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Structural analysis of the neutralising antibody repertoire to influenza virus Haemagglutinin.

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Structural analysis of the neutralising antibody repertoire to influenza virus Haemagglutinin

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Two roads diverged in a wood, and I-

I took the one less travelled by,

And that has made all the difference....

Robert Frost

ABSTRACT

Protective immunity to influenza virus correlates with levels of neutralising antibodies directed against haemagglutinin (HA), the major membrane glycoprotein of the virus. The antibodies exert selective pressure resulting in the recurrent and annual emergence of new variant viruses.

Although all five antigenic sites are implicated in antibody recognition, immunodominance is evident in the secondary antibody response, following *natural infection* of inbred mice as has been shown in this laboratory: BALB/c (H-2d) mAbs predominantly recognise HA1 198, and CBA/Ca (H-2k) mAbs, HA1 158, as deduced by sequencing the HA genes of X31 laboratory variants. Such restriction in the antibody response raises several questions concerning the mechanisms involved in repertoire selection and the structural basis of the selection process, which this investigation attempted to address using transgenic mice expressing human Ig μ chains.

The purpose of examining the memory repertoire for influenza HA in transgenic mice was to determine whether restricted V_H region gene usage and/or inability to class switch (IgM→IgG) with a resultant lack of affinity maturation would restrict the potential antibody repertoire. I have demonstrated, by structural analysis of variant virus HA genes, selected with mAbs expressing human μ chains, that there is a *preferential selection* for mutations within conserved residues that constitute part of the receptor binding site (HA1 225, HA1 226) that do not occur in previously documented laboratory variants. Also the majority of these variant viruses have amino acid substitutions at two different positions: (HA1 145, 226) or (HA1 135, 225) or (HA1 135, 158) or (HA1 145, 158).

In the second part of this study, I have examined the influence of *concomitant immunity* to X31 on re-challenge with an X31 variant virus in an attempt to mimic the human situation of recurrent infection: CBA/Ca mice were infected intranasally with X31 and their neutralising antibody repertoire investigated by hemisplenectomy, mAb production, X31 variant selection and sequencing of their HA genes. Following a rest period, mice were re-challenged with a laboratory variant, A43, differing from X31 at known antigenic sites (HA1 145, 158, del 224-230) and which was *not* recognised by the majority of mAbs from the primary challenge. The mAbs, established after re-infection with A43, showed heteroclitic reactivity for X31 and selected laboratory variants thereof, with substitutions at both conserved residues within the receptor binding site and at known antigenic sites (HA1 193, 198, 226) or HA1 (198, 223).

I have demonstrated in two different systems (a) human IgH transgenic mice, or (b) recurrent infection with a variant virus, that there is *preferential selection* of laboratory mutants containing multiple substitutions, including changes in conserved residues that constitute part of the receptor-binding pocket (HA1 225 or 226). This has led me to conclude that antibody affinity might play a determinant role in the selection of mutations that affect receptor binding function. The majority of X31 variants that I have cloned, and characterised, do indeed have altered receptor-binding specificity as shown by their resistance to horse serum inhibition of haemagglutination and/or altered binding to neoglycoproteins containing terminal α 2,3 or α 2,6 sialyl residues.

Reduced affinity of the neutralising Ab response during infection - such as in the immunocompromised, or the very young or very old, or due to previous exposure to a related virus might skew the antibody repertoire to selection of receptor-binding variants and therefore be a further determinant of antigenic drift.

Acknowledgements.

This thesis is dedicated to my dear mother.

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ABBREVIATIONS

Ab	antibody
AMV	Avian myelomablastosis virus
C gene	Constant gene
cDNA	Complementary deoxyribose nucleic acid
CDR	Complementary determining region
CMI	Cell mediated immunity
CMV	Cytomegalovirus
Cos	Cosmid
CTL	Cytotoxic T lymphocytes
d/ddATP	deoxy/dideoxy adenosine triphosphate
d/ddCTP	deoxy/dideoxy cytosine triphosphate
d/ddGTP	deoxy/dideoxy guanosine triphosphate
d/ddTTP	deoxy/dideoxy thymidine triphosphate
DCH	Delayed cutaneous hypersensitivity
ddH$_2$O	Double distilled water
ddNTP	Dideoxy nucleotide triphosphate
DMEM	Dulbecco Modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphste
DTH	Delayed-type hypersensitivity
DTT	Dithiothreitol
EBV	Epstein Barr Virus
EDTA	Ethylene diamino tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
Fab	Fragment antigen binding
FCS	Foetal calf serum

FDC	Follicular dendritic cell
gC	Glycoprotein C
H-2.	Mouse major histocompatibility complex
HA	Haemagglutinin
HA 1	Haemagglutinin subunit 1.
HA 2	Haemagglutinin subunit 2.
HAT	Hypoxanthine-aminopterin-thymidine
HT	Hypoxanthine-thymidine
HAU	Haemagglutination unit
HCMV	Human Cytomegalovirus
HI	Haemagglutination inhibition
HIV	Human Immunodeficiency virus
HSV	Herpes Simplex Virus
HuIgH	Human immunoglobulin heavy chain
i.p.	Intraperitoneal
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
LCMV	Lymphocytic choriomeningitis virus
LMP	Latent membrane protein
M	Matrix protein
mAb	Monoclonal antibody
MDCK	Madin-Darby-canine kidney
2-Me	β-Mercaptoethanol
MHC	Major histocompatibility complex
MRV	Malignant rabbit virus
MV	Measles virus
NA	Neuraminidase
NP	Nitrophenyl

NP	Nucleoprotein
NS1	Non-structural protein 1
NS2	Non-structural protein 2
ORF	Open reading frame
PA	Peanut agglutinin
PB1, PB2, PA	Influenza virus Polymerases
PCR	Polymerase chain reaction
PEC	Peritoneal exudated cells
PEG	Polyethylene glycol
PHA	phytohaemaggluitnin
PR8	A/ Puerto Rico/ 8 / 34 influenza virus strain
RAG	Recombination activating genes
RDE	Receptor destroying enzyme
RNA	Ribonucleic acid
RNAsin	RNAse inhibitor
RT	Reverse transcription
RT mix	Reverse transcriptase mix
SA	Sialic acid
SDS	Sodium dodecyl sulphate
Serpins	Serine proteinase inhibitor
SFV	Shope Fibroma virus
TAP	Transporter associated with antigen processing
TES	Tris/EDTA/SDS
TcR	T cell receptor
Tg	Transgenic mice
Th	T helper
TNF	Tumour necrosis factor
TRBC	Turkey red blood cells
Tris	Tris (hydroxymethyl) methylamine
UV	Ultraviolet

V gene	Variable gene
VSV	Vesicular stomatitis virus

CHAPTER-1

Introduction

1. GENERAL INTRODUCTION

1.1. Immune recognition

The binding of foreign antigens to complementary structures on the surface of B and T lymphocytes represents the initial step in the sequence of events leading to activation of both cellular and humoral arms of the immune system which recognise antigen rather differently. Membrane immunoglobulin (Ig), first as a receptor molecule on the surface of B lymphocytes and later as a soluble molecule, binds antigen in an unprocessed or native state. T cell receptors (TcR), on the other hand, bind antigen only in association with major histocompatibility complex (MHC) proteins (Zinkernagel and Doherty, 1974), and furthermore, the protein antigen is typically processed to a small peptide prior to its presentation to TcR.

The primary function of B cells is to produce antibodies against a vast array of antigenic substances potentially harmful to the body, whereas, T-cells regulate secretion of antibodies and limit the probability of an autoimmune response. The strategy evolved to allow higher vertebrates to produce such a large spectrum of antibodies is the generation of millions of clonally diverse B cells, each of which produces antibody molecules consisting of paired heavy and light chains and having unique antigen binding specificity. Once generated, B cells bearing their antibody receptors are seeded via the blood stream to lymphoid tissues in strategic locations throughout the body, such as the spleen, lymph nodes and specialised areas along the intestine. In these microenvironments, B cells may encounter the foreign (or self) antigens, which they recognise via their cell surface antibody receptor.

1.2. B-cell generation.

Vertebrates can produce a humoral immune response to a large number of different foreign antigens because B lymphocytes are able to synthesise

immunoglobulins having many different antigen binding specificities. The generation of B lymphocytes from stem cells occurs through an ordered program of immunoglobulin (Ig) variable (V) region gene rearrangement and subsequent selection of combinatorial variants. As a result, each newborn B cell expresses a particular pair of heavy (H) and light (L, κ and λ) chain V region (Alt, *et al.*, 1987; Tonegawa, 1983) each containing V and constant (C) genes. The diversity in structure of variable regions, the antigen binding domains of immunoglobulins, results in diversity of antigen binding specificity. Each resting B cell in the lymphocyte population expresses an Ig molecule with a single V region structure, and thus a single or limited number of binding specificities, as cell surface receptors. A subset of these cells are stimulated to grow and secrete antibody at the onset of an immune response partly as a result of clonal selection by receptor-bound antigen.

When the haemopoietic stem cells subsequently migrate to the bone marrow, this tissue becomes the permanent site of B-cell production (Osmond, 1986). B-lineage cells can be divided into three general stages of differentiation represented by pre-B cells, B cells and plasma cells. As a lymphoid stem cell differentiates to a pre-B cell, there is a μ H chain gene rearrangement, transcription, translation, and expression of cytoplasmic μ H chains in association with a surrogate light chain (λ5+V pre-B) (Karasyuama, *et al.*, 1990). Only after both chains are expressed in the cytoplasm will a complete antibody molecule appear on the cell surface. In each antibody forming cell, only one heavy chain and one light chain V region are productively rearranged and expressed (allelic exclusion). The immature B cell develops to a B cell that co-expresses IgM and IgD on the B lymphocyte surface (Goding, 1982). Up to this stage, B-lymphocyte development is antigen independent. Maturation beyond the B cell stage is thought to require activation by mitogen or an appropriate combination of antigen, T cells or T cell factors, and macrophages (McKenzie and Potter, 1979). Following encounter with antigen and activated

T cells, B-cells migrate to B-cell follicles where they interact with follicular dendritic cells (FDC). FDC specialise in binding antibody-antigen immune complexes and in inducing B-cell proliferation and differentiation. This interaction leads to the formation of a finely structured germinal centre. B-cell proliferation and differentiation leads to dark zone formation in the germinal centre, followed by the formation of the light zone (MacLennan, *et al.*, 1992). V genes undergo somatic mutation to increase affinity for antigen within days of antigenic stimulation and only during germinal centre formation. It was suggested (MacLennan and Gray, 1986) that cells are selected in germinal centres on the basis of their ability to receive signals dependent on interaction with antigen held on FDC. Cells which do not receive signals die by apoptosis. The B cell expressing high affinity antibody can follow a number of pathways: it can terminally differentiate into an immunoglobulin M (IgM) secreting plasma cell, it can switch (Shimizu and Honjo, 1984) its fully assembled V region to a downstream C-region gene to express and secrete a new class of antibody with a different C region that is associated with a different effector function (Winkelhake, 1978), or it can become a memory cell that can recognise and be restimulated by the same antigen. The antibodies made by an individual plasma cell are of single specificity and a single isotype. To appreciate the functional aspects, it is important to understand the basic structure of the antibody molecule.

