology*, **62**, 139-144.

Lonberg-Holm K. (1981). Attachment of animal viruses to cells: An introduction. pp1-20 "Receptors and recognition". Eds. Lonberg K. & Philippon L.. Publ. Chapman & Hall, London.

Lonberg K. & Philippon L. (1980). Molecular aspects of virus receptors and cell surfaces. pp789-848 "Cell membranes and viral envelopes". Eds. Blough H.A. & Tiffany J.M.. Publ. Academic Press, London.

London S.D., Rubin D.H. & Cebra J.J. (1987). Gut mucosal immunization with reovirus serotype I/L stimulates virus-specific cytotoxic T cell precursors as well as IgA memory cells in Peyer's patches. *J.Exp.Med.*, 165, 830-847.

Long E.O. & Jacobson S. (1989). Pathways of viral antigen processing and presentation to CTL: Defined by the mode of virus entry? *Immunol.Today*, 10, 45-48.

Lubeck M. & Gerhard W. (1982). Conformational changes at topologically distinct antigenic sites on the influenza A/PR/8/34 virus HA molecule are induced by the binding of monoclonal antibodies. *Virology*, 118, 1-7.

Lubeck M.D., Schulman J.L. & Pelese P. (1978). Susceptibility of influenza A viruses to guanadine is influenced by the gene coding for M protein. *J.Virology*, 28, 710-716.

Maimone M.M., Morrison L.A., Braciale V.L. & Braciale T.J. (1986). Features of target cell lysis by class I and class II MHC-restricted cytotoxic T lymphocytes. *J.Immunol.*, 137, 3639-3643.

Mandel B. (1967). The interaction of neutralized poliovirus with HeLa cells. I. Adsorption. *Virology*, 31, 238-247.

Mandel B. (1976). Neutralization of poliovirus: a hypothesis to explain the mechanism and one-hit character of the neutralization reaction. *Virology*, 69, 500-510.

Mandel B. (1984). Mechanisms of virus neutralization. Chapter 5, pp32-38 "Concepts in viral pathogenesis". Eds. Notkins A.L. & Oldstone M.B.A.. Publ. Springer-Verlag, New York.

Mandel B. (1985). Virus neutralization. Chapter 5 "Immunochimistry of viruses. The basis for serodiagnosis and vaccines." Eds. van Regenmortel M.H.V. & Neurath A.R.. Publ. Elsevier, North Holland.

Marah M. (1984). The entry of enveloped viruses into cells by endocytosis. *Biochem.J.*, 218, 1-10.

Mathiesen T., Persson M.A.A., Sundqvist V-A. & Mahren B. (1988). Neutralization capacity and antibody dependent cell-mediated cytotoxicity of separated IgG subclasses 1, 3 and 4 against herpes simplex virus. *Clin.Exp.Immunol.*, 72, 211-215.

Martinez C., del Rio L., Portela A., Domingo E. & Ortin J. (1983). Evolution of the influenza virus neuraminidase gene during drift of the N2 subtype. *Virology*, 130, 539-545.

- Matlin K.S., Reggio H., Helenius A. & Simons K. (1981). Infectious entry pathway of influenza virus in a canine kidney cell line. *J. Cell Biol.* **91**, 601-613.
- McCauley J.W. & Mahy B.W.J. (1983). Structure and function of the influenza virus genome. *Biochem. J.* **211**, 281-294.
- McGee Z.A., Johnson A.P. & Taylor-Robinson D. (1976). Human fallopian tubes in organ culture: Preparation, maintenance, and quantitation of damage by pathogenic microorganisms. *Infect. & Immunity*, **13**, 608-618.
- McMichael A.J., Gotch F.M., Dongworth D.W., Clark A. & Potter C.W. (1983). Declining T-cell immunity to influenza, 1977-82. *Lancet*, **2**, 762-764.
- Meager A. & Hughes R.C. (1977). Virus receptors. pp143-195 "Receptors and recognition", volume 4. Eds. Cuatrecasas P. & Greaves M.F.. Publ. Chapman & Hall, London.
- Mestecky J. (1987). The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J. Clin. Immunol.*, **7**, 265-276.
- Mestecky J., Kulhavy R. & Kraus F.W. (1971). Method of serum IgA isolation. *J. Immunol.*, **107**, 605-607.
- Neuman O. (1983). Detection of antiviral IgM and its problems - A review. *Our. Top. Med. Immunol.*, **104** 101-131.
- Miller N. & Hitt-Fletcher L.M. (1988). A monoclonal antibody to glycoprotein gp85 inhibits fusion but not attachment of Epstein-Barr virus. *J. Virol.*, **62**, 2366-2372.
- Miller S.D. & Jenkins M.K. (1985). *In vivo* effects of GK1.5 (anti-L3T4a) monoclonal antibody on induction and expression of delayed-type hypersensitivity. *Cell Immunol.*, **92**, 414-426.
- Mills K.H.G. (1986). Processing of viral antigens and presentation to class II-restricted T cells. *Immunol. Today*, **7**, 260-263.
- Mills K.H.G., Burt D.S., Skehel J.J. & Thomas D.B. (1988). Fine specificity of murine class II-restricted T cell clones for synthetic peptides of influenza virus haemagglutinin. Heterogeneity of antigen interaction with the T cell and the Ia molecule. *J. Immunol.*, **140**, 4083-4090.
- Mise C.A. & White D.O. (1984). The immune response to viral infection. pp87-132 "Viral pathogenicity and immunology". Blackwell Scientific Publications, Oxford.
- Minor P.D., Hart J.G. & Dimmock N.J. (1979). Influence of the host cell on proteins synthesized by different strains of influenza virus. *Virology*, **97**, 482-487.
- Mitchell D.M., McMichael A.J. & Laab J.R. (1985). The immunology of influenza. *Br. Med. Bull.*, **41**, 80-85.