1.3. The antibody molecule

An antibody molecule of the IgG subclass appears as a Y shaped object in the electron microscope that consists of two identical heavy (H) and light (L) chains that are disulphide-linked to form a bivalent H_2L_2 molecule (fig 1). The two arms of the Y (Fab fragments) can be cleaved by proteolysis and retain the capacity to interact with antigen. The amino terminal end of each
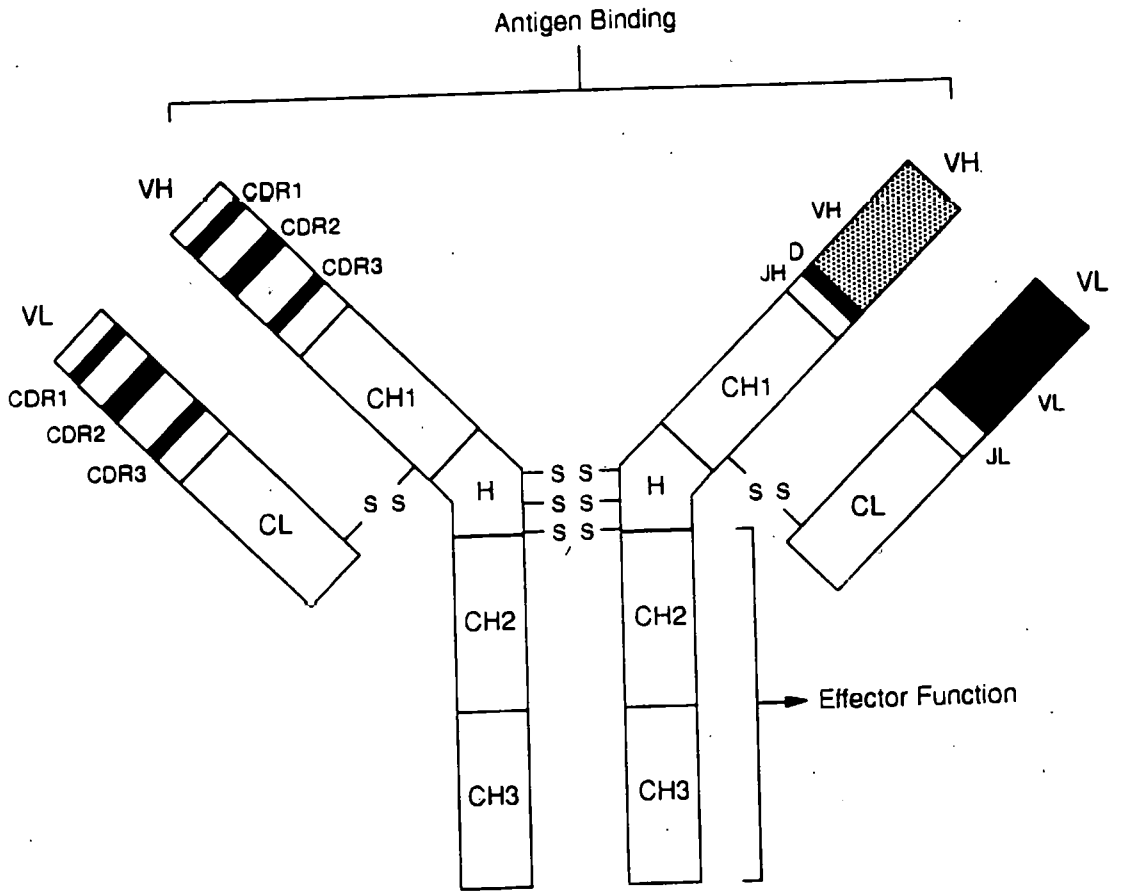
H and L chain is encoded by a variable (V) region gene and the carboxy terminal end is encoded by a constant (C) region gene. The V-region domain contains three areas of exceptional amino acid sequence variability called complementarity-determining regions (CDRs) which are the sites for antigen contact (Wu and Kabat, 1970). Surrounding the CDRs are areas of less sequence variability called framework regions. Because the CDRs are loop structures, they are more likely to be regions of high flexibility which may be an important feature of antigen binding (Tainer, *et al.*, 1985).

The V-region domains are encoded by a series of smaller genetic elements designated variable (V_H), diversity (D_H), and joining (J_H) for the heavy chains and V_L and J_L for the light chains. Each genetic element is part of a larger linkage group, and those encoding the murine heavy and both light chains, kappa (κ) and lambda (λ) V regions are located on different chromosomes (Alzari, *et al.*, 1988). Each group of genetic elements is separated in the germ-line configuration, and for the heavy chain, there are 100 to 1000 V_H (Tonegawa, 1983), approximately 12 D and 4 J genetic elements. In mouse, the κ light chain has more than 100 V and 5 J genetic elements, one of which, $J_\kappa 3$, is a pseudogene. The formation of a complete V region occurs by a site-specific recombination mechanism (Tonegawa, 1983). Before each of the many antibodies can be presented on the surface of individual antibody-forming B cells, the V region genes are rearranged and brought into close proximity with their respective C-region genes, a process that triggers their expression. Processing of pre-mRNA then joins the V and C-region genes to produce m-RNA that is translated into the complete polypeptide chains. The heavy chain genes rearrange and are expressed first in the cytoplasm of the cell along with the $C\mu$-region gene, and this is followed by the rearrangement and expression of a light chain. In each antibody-forming cell, only one heavy chain and one light chain V region are productively rearranged and expressed, a phenomenon called allelic exclusion. Since any V

can join to any (D), J and C, there are potentially over 800 different kappa chains and over 8000 possible heavy chains that can be formed, and with a combination of any L to any H chain, a potential 6.4 million different possible antibodies (excluding junctional region diversity and somatic mutation) (Alt, *et al.*, 1982; Paige, *et al.*, 1978).

The diversity of antibody specificities is contributed by a variety of factors, including the multiplicity of distinct germ-line V_H and V_L sequences that encode different primary amino-acid sequences, the combinatorial assortment of the multiple V, D, and J segments, mechanisms that modify the coding capacity of the regions where these segments are joined (thus, modifying CDR 3), somatic mutational mechanisms that modify the coding capacity of the fully assembled variable region gene (Rajewsky, *et al.*, 1987), and the combinatorial association of unique heavy and light chains to form the complete antibody. Together, these factors permit the generation of a nearly limitless variety of different antibody specificities (Tonegawa, 1983).

1.4. Mechanism of Immunoglobulin V-region assembly

The rearrangement of V region genes is a highly regulated process. V(D)J recombination appears to be directed by sequence information contained within the intervening segments that are deleted or displaced during the formation of a functional antigen-binding exon. Sequences at the end of these gene segments are known as recombination signal sequences (RSS). An RSS contains a conserved palindromic heptanucleotide sequence CACAGTG separated by a spacer of 12 or 23 bp from a less conserved AT rich nonameric sequence ACAAAAACC.

Asymmetry of these spacers guides V gene segment assembly such that only segments with different size spacers are juxtaposed (12/23 rule). Twenty-three base spacers immediately flanking 5' of V_H and 3' of J_H genes, and 12 base spacers of both 5' and 3' of D_H ensures D joins to J, then V to DJ. Direct

V-J joining is not observed at a significant frequency due to 12/32 rule, and although V-D (without J) is possible by this rule, this pairing is not found at a significant frequency relative to V-DJ.

Several years ago, the recombination activating genes, RAG1 and RAG2, were identified (Oettinger, *et al.*, 1990). Mice lacking RAG-1 and RAG-2 suffered from severe immunodeficiency disease due to their inability to initiate VDJ recombination in developing T and B cells (Oettinger, *et al.*, 1990). Elimination of either RAG-1 or RAG-2 from the germ line of a mouse is sufficient to eliminate completely recombination of both immunoglobulin and TcR genes and prevents the development of B and T lymphocytes (Mombaerts, *et al.*, 1992). Thus, either RAG-1 and RAG-2 are lymphoid specific components of the V(D)J recombinase or induce the expression of genes necessary for carrying out V(D)J recombination. Several other gene products have also been shown to be involved in V(D)J recombination. These include the XRCC5 protein, which also plays a role in DNA repair in response to DNA damage (Taccioli, *et al.*, 1994), and the SCID protein, which has been shown to be involved in both double-strand break repair and coding joint formation (Fulop and Phillips, 1990).

Once VDJ recombination has occurred, these join to the C region gene, initially to the most J_H proximal which is $C\mu$, so during early B-cell develoment, the cells always express surface membrane IgM which facilitates light chain gene assembly and expression. It was suggested that membrane μ (μ m) expressed by pre B cells in association with surrogate L chains λ_5 and VpreB on the preB cell surface may generate a signal that activates $V\kappa$ rearrangement. This complex in association with $Ig\alpha$ and $Ig\beta$ transmembrane heterodimers couples surface Ig to signal transduction machinery in the cell. Cross-linking the complex induces Ca^{2-} mobilisation and μ m generates the

regulatory signal for suppression of further V_H rearrangement and enhancement of Ig κ locus rearrangement.

PreB cells synthesise μ H chains but not κ or λ L chains and therefore complete IgM molecules are not expressed. μ chains are retained in the endoplasmic reticulum (ER) where they bind to the retention protein BiP, a 78 kDa glucose-regulated protein, and therefore cannot migrate to the Golgi to be exported. When L chains are synthesised in sufficient amounts to displace BiP,. IgM moves to the Golgi and undergoes glycosylation, processing and export to the cell surface.

1.5. Affinity maturation

1.5.1.Somatic hypermutation.

The events leading to the generation of memory B cells occur in the germinal centre and include somatic mutation, clonal expansion, Ag-driven selection, and isotype switching (Berek, 1993; Berek, *et al.*, 1991; Kroese, *et al.*, 1987; Nossal, 1992) and are associated with increase in affinity of antibodies produced.

After the Ig repertoire is created, following maturation of B lymphocytes and Ig gene rearrangement in the germinal centre, B cells undergo further diversification of the rearranged V(D)J genes via the process of somatic hypermutation followed by the selection of high-affinity variants (Griffiths, *et al.*, 1984) which are later on expanded. B cells expressing low affinity receptors are not selected and die through apoptosis (Liu, *et al.*, 1989).

Somatic hypermutation plays a central role in antibody affinity maturation and is in large part responsible for the production of the secondary repertoire. Sequencing of the V genes from hybridomas producing antigen-specific antibody revealed two types of changes during the immune response. First, immunoglobulin genes are usually expressed in mutated form with the degree of mutation increasing during the course of response. For example,

following immunisation with 2-phenyl-5-oxazolone, there were essentially no mutations at day 7 (following primary immunisation), but the extent of mutation was such that by day 14, no identical sequences were found (Berek and Milstein, 1987). Second, the repertoire of germline V genes used in the response shifted. Thus, at day 7 and to a lesser extent at day 14, a V_H Ox1/VκOx1 gene combination predominated. Following secondary immunisation, V_H/V_L gene combinations other than V_H Ox1/VκOx1 were found with greater frequency.