Morris A.G. (1988). Interferons. *Immunol., suppl.* 1, 43-45.

Morris A.G., Lin Y-L. & Askonas B.A. (1982). Immune interferon release when a clones cytotoxic T-cell line meets its correct influenza-infected target cell. *Nature (London)*, **295**, 150-152.

Morser M.J., Kennedy S.I.T. & Burke D.C. (1973). Virus-specified polypeptides in cells infected with Sealiki Forest Virus. *J.Gen.Virol.*, **21**, 19-29.

Moemann T.R. & Coffman R.L. (1987). Two types of mouse helper T-cell clone. Implications for immune regulation. *Immunol.Today*, **8**, 223-227.

Mostow S.R. & Tyrrell D.A.J. (1973). The behaviour *in vitro* of attenuated recombinant influenza viruses. *Arch.Virol.*, **43**, 385-392.

Mostow S.R., Hopkins J.A. & Wright P.F. (1979). Behavior of vaccine revertants of temperature-sensitive mutants of influenza virus in ferret tracheal organ culture. *Infect. & Immunity*, **26**, 193-196.

Murakami T. & Matsuyama T. (1988). Appearance of influenza A virus antigenic variants after treatment of infected MDCK cells with human leukocytes. *J.Gen.Virol.*, **62**, 1841-1845.

Murphy B.R., Chalhub E.G., Maminoff S.R., Kasel J. & Chanock R.M. (1973). Temperature-sensitive mutants of influenza virus. III. Further characterization of the ts-1[E] influenza A recombinant (H3N2) in man. *J.Inf.Dis.*, **128**, 479-487.

Murphy B.R., Nelson D.L., Wright P.F., Tierney E.L., Phelan M.A. & Chanock R.M. (1982). Secretory and systemic immunological response in children infected with live attenuated influenza A virus vaccines. *Infect. & Immunity*, **36**, 1102-1108.

Murti K.G. & Webster R.G. (1986). Distribution of haemagglutinin and neuraminidase on influenza virions as revealed by immunoelectron microscopy. *Virol.*, **149**, 36-43.

Neeve C.W., Hinshaw V.S. Webster R.G. (1984). Mutations in the haemagglutinin receptor-binding site can change the biological properties of an influenza virus. *J.Virol.*, **51**, 567-569.

Nakajima K., Desselberger U. & Palese P. (1978). Recent human influenza A viruses are closely related genetically to strains isolated in 1950. *Nature (London)*, **274**, 334-339.

Natali A., Oxford J.S. & Schild G.C. (1981). Frequency of naturally occurring antibody to influenza virus antigenic variants selected *in vitro* with monoclonal antibody. *J.Hyg. (Camb.)*, **87**, 185-190.

Nayak D.P., Chambers T.M. & Aldkina R.K. (1985). Defective-interfering (DI) RNAs of influenza viruses: origin, structure, expression and interference. *Curr.Top.Micro. Immunol.*, **114**, 103-151.

- Nestorowicz A., Laver G. & Jackson D.C. (1985). Antigenic determinants of influenza virus haemagglutinin. X. A comparison of the physical and antigenic properties of monomeric and trimeric forms. *J.Gen.Virol.*, **66**, 1687-1695.
- Nestorowicz A., Kawasaka Y., Bean W.L. & Webster R.G. (1987). Molecular analysis of the haemagglutinin genes of Australian H7N7 influenza viruses: Role of Passerine birds in maintenance or transmission? *J. Virol.*, **160**, 411-418.
- Nguyen T.D., Bottreau E., Bernard S., Lantier I. & Aynaud J.M. (1986). Neutralizing secretory IgA and IgG do not inhibit attachment of transmissible gastroenteritis virus. *J.Gen.Virol.*, **67**, 939-943.
- Nichol S.T., Penn C.R. & Mahy B.W.J. (1981). Evidence for the involvement of influenza A (Fowl Plague Rostock) virus protein P2 in ApG and mRNA primed *in vitro* RNA synthesis. *J.Gen.Virol.*, **57**, 407-413.
- Ochiai H., Kurokawa M., Hayashi K. & Niwayama S. (1988). Antibody-mediated growth of influenza A NWS virus in macrophagelike cell line P388D1. *J.Virol.*, **62**, 20-26.
- Chuchi J.S., Orlich M., Chuchi R., Simpson B.E.J., Garten W., Klenk H-D. & Rott R. (1989). Mutations at the cleavage site of the haemagglutinin after the pathogenicity of influenza virus A/Chick/Penn/83 (H5N2). *Virology*, **168**, 274-280.
- Oi V.T., Vuong T.M., Hardy R., Reidler J., Dangl J., Herzenberg L.A. & Stryer L. (1984). Correlation between segmental flexibility and effector function of antibodies. *Nature (London)*, **307**, 136-140.
- Owens S.L., Osebold J.W. & Zee Y.C. (1981). Dynamics of B-lymphocytes in the lungs of mice exposed to aerosolized influenza virus. *Inf.Immun.*, **33**, 231-238.
- Oxford J.S. & Schild G.C. (1976). Immunological and physicochemical studies of influenza matrix (M) polypeptides. *Virology*, **74**, 394-402.
- Oxford J.S., Concannon T., Knott R., Bates J., Batolomei O., Major D., Newman R.W., Yates P., Webster R.G. & Schild G.C. (1987). Serological studies with influenza A (H1N1) viruses cultivated in eggs or in canine kidney cell line (MDCK). *WHO Bull.*, **65**, 181-187.
- Palese P. (1977). The genes of influenza virus. *Cell*, **10**, 1-10.
- Palese P. & Schulman J. (1974). Isolation and characterization of influenza virus recombinants with high and low neuraminidase activity. *Virology*, **57**, 227-237.
- Palese P., Tobita K., Ueda M. & Compans R.W. (1974). Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology*, **61**, 397-410.