In the C57 BL/6 antibody response against nitrophenyl (NP), antibodies using heavy chain V_H 186.2 and light chain Vλ1 genes dominate the early primary response. Somatic mutation in clonotypes was observed as early as six days after primary NP immunisation (Cumano and Rajewsky, 1986; Yokochi, et al., 1982). The mutational change in hypervariable region CDR1 of tryptophan at position 33 to leucine increases antibody affinity for NP ten fold. This change was found in 75% of late primary and B memory clones. There was no further selection after somatic mutation as affinity increasing mutations emerged by the end of the second week. Early somatic mutation, in the first week (CDR3 usage) is restricted to the germinal centre pathway of development, but early ab secreting cells take a different, independent pathway of development and a secondary selection event occurs at the bifurcation of these two paths in vivo (Berek, et al., 1991).

Extensive analysis of somatic mutations in antibodies has revealed that mutations are largely confined to the variable domains specifically in the CDRs (Berek and Milstein, 1987), and rarely found in the constant domains (Gearhart and Bogenhagen, 1983). Some residues are frequently targeted (hot spots). Mutations largely occur over a region of one to two kilobases, around the rearranged V-J segments and the mutation domain extends from a 5' site within the leader intron (Both, et al., 1990).

1.5.2. Ig Class Switching.

Antibodies can be classified according to their heavy-chain constant regions into eight (for mice) or nine (for humans) classes or serological isotypes. Ig class switching can take place at any time during B-cell proliferation. At two differentiation stages, however, switching occurs at remarkable high frequencies, namely, (a) at the formation of naive B cells with a particular switch from IgM to IgM+IgD, and (b) at activation of B cells with a switch from IgM to IgG, IgA or IgE expression (Esser and Radbruch, 1990) because of the expression of a different heavy-chain constant region (C_H). $C\mu$ is the first C_H gene expressed upon generation of a functional $V_H DJ_H$ gene. After the assembly of an immunoglobulin light chain, $V_L J_L$ gene, the complete IgM molecule is then presented on the cell surface of the B cell. The next step is expression of both $C\mu$ and $C\delta$ with the same $V_H DJ_H$ gene and is achieved by a combination of differential termination of transcription and differential splicing of transcripts of the V_H -D-J_H-$C\mu$-$C\delta$ regions (Tucker, 1985). Upon activation by antigen, resting B cells proliferate, differentiate into immunoglobulin secreting plasma cells and memory cells, and at a high frequency, switch to expression of $C\gamma$, $C\varepsilon$, or $C\alpha$ genes to make IgG, IgE and IgA. The IgG class comprises four subclasses, IgG1, IgG2a, IgG2b, and IgG3 in mouse; IgG1, IgG2, IgG3 and IgG4 in humans.

The isotype pattern varies according to the mode of B-cell activation, which is dependent on the antigen, route of immunisation, regulatory cells involved and genetic background of the responder (Hocart, *et al.*, 1989; Slack, 1985). Coutelier et al, in 1987 analysed the isotype distribution in antiviral immune responses using a panel of murine DNA and RNA viruses. In most cases IgG2a was produced in a 10 to 100 fold excess over IgG1 (Coutelier, *et al.*, 1987) and this is now known to be due to the contribution of regulatory (T_H1 type) T cells that preferentially secrete γ-interferon (to be discussed later).

1.6.Cellular Immune Responses

1.6.1.T cell specificity and repertoire.

There are significant differences between B-cell and T-cell recognition of antigen. While B cells bind free antigen, T cells bind antigen that has been internally processed by proteolysis and re-expressed on the cell surface in association with molecules encoded by the major histocompatibility complex (MHC). However, it is important to note that T and B lymphocytes are complementary parts of a larger defence system, and that in particular, B cells, having encountered an antigen appropriate to their surface immunoglobulin, usually need T cell signals (in the form of lymphokines) before they can differentiate into plasma cells and secrete antibodies. Thus, in at least this way, the recognition systems are linked and interdependent.

The genes encoding MHC molecules are highly polymorphic, and in general, a T cell will not respond to its antigenic peptide bound by the wrong allele of MHC (MHC restriction). MHC molecules can be subdivided into two classes; MHC class I molecules are found on the surface of all somatic cells, while MHC class II molecules are expressed selectively on the surfaces of antigen presenting cells (APCs), such as B cells and macrophages. T cells can be subdivided into two separate functional categories: cytotoxic T lymphocytes (CTLs) bearing CD8 molecules recognise antigen in association with MHC class I and T helper cells (Th) bearing CD4 molecules recognise antigen in association with class II. Precursor T cells bearing receptors which can bind well to self peptides on self MHC alleles die in the thymus, a process called negative selection which leads to self tolerance.

T cell recognition of antigen in association with MHC is mediated by a single T-cell receptor (TcR) (Yague, *et al.*, 1985). TcR is a heterodimer made of two chains, predominantly α and β assembled in an antibody-like fashion, however, during ontogeny of the immune system, T cell receptors expressing γ

and δ chains are also found. The function of the γ:δ heterodimer is unknown, whereas TcR α:β appears to be responsible for the specificity of all cytotoxic and T helper cells. Each polypeptide consists of seven regions, an N-terminal hydrophobic leader region of 18-29 amino acids, a membrane distal V segment of 88-98 amino acids, a J region of 14-21 amino acids, C region of 87-133 amino acids, a connecting peptide of variable length, a transmembrane region of 20-24 amino acids and a small cytoplasmic tail of 5-12 amino acids. TcR (VDJ) $_\beta$ and (VJ)$_\alpha$ are homologous to Ig V regions, and TcR C $_\alpha$ and C$_\beta$ are homologous to Ig C regions. Although the exact number of hypervariable regions in TcR sequence is controversial, diversity is clearly evident in the regions equivalent to the three classic immunoglobulin hypervariable regions.

Like the Ig loci, the TcR genes are divided into an array of interchangeable coding segments scattered over large tracts of chromosomal DNA. These genes are actually composed of two parts: a variable gene and a constant gene. The variable gene is composed of either two (V and J) or three (V,D and J) gene segments and each gene family has multiple V and J gene segments and one to three constant genes. The V,D, and J gene segments are separated in the germ line and are brought together by DNA rearrangement during T lymphocyte differentiation to form the complete V gene (Chien, *et al.*, 1984). An interesting feature of T cell receptor gene organisation is the relative abundance of J region gene segments in TcR α and TcR β genes and a relative scarcity of V region. The net result of this is to greatly increase the amount of potential diversity in the amino acids encoded by the V-J interface in TcR versus the equivalent region in immunoglobulins and to significantly decrease the diversity expressed in the remainder of the V region heterodimer.

Diversity of TcR expression is generated in much the same way as for immunoglobulins, through the combinatorial joining of DNA segments and the variable addition of nucleotides at junctions (Davis and Bjorkman, 1988).

Alignment of TcR sequences during T-cell ontogeny in the thymus, leads to defined hypervariable regions, much in the same way as with antibodies, with one possible major difference: the TcR α and β sequences display only one well-clustered region of hypervariable residues (equivalent to CDR 3) (Bougueleret and Claverie, 1987). TcR heterodimers α, β, γ ,δ are usually found associated with other molecules in the CD3 complex, which includes at least four additional membrane-spanning polypeptide chains (Clevers, et al., 1988). In addition, accessory molecules such as CD4 and CD8 participate in the interaction between T cells and APCs and determine restriction to a given MHC class with CD4 T cells predominantly recognising MHC class II while CD8 T cells largely recognise MHC class I.

1.6.2. The structure and function of MHC molecules.

MHC molecules are polymorphic cell surface glycoproteins that occur in two distinct forms: class I MHC molecules (H-2 in mice, HLA-A, B, C in humans) consist of a glycosylated heavy chain of about 45kDa, which spans the cell membrane, associated non-covalently with a non-polymorphic, nonglycosylated, 12kDa light chain called β_2-microglobulin, MHC Class II (Ia in mice, HLA-D in humans) are made of two covalently linked polymorphic chains, α and β, that both span the cell membrane and associate as a heterodimer.

MHC class II molecules present antigenic peptides to CD4 positive Th cells (Unanue, 1984). The peptides bound to class II are derived mainly from proteins that have entered the endocytic pathway. MHC class I, on the other hand, binds and present those peptides to CD8 positive CTLs that are derived by proteolysis in the cytoplasm (Townsend, et al., 1989). Presentation of foreign antigens to helper T cells requires co-ordination of both the biosynthetic and endocytic pathways within specialised APCs. The endocytic pathway generates and delivers antigenic peptides to the MHC class II

molecule (Brodsky, 1990). Class II molecules assemble in the endoplasmic reticulum with a third protein, invariant chain (Ii) (Germain, 1986; Long, 1989). Ii blocks binding of endogenous antigens to the class II molecules and stabilises the unoccupied α/β dimers while targeting them through the biosynthetic pathway to an endosomal compartment (Townsend, *et al.*, 1989). After removal of Ii in the acidic and proteolytic environment of the endosomal compartment, the class II molecules bind peptides. Peptide-loaded class II complexes are then transported to the cell surface for recognition by T cells.

The peptides which bind MHC class I molecules are derived from proteins that are expressed within the cell. Peptides of 8-10 residues are transported from the cytosol into the endoplasmic reticulum (ER) by the MHC-encoded TAP (transporter-associated with antigen processing) proteins, TAP-1 and TAP-2 (Schumacher, *et al.*, 1994). Loosely assembled class I α chains and β_2-microglobulin are retained in the ER until peptide binding induces a conformational alteration, resulting in their rapid transport through the Golgi complex to the cell surface.

In addition to determining antigen presentation during an immune response, MHC molecules also play an important role in driving thymocyte development. MHC molecules on thymus epithelial cells select both positively and negatively for maturation and export to the peripheral lymphoid organs for T cell bearing receptors that will be able to react to a foreign peptide presented by the same MHC molecule (Von Boehmer, 1994).

1.6.3. Functional differences between Th1 and Th2 cells.

Activated T cells play a fundamental role in the regulation of humoral and cellular immune responses by both cell / cell contact and by secreted lymphokines. Differential production of cytokines by helper T cells (Th) during an immune response is the basis for distinguishing two subtypes Th1 and Th2 which are functionally different. Th1 responses in mice are characterised by

substantial interleukin 2 (IL-2) and γ-interferon (IFN-γ) release. Th1 responses predominate in viral infection resulting in activation of macrophages to kill intracellular parasites, in delayed-type hypersensitivity (DTH), and IgG2a, but no IgE synthesis, while responses to bacteria and parasites for the most part involve Th2 cells and the release of IL-4 and IL-5. The Th2 responses recruit IgG1 and IgE responses.

Th cell clones do not constitutively secrete lymphokines but are dependent upon antigen or mitogenic stimulation to do so (Mosmann and Coffman, 1989). TcR ligation by either anti-TcR abs or antigen/ class II results in the induction of lymphokine secretion which in turn induces the proliferation and differentiation of B cells (Lichtman, et al., 1987).