- Pallesen P., Ritchey M.B. & Schulman J.L. (1977). P1 and P3 proteins of influenza virus are required for complementary RNA synthesis. *J.Virol.*, **21**, 1187-1195.
- Parnhas P. (1984). A repulsive view of MHC-restriction. *Immunol.Today*, **5**, 89-92.
- Parnhas P. (1988). Presentation and processing of antigens in Paris. *Immunol.Today*, **9**, 65-68.
- Patterson S. & Oxford J.S. (1986). Early interactions between animal viruses and the host cell; Relevance to viral vaccines. *Vaccine*, **4**, 79-90.
- Patterson S., Oxford J.S. & Dourashkin R.R. (1979). Studies on the mechanism of influenza virus entry into cells. *J.Gen.Virol.*, **43**, 223-229.
- Patterson S., Gross J. & Oxford J.S. (1988). The intracellular distribution of influenza virus matrix protein and nucleoprotein in infected cells and their relationship to haemagglutinin in the plasma membrane. *J.Gen.Virol.*, **69**, 1859-1872.
- Peiris J.S.M. & Porterfield J.S. (1982). Antibody-dependent plaque enhancement: its antigenic specificity in relation to Togaviridae. *J.Gen.Virol.*, **59**, 291-296.
- Peiris J.S.M., Gordon S., Unkeles J.C. & Porterfield J.S. (1981). Monoclonal anti-Fc receptor IgG blocks antibody enhancement of viral replication in macrophages. *Nature (London)*, **289**, 189-191.
- Peiris J.S.M., Porterfield J.S. & Roehrig J.T. (1982). Monoclonal antibodies against the flavivirus West Nile. *J.Gen.Virol.*, **58**, 283-289.
- Penn C.R. & Mahy B.W.J. (1984). Capped mRNAs may stimulate the influenza virus polymerase by allosteric modulation. *Virus Res.*, **1**, 1-13.
- Penn C.R., Blaas D., Kuschler E. & Mahy B.W.J. (1982). Identification of the cap-binding protein of two strains of influenza A/FPV. *J.Gen.Virol.*, **62**, 177-180.
- Plotch S.J., Bouloy M. & Krug R.M. (1979). Transfer of 5'-terminal cap of globin mRNA to influenza viral complementary RNA *in vitro*. *Proc.Natl.Acad.Sci.(USA)*, **76**, 1618-1622.
- Plotch S.J., Bouloy M., Ulaanen I. & Krug R.M. (1981). A unique cap (m7GpppA)-dependent influenza virus endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell*, **23**, 847-858.
- Pons M.W., Schulze I.T. & Hirst G.K. (1969). Isolation and characterization of the ribonucleoprotein of influenza virus. *Virol.*, **39**, 250-259.
- Pomes R.D. (1981). The mechanism of neutralization of influenza virus by antibody. PhD thesis. University of Warwick.

- Poseas R.D., Schild G.C. & Dimmock N.J. (1982). Studies on the mechanism of neutralization of influenza virus by antibody: evidence that neutralizing antibody (anti-haemagglutinin) inactivates influenza virus *in vivo* by inhibiting virion transcriptase activity. *J.Gen.Virol.*, **58**, 373-386.
- Potter C.W. & Oxford J.S. (1979). Determinants of immunity to influenza infection in man. *Br.Med.Bull.*, **35**, 69-75.
- Pritchett T.J., Broesmer R., Rose U. & Paulson J.C. (1987). Recognition of sialosides by influenza virus H3 haemagglutinin. *Virology*, **160**, 502-506.
- Pumphrey R. (1986a). Computer models of the human immunoglobulins. Shape and segmental flexibility. *Immunol.Today*, **7**, 174-178.
- Pumphrey R. (1986b). Computer models of the human immunoglobulins. Binding sites and molecular interactions. *Immunol.Today*, **7**, 206-211.
- Puri J., Lonai P. & Friedlan V. (1986). Antigen-Ia interaction and the proteolytic processing of antigen: the structure of the antigen determines its restriction to the A or E molecule of the major histocompatibility complex. *Eur.J.Immunol.*, **16**, 1093-1097.
- Rajewsky K., Forster I. & Quesano A. (1987). Evolutionary and somatic selection of the antibody repertoire in the mouse. *Science*, **238**, 1088-1094.
- Ray R., Glaze B.J., Moldoveanu Z. & Compans R.W. (1988). Intranasal immunization of hamsters with envelope glycoproteins of human parainfluenza virus type 3. *J.Inf.Dis.*, **157**, 648-654.
- Rees P.J. & Dimmock N.J. (1981). Electrophoretic separation of influenza ribonucleoproteins. *J.Gen.Virol.*, **53**, 125-132.
- Reinherz M. & Narnut M.V. (1976). Preparation and characterization of influenza virus cores. *J.Gen.Virol.*, **31**, 211-220.
- Richman D.D., Yazaki P. & Hostetler K.Y. (1981). The intracellular distribution and antiviral activity of amantadine. *Virology*, **112**, 81-90.
- Richman D.D., Hostetler K.Y., Yazaki P.J. & Clark S. (1986). Fate of influenza A virion proteins after entry into subcellular fractions of LLC cells and the effect of amantadine. *Virology*, **151**, 200-215.
- Rigg R.J., Carver A.S. & Dimmock N.J. (1989). IgG-neutralized influenza virus undergoes primary, but not secondary uncoating *in vivo*. In press.
- Robertson J.S. (1987). Sequence analysis of the haemagglutinin of A/Taiwan/1/86, a new variant of human influenza A (H1N1) virus. *J.Gen.Virol.*, **68**, 1205-1208.
- Robertson J.S., Bootsman J.S., Newman R., Oxford J.S., Daniels R.S., Webster R.G. & Schild G.C. (1987). Structural changes in the haemagglutinin which accompany egg adaptation of an influenza A (H1N1) virus. *Virology*, **160**, 31-37.

- Rodgers B.C. & Mize C.A. (1982). Influenza virus replication in human alveolar macrophages. *J. Med. Virol.* **2**, 177-184.
- Roehrig J.T., Hunt A.R., Kinney R.M. & Mathews J.H. (1988). *In vitro* mechanisms of monoclonal antibody neutralization of alphaviruses. *Virol.* **165**, 66-73.
- Rogers G.N., Paulson J.C., Daniels R.S., Skehel J.J., Wilson I.A. & Wiley D.C. (1983). Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature (London)*. **304**, 76-78.
- Rogers G.N., Daniels R.S., Skehel J.J., Wiley D.C., Wang X-F., Higa H.H. & Paulson J.C. (1985). Host-mediated selection of influenza virus receptor variants. *J. Biol. Chem.* **260**, 7362-7367.
- Roitt I, Brostoff J. & Male D. (1985). "Immunology". Publ. Churchill Livingstone, Edinburgh, London, Melbourne & New York.
- Romanos M.A. & Hay A.J. (1984). Identification of the influenza virus transcriptase by affinity-labelling with pyridoxal 5'-phosphate. *Virol.* **132**, 110-117.
- Roque-Barreira M.C. & Campos-Neto A. (1985). Jacalin: An IgA-binding lectin. *J. Immunol.* **134**, 1740-1743.
- Roseman M.G. (1988). Viral receptors and drug design. *Nature (London)*. **333**, 392-393.
- Roseman M.G. & Palmenberg A.C. (1988). Conservation of the putative receptor attachment site in picornaviruses. *Virol.* **164**, 373-382.
- Rott R., Reinscher M., Orlich M. & Klenk H-D. (1980). Cleavability of haemagglutinin determines spread of avian influenza viruses in the chorioallantoic membrane of chicken embryo. *Arch. Virol.* **65**, 123-133.
- Rott R., Orlich M., Klenk H-D., Wang M.L., Skehel J.J. & Wiley D.C. (1984). Studies on the adaptation of influenza viruses to MDCK cells. *EMBO Journal*, **3**, 3329-3332.
- Rudneva I.A., Kaverin N.V., Varich N.L., Gitelman A.K., Makhov A.M., Klisarenko S.M. & Zhdanov V.M. (1986). Studies on the genetic determinants of influenza virus pathogenicity for mice with the use of reassortants between mouse-adapted and non-adapted variants of the same virus strain. *Arch. Virol.* **90**, 237-248.
- Ruigrok R.W.H., Martin S.R., Wharton S.R., Skehel J.J., Bayley P.M. & Wiley D.C. (1986a). Conformational changes in the haemagglutinin of influenza virus which accompany heat-induced fusion of virus with liposomes. *Virol.* **155**, 484-497.
- Ruigrok R.W.H., Wrigley N.G., Calder L.J., Cusack S., Wharton S.R., Brown E.B. & Skehel J.J. (1986b). Electron microscopy of the low pH structure of influenza virus haemagglutinin. *EMBO J.* **5**, 41-49.

- Ruigrok R.W.H., Aitken A., Calder L.J., Martin S.R., Skehel J.J., Wharton S.A., Weis W. & Wiley D.C. (1988). Studies on the structure of the influenza virus haemagglutinin at the pH of membrane fusion. *J.Gen.Virol.*, **69**, 2785-2795.
- Russell P.H. (1986a). Newcastle disease virus and two influenza viruses: Differing effects of acid and temperature on the uptake of infectious virus into bovine and canine kidney cell lines. *Arch.Virol.*, **88**, 159-166.
- Russell P.H. (1986b). The synergistic neutralization of Newcastle disease virus by two monoclonal antibodies to its haemagglutinin-neuraminidase protein. *Arch.Virol.*, **90**, 135-144.
- Scharle P.A. & Gerhard W. (1986). Functional analysis of influenza-specific helper T cell clones *in vivo*. T cells specific for internal viral proteins provide cognate help for B cell responses to haemagglutinin. *J.Exp.Med.*, **164**, 1114-1128.
- Schiff L.J. (1974). Studies on the mechanisms of influenza virus infection in hamster trachea organ culture. *Arch.Virol.*, **44**, 195-204.
- Schild G.C. & Neuman R.W. (1969). Immunological relationships between the neuraminidase of human and animal influenza viruses. *WHO Bull.*, **41**, 437.
- Schild G.C., Oxford J.S., de Jong J.C. & Webster R.G. (1983). Evidence for host-cell selection of influenza virus antigenic variants. *Nature (London)*, **303**, 706-709.
- Schild G.C., Oxford J.S. & Webster R.G. (1984). Evidence for host cell selection of antigenic variants of influenza A (H1N1) and B viruses: antigenic differences between the haemagglutinins of viruses grown in eggs and mammalian cells. pp164 "The molecular virology and epidemiology of influenza". Eds Stuart-Harris C.H. & Potter C.W.. Publ. Academic Press, London.
- Schlegel R. & Wade M. (1983). Neutralized vesicular stomatitis virus binds to host cells by a different "receptor". *Biochem. & Biophys. Res. Commun.*, **114**, 774-778.
- Scholtissek C. (1986). Influenza A viruses with non-cleaved haemagglutinin are not internalized after adsorption. *Arch.Virol.*, **90**, 159-163.
- Scholtissek C. & Faulkner G.P. (1979). Amantadine-resistant and -sensitive influenza A strains and recombinants. *J.Gen.Virol.*, **44**, 807-815.
- Scholtissek C., Rhode W., von Hoyningen V. & Rott R. (1978). On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology*, **87**, 13-20.
- Scholtissek C., Vallbracht A., Flehig B. & Rott R. (1979). Correlation of pathogenicity and gene constellation of an influenza virus. II. Highly virulent recombinants derived from non-neurovirulent or weakly neurovirulent parent virus strains. *Virology*, **92**, 492-500.