1.6.4. Cellular Interactions in the Humoral Immune response.

Specific recognition of foreign antigen by cell surface immunoglobulin (Ig) and consequent cross-linking induces B cells to proliferate and differentiate into either plasma cells, which produce soluble immunoglobulin, or into memory B cells which can respond to subsequent encounter with the same antigen. The proliferation and differentiation of resting B cells requires contact dependent, antigen specific, major histocompatibility complex (MHC) class II restricted interaction with Th cells. In contrast, the growth and differentiation of activated or large B cells is mainly mediated by lymphokines that act in an antigen non-specific, genetically unrestricted fashion (Noelle, 1992). Stimulation of T lymphocytes in turn requires both TcR engagement (including the CD4 and CD8 co-receptors) with an antigenic-peptide MHC complex and an additional, contact dependent, costimulatory signal delivered by an antigen presenting cell (APC) (Schwartz, 1990). Costimulation is potentially mediated by a large set of non polymorphic cell surface molecules interacting with counter-receptors expressed by T cells.

CD40 and CD80, both interacting with T cell specific CD40L (gp39), CD28 and/or CTLA-4 represent the best characterised costimulatory molecules expressed by APC (Fig 2). Once active, the T cells in turn promote B-cell activation, both by releasing T-cell-derived cytokines such as IL-2 and IFN-γ by Th1 cells and IL-4 and IL-5 by Th2 cells as well as by direct intercellular contact (MacLennan, et al., 1992).

CD40, a surface glycoprotein is related to the receptors for tumor necrosis factor-α (TNF-α) and is expressed on pre-B cells, mature B cells and certain accessory cells. Cross-linking CD40 promotes B cell proliferation (Clark and Lane, 1991), prevents apoptosis of germinal centre B cells and promotes immunoglobulin class switching (Jabara, et al., 1990). When the CD40-CD40L interaction is blocked in vitro with soluble CD40 or monoclonal antibodies to CD40L (gp39), B cells cannot proliferate or produce immunoglobulin in response to T-cell signals.

Recently, the gene encoding CD40L has been shown to be defective in patients with hyper-IgM syndrome. Although these patients have IgM-producing B cells, they do not form germinal centres in response to foreign antigen. Their B cells are capable of switching from IgM to IgG or IgE production in vitro when exposed to IL-4 and monoclonal antibodies against CD40, but they do not switch immunoglobulin class in vivo (Hill and Chapel, 1993). T cells and CD40L-CD40 interactions are thus essential.

Whereas the CD40-CD40L interaction enables the B cell to respond to an activated T cell, the interaction between CD80 and CD28 allows peripheral T cells to respond to an activated B cell by dividing and producing cytokines required for T cell differentiation. Of the ligands for the CD80 family, CD28 is found on resting and activated T cells whereas, CTLA-4 is found only on activated T cells. The binding of CD80 to CD28 on T cells previously stimulated through their antigen receptors increases IL-2 production and T-cell

CD11a/18
(LFA-1)

CD54
(ICAM-1)

CD40

CD40L
(gp39)

CD19

CD45RO

CD79b
CD79a
sIgM

CD6

B

CD22

?

T

CD5

CD72

CD4

Class II

CD3/TCR

CD58

CD2

CD59

CD80
(B7/BB1)

CD28

B70/B7-2

CTLA-4

CD20

CD43

CD54

CD11a/18

CD106
(VCAM)

CD49d/29
(VLA-4)
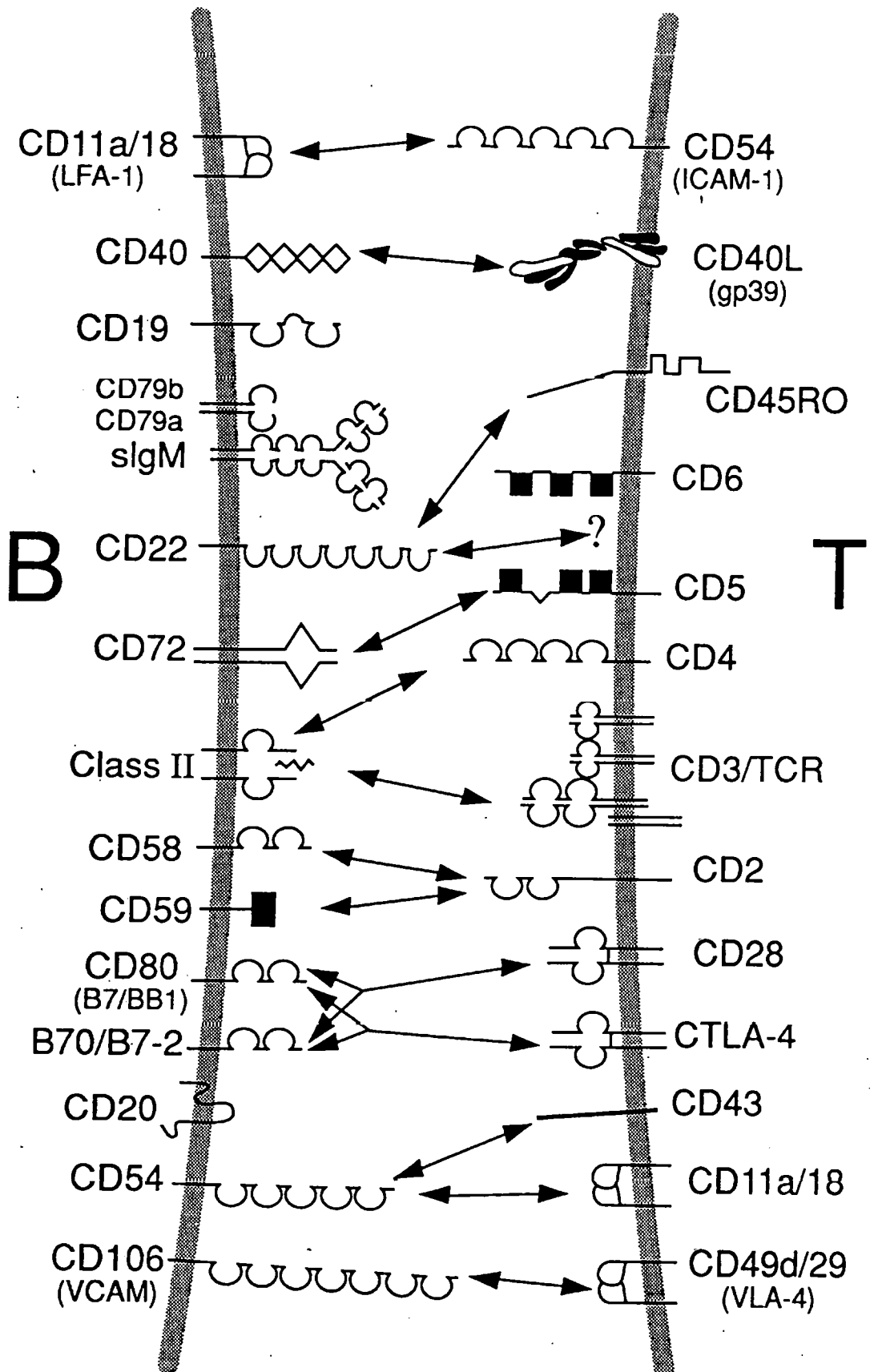
proliferation (Linsley, *et al.*, 1991). Interference with this signal in vitro can block T-cell proliferation and B cell maturation induced by T-cell cytokines. CD80 expression is induced or stimulated after cross-linking MHC class II with monoclonal antibodies or during autoreactive T-cell interaction with B cells (Nabavi, *et al.*, 1992). This cross-linking may thus induce CD80 expression in B cells so that CD80 can, in turn, signal to the T cell via CD28. It has been shown that simultaneous signalling through TcR and CD28 results in the synergistic activation of two mitogen-activated protein kinases, JNK1 and JNK2 (Su, *et al.*, 1994).

The CD40L-CD40 and CD28/CTLA-4-CD80 ligand pairs are not the only means by which T cells and B cells interact. The CD11a/18-CD54 ligand pair is also likely to play a part; an active form of CD11a/18 is rapidly induced on crosslinking the T cell receptor complex (Dustin and Springer, 1989). Antigen-specific T-cell activation rapidly induces a CD11a/18-CD54-dependent signal to the antigen presenting B cells and crosslinking CD40 on B cells promotes allogeneic T cell proliferation via CD11a/18-CD54-dependent interactions (Barrett, *et al.*, 1991).

Patients with leukocyte adhesion defects do not express CD11a/18 on their T or B cells. They make both IgM and IgG antibodies in response to specific antigen, but have depressed antibody titres, indicating that their production of memory B cells may be impaired (Ochs, *et al.*, 1993).

A number of surface molecules (e.g. CD19 and CD22) are associated with cell-surface IgM and are rapidly phosphorylated on tyrosine residues after they become cross-linked (Clark, 1993). CD22 may be required for normal signalling, as intracellular calcium is not elevated from cross-linking surface immunoglobulin in CD22-negative B cells. CD19 is expressed on B cells throughout their maturation. It interacts with various molecules on other B cells, including the receptor for Cd3 component of complement, CD21/Cd2,

indicating that it may facilitate a complement-mediated signalling pathway. When cross-linked, CD19 induces calcium mobilisation in both pre-B and mature B cells.

Antigen specific T-cell activation is dependent on interactions between T cells and APCs, specifically B cells. Once active, the T cells in turn promote B-cell activation, both by releasing T-cell-derived cytokines such as IL-2 and IFN-γ (by Th1 cells) and IL-4 and IL-5 (by Th 2) and by direct intercellular contact (MacLennan, *et al.*, 1992). T cells play an essential role not only in the activation of resting B cells, but also in isotype switching (Kong and Morse, 1976) and affinity maturation of the antibody response; *The more T-independent the response, the poorer the affinity maturation* (a particularly relevant feature for the subject matter of this thesis). T cells also play a major role in generating memory B cells which can respond more effectively to subsequent challenge with antigen (Feldbush, *et al.*, 1986). Once B cells have been activated by first T cells and later by follicular dendritic cells, they express an array of surface receptors, specifically CD40 and CD80, and can present antigen to T cells effectively.

1.7. Viral Immune Evasion Strategies

Latent and / or persistent infections are part of the life style of many viruses. This capacity to maintain a long-term relationship with the host presupposes viral mechanisms for circumventing antiviral defences. Viruses that infect vertebrate hosts achieve sustained host-to-host transmission by using specific strategies that evade or subvert the consolidated activities of the anti-viral immune and inflammatory responses.

Certain RNA viruses, including influenza virus and human immunodeficiency virus, escape humoral or cellular immunity by mutating their cell surface glycoproteins as a consequence of the error-prone RNA polymerase. A strategy particularly common among DNA viruses, which are less capable of frequent and rapid mutations, is to synthesise proteins that modify host immune attack. The larger DNA viruses are of particular interest because they encode proteins that are not required for virus replication in tissue culture, but instead allow for virus propagation in host tissues that are normally visible to the immune and inflammatory systems of the host. Several mechanisms have been reported of virus modulation of the host response to infection which can be divided into the following categories;

1.7.1. Latency.

Latency is defined as a type of persistent infection in which the viral genome is present in the host but infectious virus is not produced except during intermittent episodes of reactivation. Viruses that are considered latent evade immune responses by hiding in privileged sites or changing patterns of cytopathogenicity, growth kinetics or tissue tropism. It is a common feature of herpesviruses especially Herpes Simplex virus type 1 (HSV-1), Cytomegalovirus (CMV), and Epstein-Barr virus (EBV) that acute infection is not terminated by clearance of infectious virus and the viral genome but is

followed by a phase of persistent virus replication which is often confined to a particular tissue site.