- Scholtissek C., Müller K., Herzog S. & Freese K. (1988). Multiplication of influenza A viruses with cleavable and non-cleavable haemagglutinin in chicken embryo membranes or organs, and cell cultures derived therefrom. *J.Gen.Virol.*, **69**, 2155-2164.
- Schulman J.L. (1969). The role of antineuraminidase antibody in immunity to influenza virus infection. *WHO Bull.*, **41**, 647-650.
- Schulze I.T. (1972). The structure of influenza virus. II. A model based on the morphology and composition of subviral particle. *Virology*, **47**, 181-196.
- Schwartz R.H. (1985). T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Ann.Rev.Immunol.*, **3**, 237-261.
- Sears S.D., Clements M.L., Betts R.F., Maassab H.F., Murphy B.R. & Snyder M.H. (1988). Comparison of live, attenuated H1N1 and H3N2 cold-adapted and avian-human influenza A reassortant viruses and inactivated virus vaccine in adults. *J.Inf.Dis.*, **158**, 1209-1219.
- Seto J.T. & Rott R. (1966). Functional significance of sialidase during influenza virus multiplication. *Virology*, **30**, 731-737.
- Shapiro G.I., Gurney Jr. T. & Krug R.M. (1987). Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. *J.Virology*, **61**, 764-773.
- Shaw M.W. & Compans R.W. (1978). Isolation and characterization of cytoplasmic inclusions from influenza A virus infected cells. *J.Virology*, **25**, 608-615.
- Shimizu K., Mukaigawa J., Oguro M., Ono Y., Nakajima K. & Kida H. (1985). Inhibition of transcriptase activity of influenza A virus *in vitro* by anti-haemagglutinin antibodies. *Vaccine*, **3** (suppl.), 207-210.
- Shore S.L., Cromeans T.L. & Romano T.J. (1976). Immune destruction of virus-infected cells early in the infectious cycle. *Nature (London)*, **266**, 695-696.
- Simons K., Bennett M. & Nees A.W. (1988). Sorting in the trans-golgi network using glycoproteins as probes. *J.Cell.Biochem.*, suppl.12C, 11.
- Skehel J.J. (1972). Polypeptide synthesis in influenza virus-infected cells. *Virology*, **49**, 23-36.
- Skehel J.J. (1973). Early polypeptide synthesis in influenza virus-infected cells. *Virology*, **56**, 394-399.
- Skehel J.J. & Schild G.C. (1971). The polypeptide composition of influenza A viruses. *Virology*, **44**, 396-408.
- Skehel J.J., Hay A.J. & Armstrong J.A. (1977). On the mechanism of inhibition of influenza virus replication by amantadine chloride. *J.Gen.Virology*, **38**, 97-110.

- Skehel J.J., Bayley P.M., Brown E.B., Martin S.R., Waterfield M.D., White J.M., Wilson I.A. & Wiley D.C. (1982). Changes in the conformation of influenza virus haemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc. Natl. Acad. Sci.*, **79**, 968-972.
- Skehel J.J., Stevens D.J., Daniels R.S., Douglas A.R., Knesow M., Wilson I.A. & Wiley D.C. (1984). A carbohydrate side chain on haemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. *Proc. Natl. Acad. Sci. (USA)*, **81**, 1779-1783.
- Skinner M.A., Langlois A.J., McDaniel C.B., McDougal J.S., Bolognesi D.P. & Matthews T.J. (1988). Neutralizing antibodies to an immunodominant envelope sequence do not prevent gp120 binding to CD4. *J. Virol.*, **62**, 4195-4200.
- Smith G.L. & Hay A.J. (1982). Replication of the influenza virus genome. *Virology*, **118**, 96-108.
- Smith G.L., Levin J.Z., Palese P. & Moss B. (1987). Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses. *Virology*, **160**, 336-345.
- Snyder M.H., Stephenson E.H., Young H., York C.G., Tierney E.L., London W.T., Chanock R.M. & Murphy B.R. (1986). Infectivity and antigenicity of live avian-human influenza A reassortant virus: Comparison of intranasal and aerosol routes in Squirrel Monkeys. *J. Inf. Dis.*, **154**, 709-712.
- Springg D.R. (1986). Mx genes, interferon and enlightenment. *J. Inf. Dis.*, **154**, 361-362.
- Staeli P., Heller O., Boll W., Lindenmann J. & Weissmann C. (1986). Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. *Cell*, **44**, 147-158.
- Stegman T., Hoekstra D., Scherphof G. & Wilchut J. (1985). Kinetics of pH-dependent fusion between influenza virus and liposomes. *Biochem.*, **24**, 3107-3113.
- Stephenson J.R. & Dimmock N.J. (1975). Early events in influenza virus multiplication. I. Location and fate of the input RNA. *Virology*, **65**, 77-86.
- Stephenson J.R., Hudson J.B. & Dimmock N.J. (1978). Early events in influenza virus multiplication. II. Penetration of virus into cells at 4°C. *Virology*, **86**, 264-271.
- Stridh S., Oberg B., Chattopadhyaya J. & Josephson S. (1981). Functional analysis of influenza RNA polymerase activity by the use of caps. oligonucleotides and polynucleotides. *Antiviral Res.*, **1**, 97-105.
- Sugiura A. & Ueda M. (1980). Neurovirulence of influenza virus in mice. I. Neurovirulence of recombinants between virulent and avirulent virus strains. *Virology*, **101**, 440-449.
- Sullivan J.L., Mayner R.E., Barry D.W. & Ennis F.A. (1976). Influenza virus infection in nude mice. *J. Inf. Dis.*, **133**, 91-94.