HSV is a common neurotropic virus which in humans and experimental animals establishes latent infection of sensory ganglion neurons. Following entry into the host, virus undergoes primary replication in the skin or mucosae. The virus then gains access to the distal axon terminals of sensory neurons and travels by axonal transport to neuronal cell bodies in sensory ganglia, where limited replication may occur and viral gene expression is repressed, leading to a latent state. The host immune response curtails viral replication in the ganglia and potentially lethal spread to the brain. A primary immune response, however, is unable to prevent establishment of latent HSV infection in ganglionic neurons. Latent viral genomes existing in a non replicating state are reactivated by unknown mechanisms and depending on conditions of local immunity, reactivation may result in recurrent skin lesions (Roizman and Sears, 1987).

Recovery from acute HSV infections requires T cell responses and it has been shown that MHC class II-restricted CD4 T cells are primarily responsible for clearing HSV from skin, whereas MHC class I restricted cytotoxic T cells control HSV replication in the nervous system (Nash, *et al.*, 1987).

Reactivation of infectious virus from latent viral genomes is the initiating event in the pathogenesis of CMV disease during immunodeficiency, bone marrow and/ or organ transplantation including liver, kidney, heart and lung transplantation (Ho, 1991). In a murine model of CMV latency, the lungs were identified as a major site of latent infection, since a high-copy of viral genome was detectable in lung tissue even after it was cleared to an undetectable level in blood and bone marrow (Balthesen, *et al.*, 1993). A comparison of lungs and the spleen, the previously most thoroughly

investigated site of murine CMV latency, revealed a 10-fold-higher burden of latent viral genome for the lungs.

Activation of B cells causes a switch from latent to lytic EBV infection. In latently infected B lymphocytes a specific origin of viral DNA synthesis and a set of viral proteins (EBNAs) without known DNA polymerase activity appear to be responsible for the limited amplification and maintenance of the EBV genome in an episomal state (Yates, *et al.*, 1985). Two EBV latent membrane proteins (LMP-1 and LMP-2) influence B cell activation and hence virus replication. Crosslinking of the B cell surface antigens, such as IgM, MHC class II or CD19, causes signal transduction and an intracellular calcium flux that leads to cell activation. This process is downregulated by LMP-2A, probably because of its association with cytoplasmic tyrosine kinases, and hence cell activation and progression into lytic replication is restricted. This may be an advantage to the virus in the presence of a vigorous antibody response that may neutralise the virus infectivity.

Current opinion on AIDS pathogenesis divides the clinical course of HIV infection into a latent phase, in which the virus is silent, and a progressive phase, in which HIV replication is induced with a rapid loss of CD4 + T cells. During this latent or subclinical phase of infection, HIV continues to replicate at low levels despite an often vigorous but ineffective host immune response. When an individual is infected with HIV, a mixture of related HIV clones is transmitted. Cells infected with virulent, high expressing HIV variants are selectively eliminated by the uncompromised host immune system (Asjo, *et al.*, 1988). Low virulence variants may escape immune response because of their low-level expression or their monocytotropism (Popovic and Gartner, 1987). It has been shown that monocytes, macrophages and dendritic cells can be infected with HIV and support HIV replication in vitro and in vivo (Plata, 1987). First, monocytes-macrophages may be persistently infected and function as a reservoir for virus dissemination to lungs and brain (Koenig, *et al.*, 1988)].

Second, HIV infection may induce severe defects in the accessory functions of monocytes and peripheral blood-derived dendritic cells from symptomatic and asymptomatic HIV individuals. Shearer and co workers demonstrated a selective loss of T-cell reactivity in asymptomatic HIV-infected individuals with respect to both proliferation and generation of cytotoxic T-lymphocytes in response to nominal antigen presented by self MHC molecules (Shearer, *et al.*, 1986).

1.7.2. Immunosupression.

Viruses can perturb the host immune system in general by one of the two basic mechanisms: disordered immune regulation may be a direct consequence of viral replication in immunocompetent cells. For example, cell dysfunction due to infection of helper T lymphocytes could lead to immunosuppression or the establishment of persistent virus infection could involve virus-specific cytotoxic T lymphocytes (CTL). The majority of the viruses, e.g. measles virus, undergo restricted infection in unstimulated lymphocytes. Productive infection frequently follows mitogenic stimulation. With other viruses, e.g. lymphocytic choriomeningitis virus (LCMV), replication is restricted so that recovery of infectious virus requires cocultivation with permissive cells.

As mentioned above, measles virus infection is associated with suppression of immune functions both in vivo and in vitro. Infection with measles virus (MV) is accompanied by marked and prolonged abnormalities of cell mediated immunity (CMI), which contributes to increased susceptibility to secondary infections. Destruction of cells infected by measles virus is due to positioning in the cell membrane of cleaved fusion protein of the virus. Infected adjacent cells form syncytia that cannot survive. During acute infection, measles virus replicates in lymphoid tissues. In vivo, sensitisation and

expression of delayed-type hypersensitivity (DTH) responses are inhibited for several weeks after acute measles virus infection.

The loss of delayed cutaneous hypersensitivity (DCH) to tuberculin antigen during measles virus infection was first described in 1908 by Von Pirque and has been shown both during natural infection and after live attenuated measles virus immunisation. It was observed that the tuberculin skin test response of immune individuals was depressed during the time course of acute measles virus infection (von Pirquet, 1908).

The effect of measles virus on phytohaemagglutinin (PHA) induced stimulation of human peripheral blood mononuclear cells was investigated by Sullivan et al. It was noted that medium which had several days contact with uninfected monolayers as well as unpurified MV preparations produced significant inhibition of ^{3}H-thymidine incorporation by PHA-stimulated lymphocytes. When partially purified MV preparation was used, marked inhibition was observed. Both T and B lymphocytes taken from the peripheral blood during natural infection express viral antigen after mitogenic stimulation in vitro (Sullivan, et al, 1975).

As with other viruses that disturb CMI such as HIV, monocytes and macrophages are prime targets of MV in natural infection (Esolen, et al., 1993), which are thought to be principal IL-12 producing cells in vivo. IL-12 is crucial to the development of CMI, being a potent inducer of IFN-γ from T and NK cells, required for the development of Th1 responses and necessary for DTH responses. It was proposed that one of the mechanisms of immunosuppression by MV might be down regulation of IL-12 production (Karp, et al., 1996). This was demonstrated by infecting human moncytes with MV, followed by stimulation with bacterial antigens which resulted in down-regulation of IL-12 induction.

Retroviruses of murine, avian, feline and human origin are immunosuppressive as well as oncogenic in their hosts. In several experimental models with C-type retroviruses, virions cause immunosuppression as well as neoplasms in a dose dependent manner. Since immune mechanisms may limit the development or spread of cancer, clinically apparent tumours may develop when transformed cells acquire the means to escape host immunological defence mechanisms. Studies in the murine and feline retrovirus systems suggest that the 15,000-dalton envelope protein (p15E) of the virion may contribute to immune suppression by interfering with normal lymphocyte function. It was reported that a partially purified 15-kd structural protein of FeLV inhibited the proliferation of feline lymphocytes induced by concanavalin A (Con A) (Mathes, et al., 1978). The inhibition was dose dependent and occurred when the protein was added as late as day 3 of a 4 day culture. In contrast, another structural protein of the virus, p27, was not inhibitory. The same group also demonstrated that both UV inactivated FeLV and p15E suppressed the proliferation of human lymphocytes to Con A (Copelan, et al., 1983). Suppression was not mediated by monocytes, as pre-treated monocytes were able to secrete IL-1 and serve as accessory cells when cultured with untreated T lymphocytes.

Although the selective loss of CD4+ helper T cells in the relatively later stages of HIV infection, is the main mechanism by which HIV induces immunodeficiency, it has been claimed that the deficient antigen responsiveness of CD4+ lymphocytes from HIV-infected individuals is due in part to gp 120-induced inhibition of CD4 function (Diamond, et al., 1988). gp120 binds to CD4 (the receptor for HIV-1) with high affinity thereby facilitating entry of the virus into the cell and syncytium formation between infected and uninfected cells.

1.7.3. Down-regulation of MHC expression.

Viral peptides from within infected cells are presented on the cell surface in association with MHC class I. Cells bearing such complexes are recognised and lysed by cytotoxic T cells (CTL). CD8+ T cells that recognise peptides derived from the viral proteins synthesised in the cytoplasm of the infected cells are considered, in certain quarters, to play an important role in antiviral defence. However, viruses like adenoviruses, CMV and HSV have found means to interfere with immune recognition by down-regulation of MHC class I.

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that persists in the host and can cause severe disease in immunocompromised individuals or the foetus. Analysis of the nucleotide sequence of the virus genome has revealed the presence of an open reading frame whose translated product has homology with the heavy chain of MHC class I molecules of higher eukaryotes (Beck and Barrell, 1988). The observed sequence homology was given additional significance by another observation that HCMV virions can bind β_2 microglobulin (β_2m), the light chain of MHC class I (McKeating, *et al.*, 1987). Browne, in 1990 did a notable experiment by expressing both the HCMV class I homologue and the human β_2m gene in recombinant vaccinia virus and showed that in recombinant-vaccinia-infected cells, no synthesis of mature cellular class I occurred, while mRNA levels were unaltered (Browne, *et al.*, 1990). Thus, HCMV may evade immune recognition by preventing the maturation of host class I molecules and rendering the infected cells unrecognisable by cytotoxic T cells.

A similar mechanism of decrease in MHC class I expression is used by adenovirus 2: the E3-gene-encoded 19kDa protein of Ad2 binds to class I heavy chains and prevents transport to the cell surface, which in turn results in

reduced recognition and reduced lysis of infected cells by CTLs (Andersson, *et al.*, 1987). Andersson et al., examined the biosynthesis of the complex between class I and Ad-2 glycoprotein E19 in adenovirus-infected HeLa cells by immunoprecipitation and SDS-gel electrophoresis. At 2 hours after infection, small amounts of E19 were already co-precipitated with MHC class I heavy chains and β_2 microglobulin. Later on larger amounts of the E19 were found bound to class I, as evidenced by the antibodies against class I, whereas, antibodies against E19 protein bound only minor amounts of class I heavy chains. The rate of class I synthesis did not vary significantly, but while the electrophoretic migration of β_2 microglobulin remained the same, the mobility of the heavy chain increased such that it became identical to that of core-glycosylated heavy chains derived from infected HeLa cells suggesting that the heavy chains, on complex formation with E19 fail to become terminally glycosylated. Thus, interference with antigen presentation by means of direct protein-protein interaction with MHC is another strategy by which some viruses evade immune surveillance.