Superti F., Seganti L., Ruggeri F.M., Tinari A., Donelli G. & Orsi N. (1987). Entry pathway of vesicular stomatitis virus into different host cells. *J.Gen.Virol.*, **68**, 387-399.

Sweet C. & Smith H. (1980). Pathogenicity of influenza virus. *Microbiol.Rev.*, **44**, 303-330.

Sweet C., Bird R.A., Toms G.L., Woodward C.G. & Smith H. (1977). Thermal stability and interaction with ferret inflammatory exudates of two clones of influenza virus of differing virulence for both ferrets and man. *B.J.Exp.Pathol.*, **57**, 635-643.

Sweet C., McCartney J.C., Bird R.A., Cavanagh D., Collie M.H., Hussein R.H. & Smith H. (1981). Differential distribution of virus and histological damage in the lower respiratory tract of ferrets infected with influenza viruses of differing virulence. *J.Gen.Virol.*, **54**, 103-114.

Sweet C., Bird R.A., Hussein R.H. & Smith H. (1984). Differential replication of attenuated and virulent influenza viruses in organ cultures of ferret bronchial epithelium. *Arch.Virol.*, **80**, 219-224.

Tashiro M., Ciborowski P., Rainacher M., Pulverer G., Klensk H-D. & Rott R. (1987). Synergistic role of Staphylococcal proteases in the induction of influenza virus pathogenicity. *Virology*, **157**, 421-430.

Taylor H.P. (1986). The interaction of influenza virus with neutralizing antibody. PhD thesis, University of Warwick.

Taylor H.P. & Dimmock N.J. (1985a). Mechanism of neutralization of influenza virus by secretory IgA is different from that of monomeric IgA or IgG. *J.Exp.Med.*, **161**, 198-209.

Taylor H.P. & Dimmock N.J. (1985b). Mechanism of neutralization of influenza virus by IgM. *J.Gen.Virol.*, **66**, 903-907.

Taylor H.P., Armstrong J. & Dimmock N.J. (1987). Quantitative relationships between an influenza virus and neutralizing antibody. *Virology*, **159**, 288-298.

Taylor J.M., Illenseer R., Litwin S., Herring L., Broni B. & Krug R.M. (1977). Use of specific radioactive probes to study transcription and replication of the influenza virus genome. *J.Virol.*, **21**, 530-540.

Taylor P.M. & Askonas B.A. (1983). Diversity in the biological properties of anti-influenza cytotoxic T cell clones. *Eur.J.Immunol.*, **13**, 707.

Thomas A.A.M., Brioen P. & Boeye A. (1985). A monoclonal antibody that neutralizes poliovirus by cross-linking virions. *J.Virol.*, **54**, 7-13.

Thomas A.A.M., Vrijen R. & Boeye A. (1986). Relationship between poliovirus neutralization and aggregation. *J.Virol.*, **52**, 479-485.

- Thomas B.D., Skehel J.J., Mills K.H.G. & Graham C.M. (1987). A single amino acid substitution in influenza haemagglutinin abrogates recognition by monoclonal antibody and a spectrum of subtype-specific L3T4+ T cell clones. *Eur. J. Immunol.*, **17**, 133-136.
- Tomasini J.E. & Colonna R.J. (1986). Isolation of a receptor protein involved in attachment of human rhinoviruses. *J. Virol.*, **58**, 290-295.
- Tomes G.L., Bird R.A., Kingsman S.M., Sweet C. & Smith H. (1976). The behaviour in ferrets of two closely related clones of influenza virus of differing virulence for man. *B.J. Exp. Pathol.*, **57**, 37-48.
- Tomes G.L., Davies J.A., Woodward C.G., Sweet C. & Smith H. (1977). The relationship of pyrexia and nasal inflammatory response to virus levels in nasal washings of ferrets infected with influenza viruses of differing virulence. *B.J. Exp. Pathol.*, **58**, 444-458.
- Townsend A.R.M. & Skehel J.J. (1982). Influenza A specific cytotoxic T-cell clones that do not recognize viral glycoproteins. *Nature (London)*, **300**, 655-657.
- Townsend A.R.M., Skehel J.J., Taylor P.M. & Palese P. (1984a). Recognition of influenza A virus nucleoprotein by an H-2-restricted cytotoxic T-cell clone. *Virology*, **133**, 456-459.
- Townsend A.R.M., McMichael A.J., Carter N.P., Huddlestone J.A. & Brownlee G.G. (1984b). Cytotoxic T cell recognition of the influenza nucleoprotein and haemagglutinin expressed in transfected mouse L cells. *Cell*, **39**, 13-25.
- Townsend A.R.M., Gotch F.M. & Davey J. (1985). Cytotoxic T cells recognize fragments of the influenza nucleoprotein. *Cell*, **42**, 457-467.
- Townsend A.R.M., Rothbard J., Gotch F.M., Bahadur G., Wraith D. & McMichael A.J. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell*, **44**, 959-968.
- Unanue E.R. (1984). Antigen-presenting function of the macrophage. *Ann. Rev. Immunol.*, **2**, 395-428.
- Ulanen I., Broni B. & Krug R.M. (1981). Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m7GpppNs) on RNAs and in initiating viral RNA transcription. *Proc. Natl. Acad. Sci. (USA)*, **78**, 7355-7359.
- Ulanen I., Broni B. & Krug R.M. (1983). Influenza virus temperature-sensitive cap (m7GpppNs)-dependent endonuclease. *J. Virol.*, **45**, 27-35.
- Underdown B.J. & Schiff J.M. (1987). Immunoglobulin A: strategic defense initiative at the mucosal surface. *Ann. Rev. Immunol.*, **4**, 369-417.
- Unkeless J.C., Scigliano E. & Freedman V.H. (1988). Structure and function of human and murine receptors for IgG. *Ann. Rev. Immunol.*, **6**, 251-281.

- van Campen H., Easterday B.C. & Hinshaw V.S. (1989). Destruction of lymphocytes by a virulent influenza A virus. *J.Gen.Virol.*, 70, 467-472.
- van Wyke K.L., Hinshaw V.S., Bean Jr W.J. & Webster R.G. (1980). Antigenic variation of influenza A virus nucleoprotein detected with monoclonal antibodies. *J.Virol.*, 35, 24-30.
- van Wyke K.L., Bean Jr W.J. & Webster R.G. (1981). Monoclonal antibodies to the influenza virus nucleoprotein affecting RNA transcription. *J.Virol.*, 39, 313-317.
- Varghese J.N., Laver W.G. & Colman P.M. (1983). Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9A resolution. *Nature (London)*, 303, 35-40.
- Vasantha S., Coelingh K.L., Murphy B.R., Dourmashkin R.R., Hammer C.H., Frank M.M. & Frazer L.F. (1988). Interactions of a nonneutralizing IgM antibody and complement in parainfluenza virus neutralization. *Virology*, 167, 433-441.
- Vitala J. & Jarnefelt J. (1985). The red cell surface revisited. *TIBS*, 10, 392-395.
- Virelizier J.L. (1975). Host defences against influenza virus: the role of anti-haemagglutinin antibody. *J.Immunol.*, 115, 434-439.
- Waldman R.H., Kamei J.A., Fulk R.V., Togo Y., Hornick R.B., Heiner G.G. & Dawkins Jr A.T. (1968). Influenza antibody in human respiratory secretions after subcutaneous or respiratory immunization with inactivated virus. *Nature (London)*, 218, 594-595.
- Waldman R.H., Wrigley F.M. & Small Jr P.A. (1970). Specificity of respiratory secretion antibody against influenza virus. *J.Immunol.*, 105, 1477-1483.
- Waldman R.H., Spencer C.S. & Johnson III J.E. (1972). Respiratory and systemic cellular and humoral responses to influenza virus vaccine administered parenterally or by nose drops. *Cell Immunol.*, 3, 294-300.
- Wang M-L., Skehel J.J. & Wiley D.C. (1986). Comparative analyses of the specificities of anti-influenza haemagglutinin antibodies in human sera. *J.Virol.*, 57, 124-128.
- Webster R.G. & Laver W.G. (1967). Preparation and properties of antibody directed specifically against the neuraminidase of influenza virus. *J.Immunol.*, 99, 49-55.
- Webster R.G. & Laver W.G. (1980). Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus haemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. *Virology*, 104, 139-148.
- Webster R.G. & Pereira H.G. (1968). A common surface antigen in influenza viruses from human and avian sources. *J.Gen.Virol.*, 3, 201-208.

- Webster R.G., Campbell C.H. & Granoff A. (1971). The "in vivo" production of "new" influenza viruses. I. Genetic recombination between avian and mammalian influenza viruses. *Virology*, **44**, 317-328.
- Webster R.G., Campbell C.H. & Granoff A. (1973). The "in vivo" production of "new" influenza viruses. III. Selection of recombinant influenza viruses under simulated conditions of natural transmission. *Virology*, **51**, 149-162.
- Webster R.G., Hineshaw V.S. & Laver W.G. (1982). Selection and analysis of antigenic variants of the neuraminidase of N2 influenza viruses with monoclonal antibodies. *Virology*, **117**, 93-104.
- Webster R.G., Brown L.E. & Jackson D.C. (1983). Changes in the antigenicity of the haemagglutinin molecule of H3 influenza virus at acidic pH. *Virology*, **126**, 587-599.
- Webster R.G., Laver W.G. & Air G.M. (1983). Antigenic variation among type A influenza viruses. Chapter 5 pp127-188 "Genetics of influenza viruses". Eds. Palese P. & Kingsbury D.W.. Publ. Springer-Verlag, New York
- Webster R.G., Reay P.A. & Laver W.G. (1988). Protection against lethal influenza with neuraminidase. *Virology*, **164**, 230-237.
- Weis W., Brown J.H., Quack S., Paulson J.C., Skehel J.J. & Wiley D.C. (1988). Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature (London)*, **333**, 426-431.
- Wells M.A., Albrecht P. & Ennis F.A. (1981). Recovery from a viral respiratory infection. I. Influenza pneumonia in normal and T-deficient mice. *J. Immunol.*, **126**, 1036-1041.
- Westerberg S.C., Smith C.B., Wiley B.B. & Jensen C.G. (1972). Pathogenesis of influenza virus infection in acute tracheal organ cultures. *Proc.Soc.Exp.Biol.Med.*, **140**, 846-851.
- Wetz K., Willingmann P., Zeichhardt H. & Habermehl K-O. (1986). Neutralization of poliovirus by polyclonal antibodies requires binding of a single IgG molecule per virion. *Arch.Virology*, **91**, 207-220.
- White J., Helenius A. & Gething M-J. (1982). Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. *Nature (London)*, **300**, 658-659.
- WHO memorandum. (1980). A revision of the system of nomenclature for influenza viruses: A WHO memorandum. *Bull.WHO*, **28(4)**, 585-591.
- Wiley D.C. & Skehel J.J. (1987). The structure and function of the haemagglutinin membrane glycoprotein of influenza virus. *Ann.Rev.Biochem.*, **56**, 365-394.
- Wiley D.C., Wilson I.A. & Skehel J.J. (1981). Structural identification of the antibody-binding sites of Hong Kong haemagglutinin and their involvement in antigenic variation. *Nature (London)*, **289**, 373-378.