Herpes Simplex Virus (HSV) also blocks presentation of viral peptides to MHC class I restricted T cells. Antigenic peptides are generated in the cytoplasm by proteosomes (Rock, *et al.*, 1994) and translocated into the lumen of the endoplasmic reticulum (ER) by TAP1 and TAP-2 (Townsend and Trowsdale, 1993). HSV expresses an immediate early protein, ICP47 which lacks a recognisable signal sequence. ICP47 binds to TAP and prevents peptide translocation into the ER. By expressing ICP47 in HeLa cells, Fruh et al showed that ICP47 efficiently inhibits peptide transport such that nascent class I molecules fail to acquire antigenic peptides. Furthermore, ICP47 colocalises and physically associates with TAP within the cell (Fruh, *et al.*, 1995). Thus, interference with peptide translocation into the ER is a further mechanism by which viruses may evade immune recognition.

1.7.4. Inhibition of Cytokine function.

Cytokines play a fundamental role in the inflammatory and immune response to infection and may directly destroy virus-infected cells. Viruses have devised an array of strategies to abrogate cytokine function. A strategy used by some herpes viruses and poxviruses is to encode cytokine receptor (s) that bind and sequester the cognate cytokine and prevent it from reaching the natural receptor and triggering signal transduction. Poxviruses express receptors for interleukin-1β (IL-1β), TNF and interferon-γ. In each case, the protein is secreted from the infected cells and is homologous only to the external cytokine-binding domain of the cellular receptor, lacking the transmembrane domain and cytoplasmic region.

An IL-1β receptor has been detected in cowpox and many strains of vaccinia virus. IL-1 is produced in response to infection and tissue injury. It is involved in the regulation of the inflammatory and immune responses and in the activation of a broad spectrum of systemic effects that contribute to host defence (Dinarello, 1988). Two classes of IL-1 receptors have been identified that bind both IL-1α and IL-1β with similar affinity (Dower and Urdal, 1987). The 80kDa type1 IL-1 receptor is found on T cells and fibroblasts, while the type II IL-1 receptor is present on B cells and macrophages. It was reported that vaccinia virus BR15 ORF (open reading frame) is actively transcribed, translated, and encodes secretory glycoprotein in the supernatants of vaccinia-virus infected cells that functions as a soluble IL-1 receptor. Unlike the membrane-bound cellular IL-1 receptors (type I and II), which bind IL-1α, IL-1β and the IL-1 receptor antagonist protein, the virus IL-1 receptor binds only IL-1β, emphasising the importance of this cytokine in poxvius infections and suggesting that it can interfere with the immune responses by blocking the effects of this cytokine. This was shown in binding experiments to radioiodinated ILs and was corroborated in competition assays with unlabeled

cytokines and by blocking the interaction of the ILs with the natural receptors on cells (Alcami and Smith, 1992).

Similarly other poxviruses, notably shope fibroma virus (SFV), myxoma virus, and malignant rabbit virus (MRV) encode proteins with homologies to cellular receptors for tumor necrosis factor (TNF). TNF is a potent cytokine produced mainly by activated macrophages and plays an important role in inflammatory, immunoregulatory, proliferative and antiviral responses and also synergises with interferons to augment the antiviral state (Beutler and Cerami, 1988). The T2 open reading frame of both SFV and myxoma virus possesses significant homology to the ligand binding domains of the murine and human TNF (α, β) receptors (Upton, *et al.*, 1991). TNF-binding protein T2 competitively inhibits TNF binding to its cell surface receptor. Targeted disruption of both copies of the myxoma T2 gene revealed that the absence of T2 expression caused significant attenuation of myxomatosis in rabbits (Upton, *et al.*, 1991). To characterise the T2-TNF interaction, myxoma T2 protein and rabbit, mouse and human TNF-α were expressed independently from vaccinia virus vectors. Growth of the TNF-α expressing cells was significantly attenuated in TNF-hypersensitive cells (L929-8 cells), and these cells were rapidly lysed by all three TNF-αs. When the ability of the myxoma T2 protein to inhibit biological activities of TNF-α was assayed, T2 protein protected L929-8 cells were lysed by rabbit but not human or mouse TNF-α.

Myxoma virus also expresses an IFN-γ receptor that can protect cells from the anti-viral effects of IFN. IFN-γ is a potent immunomodulatory cytokine produced by activated T lymphocytes and functions to combat viral infections by inducing anti-viral pathways and by modulating cellular immune responses to viral challenge. It also possess the ability to enhance expression of MHC class I and II, resulting in an increase of viral antigen presentation, and

furthermore, IFN-γ induces the secretion of the ligand binding domain of the low density lipoprotein receptor, which interferes with assembly and budding of certain enveloped viruses. Poxviruses were the first viruses found to be capable of interrupting the extracellular ligand-dependent triggering of the IFN-γ receptors, thus preventing signal transduction from an extracellular location. M-T7, the most abundantly secreted protein from myxoma virus-infected cells, was shown to be expressed in significant amounts as a typical poxvirus early gene product, efficiently secreted at early times of infection, and to function as a stable inhibitory protein in infected cell supernatants until later times of infection. It contains significant sequence similarity to the ligand binding domain of the mammalian IFN-γ receptors and functions as a soluble homologue which can specifically bind and inhibit the biological activities of rabbit IFN-γ but not human or murine IFN-γ (Mossman, *et al*, 1995).

1.7.5. Intracellular blockade of cytokine synthesis and function.

As well as expressing cytokine receptors, some viruses have also developed measures to block the production of mature cytokines and to modify the consequences of cytokine-binding to the receptors. Recently, seven distinct serine proteinase inhibitor genes have been discovered in the poxvirus genome all of which encode proteins belonging to the superfamily of serine proteinase inhibitors (serpins) (Turner, *et al.*, 1995). Viruses such as vaccinia, cowpox and rabbitpox, each encode three known serpins that have been designated SPI-1,-2, and-3. Cowpox virus expresses a 38kDa intracellular protein (crmA) with sequence similarity to SPI-1 which inhibits the pro-IL-1β converting enzyme (ICE) that cleaves the intracellular 30kDa IL-1β precursor into the 17kDa mature secreted form.

Recombinant BCRF1 protein of Epstein Barr virus (EBV) mimics the activity of another cytokine Interleukin-10 (IL-10) suggesting its role in the interaction with the immune system. IL-10 is produced by Th2 cells and

inhibits synthesis of cytokines, specifically IFN-γ by activated Th-clones. Because Th1 cells preferentially mediate delayed-type hypersensitivity (DTH) and macrophage activation, whereas Th2 cells provide help for B cells, IL-10 may represent a mechanism whereby Th2 cells can inhibit the effector functions of Th1 cells. It has been shown that the mature, secreted IL-10 polypeptide has approximately 70% homology to BCRF1 (Baer, *et al.*, 1984). Hsu et al cloned and expressed the BCRF1 gene, and demonstrated that the expressed BCRF1 protein, like IL-10, inhibits IFN-γ synthesis by activated lymphocytes (Hsu, *et al.*, 1990).

Human adenoviruses encode three proteins, E3-14.7K, E3-10.4K/ 14.5, and E1B-19K, that protect virus-infected cells from cytolysis by TNF (Wold and Gooding, 1991). Little is known about the mechanism of action of these adenoproteins, except that all three act at steps subsequent to TNF binding to its receptor.

1.7.6. Inhibition of complement cascade.

The complement system consists of more than twenty plasma proteins that participate in host defences against infectious agents and may be activated in two ways: the classical pathway requires a specific antibody-antigen interaction, while the alternative pathway may be activated in the absence of antibody by certain antigens, such as bacterial cell wall polysaccharides. Each pathway contains a cascade of enzymatic reactions that greatly amplifies the original signal and leads to the formation of a membrane attack complex that damages the surface membrane of envelope viruses or infected cells. Several poxviruses and herpes viruses have evolved defences against it by encoding proteins that have amino acid similarity with proteins of the complement system.

Inhibition of both classical and alternative pathways of complement activation is mediated by the enveloped glycoprotein gC-1 of herpes simplex

virus. Glycoprotein C-1 binds the C3b fragment of complement component C3 which is central to both classical and alternative pathways, thus preventing both complement-mediated virus neutralisation and cytolysis of virus-infected cells (Harris, *et al.*, 1990).

Herpes viruses have adopted an additional mechanism to counter antibody dependent complement-mediated attack by encoding a pair of proteins, gE and gI. These proteins bind the Fc region of IgG by bipolar bridging i.e. antibody binding by its Fab end to HSV antigen and by its Fc end to the viral Fc receptor. This bipolar bridging of antibody modifies effector functions mediated by the Fc region, including antibody-dependent cellular cytotoxicity, binding of C1q, and antibody mediated complement-enhanced virus neutralisation (Dubin, et al, 1991).

Vaccinia virus evades the consequences of complement activation by encoding two proteins that have four copies of the 60-70 amino acid motif called the consensus sequence (SCR) that is typical of complement control protein. One of these is a 35-kDa protein (VCP) that is secreted from the infected cell and has closest sequence similarity with a human protein C4b. It binds to C3b and C4b fragments of the third and fourth complement components, blocks activation of both the alternative and classical pathways, and protects intracellular virus from complement-mediated neutralisation of infectivity (Kotwal, *et al.*, 1990). The second protein is present on the surface of the virus infected cells and extracellular virus particles.

The immune system, in conjunction with all its effector mechanisms aims to recognise and eliminate viruses and virally infected cells. However, the above mentioned examples give a clear picture of an array of strategies by which viruses perturb immune mechanisms and ensure their survival.

Antigenic variation, a common strategy of immune evasion by RNA viruses, has been most extensively studied for influenza virus. Since this is the

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Fig 3: Diagram of the influenza virus.

Adapted from Oxford and Hockley, 1987.

The viral segmented genome consists of eight strands of negative polarity RNA which encode ten different viral proteins including the antigenic surface glycoproteins haemagglutinin and neuraminidase. Haemagglutinin is present on the surface as a trimer and neuraminidase as a tetramer. M2 is a membrane protein which forms ion channels (Oxford and Hockley, 1987).

differential splicing to generate transcripts for the structural proteins M1 and M2, and the non-structural proteins NS1 and NS2, respectively (Lamb and Choppin, 1983; Brand and Skehel, 1972) (fig 3).Viral mRNA synthesis is catalysed by viral nucleocapsids (Inglis, et al., 1976) (Plotch, et al., 1981), which consist of individual vRNAs associated with four viral proteins: the nucleocapsid (NP) protein and the three P (PB1, PB2, and PA) proteins (Inglis, et al., 1976; Ulmanen, et al., 1981). The P proteins are responsible for viral mRNA synthesis and appear in the form of a complex that start at 3' ends of the vRNA templates and moves down the templates in association with the elongating mRNAs during transcription (Braam, et al., 1983).

1.8.2. Influenza virus replication.

Influenza viruses bind to cells by interacting with membrane receptor molecules containing sialic acid (Gottschalk, 1959). Viral entry and membrane fusion is mediated through haemagglutinin. Binding of virus particles to their receptors is followed by endocytosis after which fusion of virus and endosomal membranes at acidic pH occur. The transcription complex released into the cell as a result of membrane fusion is transferred to the nucleus by cellular processes which recognise signals on the virus proteins similar to those on the nuclear proteins of the cell. The complex consists of five virus proteins PA, PB1, NP and MP and eight RNA molecules of the virus genome. The transcription complex has two functions; to synthesise the early virus messenger RNAs required for virus replication to begin, and to produce the complementary RNAs involved as templates in the replication of virus genome RNAs.

1.8.3. Influenza virus budding.

Influenza virus contains a lipid membrane that it obtains during maturation by budding from the plasma membrane of an infected cell (Wiley, 1985). In early microscopic studies of the maturation of influenza virus in the chorioallantoic membrane of infected chicken embryos (Murphy and Bang,

1952), it was observed that virus budding occurred at the free luminal cell surfaces. Similar polarity in enveloped virus maturation has been observed in epithelial cells grown in vitro (Rodriguez, Boulan and Sabatini, 1978). Monolayers of the epithelial Madin-Darby canine kidney (MDCK) line form tight junctions between adjacent cells and exhibit an electrical potential between the upper and lower surfaces (Cereijido, *et al.*, 1978; Leighton, *et al.*, 1970; Misfeld, *et al.*, 1976). It has been reported (Rodriguez Boulan and Sabatini, 1978) that vesicular stomatitis virus (VSV) buds exclusively from the basolateral membranes in these cells, whereas influenza and parainfluenza viruses bud exclusively from the free apical surface.

1.8.4. Structure, function, and antigenicity of the Haemagglutinin of influenza.

Haemagglutinin is one of the major surface glycoproteins of the influenza virus that interacts with neutralising antibodies; alterations in the molecule enable the virus to escape immune surveillance and cause epidemics of the disease. HA has two functions in virus replication; it binds virus to cell-surface glycoconjugates by recognising terminal sialic acid residues of carbohydrate side chains, and, following endocytosis of bound virus, it mediates the fusion of the virus and endosomal membranes required for transfer of the virus genome-transcriptase complex into the cell (Wiley and Skehel, 1987). To better understand why this virus has eluded our control efforts, it is necessary to understand the three-dimensional structure and function of the HA molecule and the immune responses to this protein.

1.8.5. Structure of Haemagglutinin.

Haemagglutinin (HA) is a homotrimer (Wiley, *et al.*, 1977) and is synthesised in the rough endoplasmic reticulum of infected cells as a single polypeptide chain (HA0) of around 550 amino acids. During passage to the plasma membrane, the molecule is glycosylated in several positions and is subsequently cleaved into two disulphide-linked subunits, HA1 and HA2 by

host cell proteases. Bromelain digestion cleaves HA on virus membranes at HA2 175 and results in the release of the entire ectodomain (BHA) as a soluble trimer (Brand and Skehel, 1972).

The three dimensional structure of X31 B-HA has been determined to 3Å by X-ray crystallography (Wilson, *et al.*, 1981). Basically, each monomer consists of a globular membrane distal region consisting of HA1 on top of an elongated stem consisting of all of HA2 and parts of HA1. Residues 63-305 form the distal globular domain, which contains an eight stranded anti-parallel β-sheet and two short α-helices. This framework supports the receptor-binding site present in this region. The receptor binding site is a shallow concave pocket of highly conserved amino acid residues surrounded by regions that vary with changes in antigenicity. The major forces stabilising the haemagglutinin's trimeric subunit interactions arise from a triple-stranded coiled-coil in the fibrous region of the molecule.

1.8.6. Antibody recognition sites of Influenza HA.

As mentioned earlier, the conserved receptor binding pocket is surrounded by highly antigenic regions against which neutralising antibodies are directed and resistance to infection correlates with the levels of serum anti-HA antibodies (and secretory IgA) which are subtype specific (Dowdle, *et al.*, 1973; Hobson, *et al.*, 1972). The antibody recognition sites have been deduced from structural analysis of laboratory escape mutants, selected with neutralising monoclonal antibodies *in ovo*, and differing from the immunising virus by one or occasionally two amino acid substitutions in HA molecule (Gerhard, *et al.*, 1981; Laver, *et al.*, 1979). The molecular locations of these substitutions define five antigenically important sites designated as A-E (fig 4) corresponding to most surface exposed regions (Caton, *et al.*, 1982; Gerhard, *et al.*, 1981; Lubeck and Gerhard, 1981; Underwood, 1982; Wiley, *et al.*, 1981). Each one of the proposed antigenic sites A, B, C, D and E has

Fig 4: Schematic representation of an H3 HA monomer, showing major antibody binding regions.

Adapted from Skehel et al., 1984 and after Wharton et al., 1989.

The HA monomer consists of disulphide linked HA1 (blue) and HA2 (red) subunits, both encoded by the HA gene as HA0, and cleaved to HA1 and HA2. The HA spike consists of a globular head region supported on a fibrous stem (containing only HA2 residues) anchored to the viral membrane at the base of the molecule. The globular head region, consisting mainly of HA1 residues, houses the receptor binding site at the trimer interface, to the right of the molecule (antigenic site D) and the major antigenic sites A-E. Carbohydrate is shown in green (Skehel, *et al.*, 1984; Wharton, *et al*, 1989).

A B D E C

160
130 150 200
50 220
110 230 210
190 100
120 0 240
360 110
60 90 70 80
150 300
280 310 90
290 60 100
40 30
50 320 110
20
20 40
10 120
150 140 30
100 170
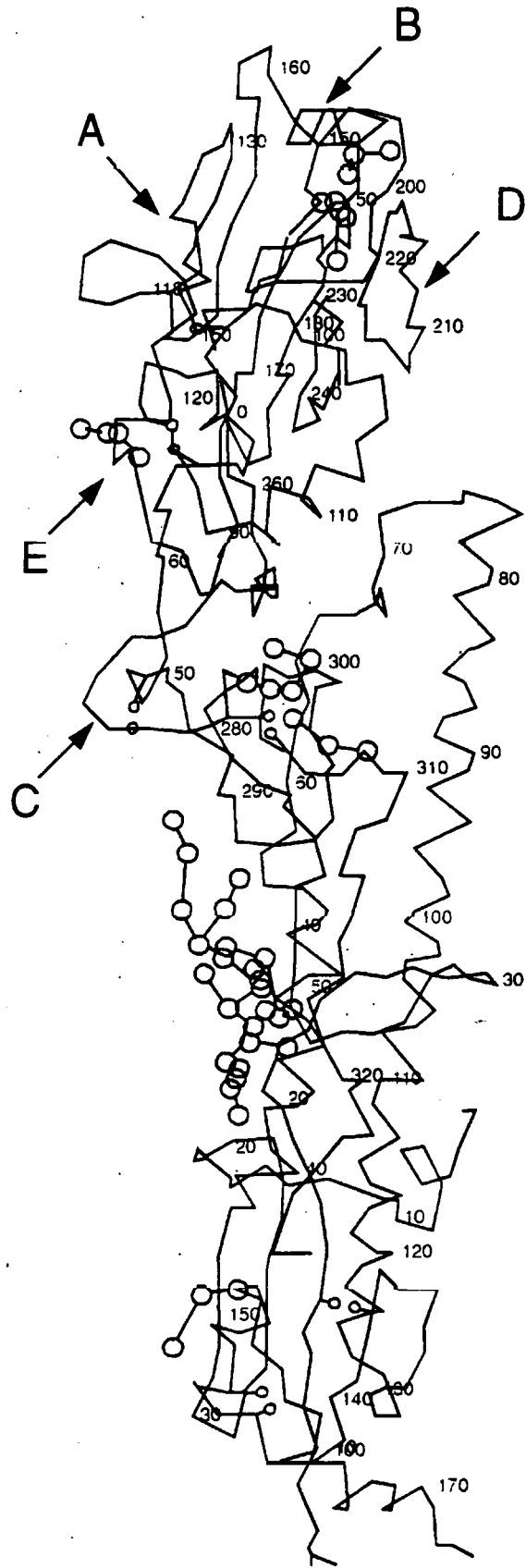
Fig 5: Structure of the receptor binding pocket of the HA molecule (Weis, *et al.*, 1988).

Leu 226

225

224

Tyr 98

138

137

Ser 136

135

H

N

HO

O

O

OH

H RO

OH

2

3

4

6

5

7

8

9

OH

OH

NH

O

CH$_3$

N

H

O

HO

O

O

HO

HN

N

N

H

His 183

Glu 190

Leu 194

Tyr 195

Thr 155

OH

Trp 153
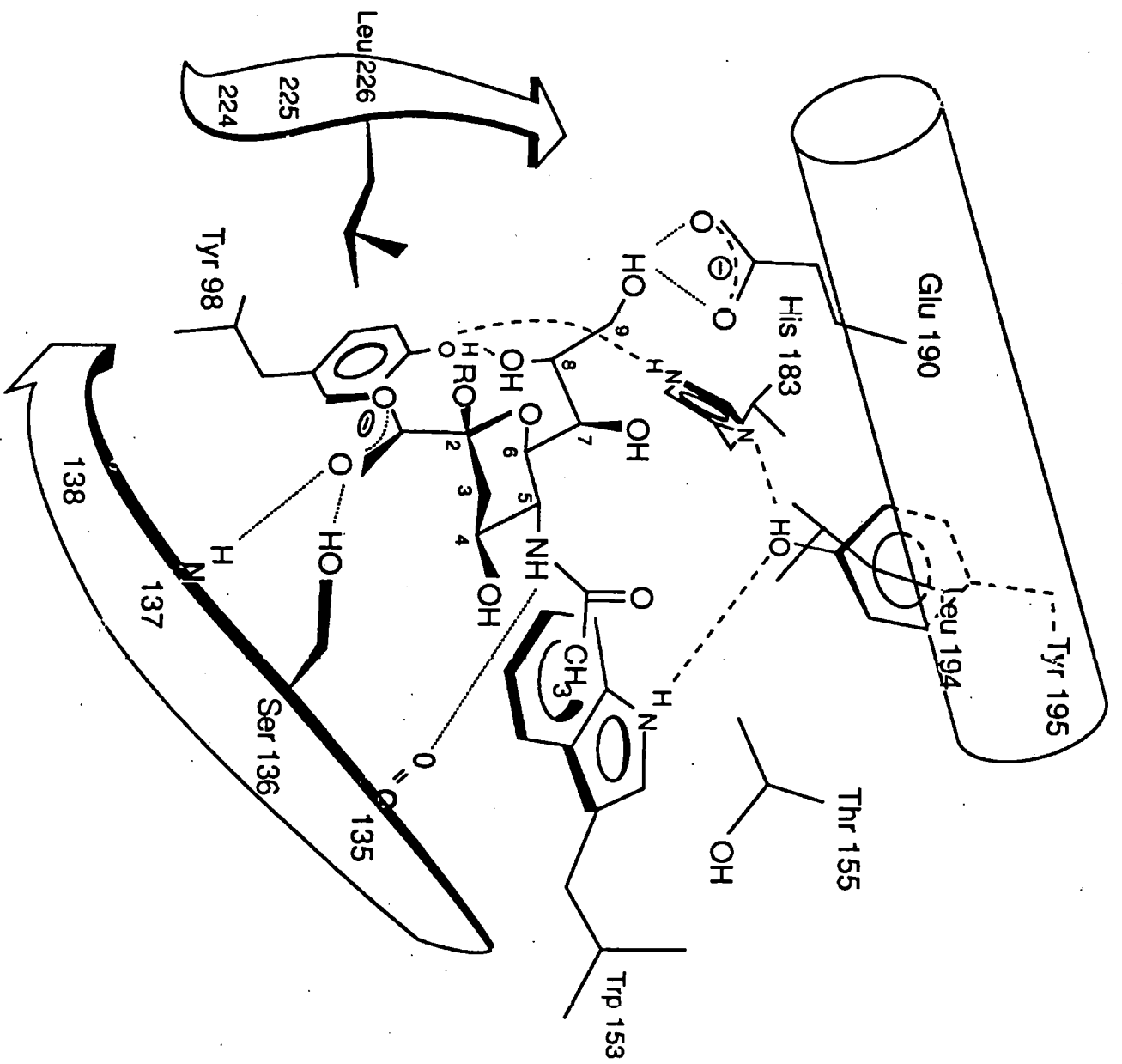
1.8.7. Structure of the receptor-binding pocket.