- Williams A.F. (1984). The T-lymphocyte antigen receptor - elusive no more. *Nature (London)*, **301**, 108-109.
- Williams A.F. (1985). Immunoglobulin-related domains for cell surface recognition. *Nature (London)*, **314**, 579-580.
- Wilson I.A., Skehel J.J. & Wiley D.C. (1981). Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3A resolution. *Nature (London)*, **289**, 366-373.
- Wohlfart C. (1988). Neutralization of adenoviruses: kinetics, stoichiometry, and mechanisms. *J.Virol.*, **62**, 2321-2328.
- Wohlfart C., Svensson U.K. & Everitt E. (1985). Interaction between Hela cells and adenovirus type 2 virion neutralized by different antisera. *J.Virol.*, **56**, 896-903.
- Wolstenholme A.J., Barrett T., Nichol S.T. & Mahy B.W.J. (1980). Influenza virus-specific RNA and protein synthesis in cells infected with temperature-sensitive mutants defective in the genome segment encoding nonstructural protein 1. *J.Virol.*, **35**, 1-7.
- Wrath D.C. (1987). The recognition of influenza A virus-infected cells by cytotoxic T lymphocytes. *Immunol.Today*, **8**, 239-246.
- Wrath D.C. & Vessey A.E. (1986). Influenza virus-specific cytotoxic T-cell recognition: Stimulation of nucleoprotein-specific clones with intact antigen. *Immunol.*, **59**, 173-180.
- Wrigley N.G., Brown E.B., Daniels R.S., Douglas A.R., Skehel J.J. & Wiley D.C. (1983). Electron microscopy of influenza haemagglutinin-monovalent antibody complexes. *Virology*, **131**, 308-314.
- Yap K.L. & Ada G.L. (1978a). The recovery of mice from influenza virus infection: Adoptive transfer of immunity with immune T lymphocytes. *Scand.J.Immunol.*, **7**, 389-397.
- Yap K.L. & Ada G.L. (1978b). The recovery of mice from influenza A virus infection: Adoptive transfer of immunity with influenza virus-specific cytotoxic T lymphocytes recognizing a common virion antigen. *Scand.J.Immunol.*, **8**, 413-420.
- Yap K.L., Ada G.L. & Mackenzie I.F.C. (1978a). Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature (London)*, **273**, 238-239.
- Yewdell J.W. & Hackett C.J. (1987). The specificity and function of T lymphocytes induced by influenza A viruses. pp1-66 "The influenza viruses". Ed. Krug R.M.. Publ. Plenum, New York.
- Yewdell J.W., Gerhard W. & Bachi T. (1983). Monoclonal anti-haemagglutinin antibodies detect irreversible antigenic alterations that coincide with the acid activation of influenza virus A/PR/8/34-mediated haemolysis. *J.Virol.*, **48**, 239-248.

- Yoden S., Kida H. & Yanagawa R. (1982). An avian influenza virus of which infectivity is neutralized by antisera lacking hemagglutination-inhibition activity. *Arch.Virol.*, **74**, 205-210.
- Yoden S., Kida H., Kusabara M., Yanagawa R. & Webster R.G. (1986). Spin-labelling of influenza virus hemagglutinin permits analysis of the conformational change at low pH and its inhibition by antibody. *Virus Res.*, **4**, 251-261.
- Yoshimura A., Kuroda K., Kawasaki K., Yamashina Y., Maeda T. & Ohnishi S-I. (1982). Infectious cell entry mechanism of influenza virus. *J.Virol.*, **43**, 284-293.
- Young J.F., Desselberger U. & Palese P. (1979). Evolution of human influenza A viruses in nature: Sequential mutations in the genomes of new H1N1 isolates. *Cell*, **18**, 73-83.
- Zabedee S.L. & Lamb R.A. (1988). Influenza A virus M2 protein: Monoclonal antibody restriction of virus growth and detection of M2 in virions. *J.Virol.*, **62**, 2762-2772.
- Zabedee S.L., Richardson C.D. & Lamb R.A. (1985). Characterisation of the influenza virus M2 integral membrane protein and expression at the infected-cell surface from cloned cDNA. *J.Virol.*, **56**, 502-511.
- Zinkernagel R.M. & Doherty P.C. (1979). MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function and responsiveness. *Adv.Immunol.*, **27**, 52.
- Zvonarjev A.Y. & Ghandon Y.Z. (1980). Influence of membrane (M) protein on influenza A virus virion transcriptase activity *in vitro* and its susceptibility to Rimantadine. *J.Virol.*, **33**, 583-586.
- Zweerink H.J., Courtneidge S.A., Skehel J.J., Crumpton M.J. & Askonas B.A. (1977). Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. *Nature (London)*, **267**, 354-356.
- Zweerink H.J., Courtneidge S.A., Skehel J.J., Crumpton M.J. & Askonas B.A. (1977). Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. *Nature (London)*, **267**, 354-356.

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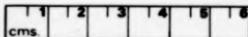
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