The HA of the influenza virus is responsible for binding the virus to cell-surface sialic acid receptors during infection. The region of the haemagglutinin involved in receptor binding has been deduced from crystallographic studies to involve a shallow, concave pocket of several highly conserved amino acid residues at its membrane distal surface (Weis, *et al.*, 1988).While changes have occurred all around the receptor binding site, the pocket itself has not changed since 1968, although receptor-binding variant viruses have been selected in the laboratory (Rogers and Paulson, 1982; Temoltzin-Palacois and Thomas, 1994). Sialic acid occupies the entire pocket, indicating that it is the dominant component of the influenza virus cellular receptor.

The binding site is a depression, the bottom of which is formed by Tyr 98 and Trp 153. Glu 190 and Leu 194 project down from the short α-helix to define the rear of the site with His 183 and Thr 155. Residues 134-138 form the right side of the site, and residues 224-228 form the left side (Weis, *et al.*, 1988) (Fig 5). Comparison of receptor specificities of influenza viruses of the H3 subtype has revealed at least three distinct specificities based on preferential binding to either one or both of the sialylated oligosaccharides containing SA α,2-6Gal or SA α,2-3 Gal, and showed sensitivity to inhibition by α_2 -macroglobulin present in the horse serum. Avian and equine H3 isolates, on the other hand, preferentially bind SA α, 2-3 Gal and are resistant to horse serum inhibition.(Rogers and Paulson, 1983; Rogers, *et al.*, 1983a; Rogers, *et al.*, 1983b). Virus specificity for these different sialylated receptors was identified by selecting variants of H3 strains with different binding specificities.

1.8.8. Antigenic Variation

One of the hallmarks of influenza virus is its ability to undergo unpredictable and rapid antigenic variation. Both of the surface glycoproteins HA and NA are protective antigens that undergo amino acid substitutions to

evade immune recognition. Although, antibodies can be, and are, made against any structural protein of the influenza virus, it is antibodies that are directed against HA that neutralise virus infectivity. These neutralising antibodies focus on sites that are clustered around the receptor binding pocket, thus preventing attachment of the virus to the host cell sialic acid receptors (Wilson, *et al.*, 1981).

Antigenic variation alters the nature of the surface proteins such that they cannot be recognised by host defence systems but without altering their structure enough to affect other essential functions of these proteins. Antigenic variation in HA involves two separate processes, antigenic shift and antigenic drift.

Antigenic shift occurs only for type A influenza viruses, when a virus HA of a novel subtype is newly introduced into the human population resulting in new epidemic or pandemic. These are thought to arise by recombination between isolates of different species e.g. avian and porcine (Webster, *et al.*, 1971) in either one or both of the glycoproteins with up to 80% sequence change (Webster, *et al.*, 1982). Large segments of the population have no immunity to these new viruses, because there is little or no serologic relation between the haemagglutinin of the new viruses and those of their predecessors. Perhaps the best example of an influenza pandemic caused by reassortment occurred in 1968, when the H2N2 viruses were displaced by the H3N2 Hong Kong subtype. As determined by molecular analysis, the new H3N2 virus contained seven of eight genes from the preceding H2N2 strain. Only the H3 haemagglutinin was different (Nakajima, *et al.*, 1982).

Antigenic drift is less drastic and occurs continually. It results from accumulation of amino acid substitutions primarily in the HA1 chain. Changes within subtypes are caused by mutations in the RNA genome at sites coding for amino acids in exposed areas of either haemagglutinins or of neuraminidases. Such changes result in alterations of a subtype's antigenicity, and they can give

the new, altered strain a selective growth advantage over the parent strain, evading antibody neutralisation during interpandemic years. Gradual accumulation of these subtle changes in antigenicity, however, sporadically yield epidemic strains.

The molecular location of antigenic variation has been investigated using HA-specific monoclonal antibody (mAb) selected variant viruses, and single amino acid substitution differences to wild type viruses have identified key residues in the 3D structure confirmed by electron microscopy of antibody-HA complexes (Wrigley, et al., 1983). An antibody that recognises any of the five antigenic regions is sufficient for virus neutralisation. These mAb-selected variants have single amino acid substitutions corresponding to the residues that frequently change in natural variants, confirming the importance of antibody selection in antigenic variation (Underwood, 1982). The frequency of selection of antigenic variants by monoclonal antibodies is 10^{-4} -10^{-5} (Yewdell, et al., 1979).

1.8.9. Antibody recognition of influenza HA.

There have been some conflicting reports in the literature concerning the antibody repertoire for influenza HA. Initial studies on antibody recognition of influenza HA were persued by immunisation of BALB/c mice with PR8 (H1N1) virus, and specificity assigned by haemagglutination inhibition reactivity patterns with natural variants. In such studies, extreme diversity in the secondary antibody response to influenza HA was found (Staudt and Gerhard, 1983).

Clarke and colleagues also reported highly diverse specificities in the secondary antibody response to influenza HA site Sb (equivalent of H3 site B) in contrast to the primary antibody responses that were structurally and functionally similar (Clarke, et al., 1985). The secondary antibody repertoire reflects the expansion of clones present in the primary repertoire (Cancro, et al., 1978), and it has been suggested that the increase in diversity is due to the

use of both larger repertoire of antibody V-region genes and somatic mutation (McKean, *et al.*, 1984).

Underwood, studying the secondary response of BALB/c mice immunised i.p. with H3N2 virus, showed the highest proportion of antibodies were directed against the top of the HA molecule (site B) and the second most common site was the trimer interface, suggesting that the antibody repertoire of individual mice might be restricted to one or two target areas .

1.8.10. Immunodominance in the antibody response against influenza HA.

Previous studies from this laboratory have shown the secondary antibody response to be unexpectedly limited following natural infection with X31 virus (H3N2). It was shown that there was striking *immunodominance* in the neutralising antibody response of CBA/Ca mice (H-2^k haplotype) (Smith, *et al.*, 1991; Temoltzin-Palacois and Thomas, 1994), BALB/c mice (H-2^d haplotype) and BALB/k congenic mice (Patera, *et al.*, 1995) following natural intranasal infection with influenza virus; a majority of neutralising mAbs, established from individual donors focused on a single antigenic site. This was deduced by sequencing the HA genes of mAb selected laboratory mutants which were found to differ from wild type X31 virus by the same single amino acid change, HA1 158 G→E in the H-2^k haplotype and HA1 198 A→E in the H-2^d haplotype. Interestingly, in a further analysis of antibody recognition specificities for MHC congenic BALB/k mice, there was codominance in the selection of laboratory variants, with single residue changes at either HA1 158 or HA1 198, dependent on the donor origin of the selecting mAb. Sequence analysis of heavy and light chain gene usage indicated that despite immunodominance of the two residues, there was extensive progenitor B cell diversity contributing to the memory response. Such immunodominance is not evident in the neutralising antibody response following immunisation with inactivated virus.

In a further analysis of immunodominance, it was demonstrated that the introduction of a few mutations in the globular head region of the HA1 subunit, previously shown to be immunodominant, provided structural constraints on further antigenic change in influenza under immune pressure of neutralising antibodies (Temoltzin-Palacois and Thomas, 1994). It is interesting to note that such constraints resulted in the selection of a receptor-binding variant virus (HA1 190 G→D, 226 L→Q); similar amino acid change has also been shown in the recent human isolates of the H3 subtype (A/Beijing/92) which might indicate similar pressures for immune evasion.

1.8.11. Commonality of B-cell and T-cell recognition site.

The neutralising antibody response to influenza is a thymus-dependent event that requires cognate B-cell and T-cell recognition. Initial studies in this area indicated that T cells recognise relatively conserved regions of the molecule distinct from the antibody binding sites however, immunodominance is also observed in the BALB/c and CBA/Ca T-cell response to influenza virus HA after natural infection. Despite the potential diversity of the responses, the BALB/c (H-2^d) Th cells recognise predominantly HA1 177-199 (Barnett, *et al.*, 1989) corresponding to site B, or 56-76 (Graham, *et al.*, 1989), and the CBA/Ca (H-2^k) Th cells recognise HA1 118-138, or 226-245 or 54-63 (Burt, *et al.*, 1989). HA specific and class II restricted T cell clones elicited by natural infection recognise regions of the HA1 subunit that have featured in antigenic drift. This extensive commonality between antibody and T cell recognition specificities may be a consequence of events following natural infection in which the memory B cell plays a selective role in the presentation of antigenic peptides to T cells. The antigenic properties of HA have been extensively analysed using natural variants and mAb-selected mutants to determine the location of antibody binding sites. The CD4+ Th cell recognition patterns have also been studied and shown to coincide with antibody binding sites (Burt, *et al.*, 1989; Graham, *et al.*, 1989; Mills, *et al.*, 1986; Thomas, *et al.*, 1987), thus

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amino acid changes within these antigenic sites abrogate both T-cell and B-cell recognition.

1.8.12. The aim of this investigation.

The main objective of the present studies was to extend the previous findings of immunodominance by using two different strategies: Firstly, analysing the memory antibody repertoire using a transgenic murine system in which an IgM to IgG class switch, and concomitant affinity maturation, was absent and in which the V_H gene usage was restricted. The transgenic mouse contained a HuIgH minilocus with human V_H (V_H 26), D_H (D_{Q5}), all of the J_H and secretory $C\mu$ elements in association an additional V_H (mouse V_H 186.2) with targeted disruption of mouse $C\mu$. The question I addressed was how diverse or restricted is the memory response? Since IgM antibodies are potentially low affinity antibodies compared to moderate affinity IgG antibodies, the next question was to whether inability to class switch would make any difference to their recognition specificity.

Secondly, an analysis of memory repertoire was made in CBA/Ca mice that were infected with X31 virus, underwent *hemisplenectomy*, and rechallenged with a variant of X31 virus that had amino acid substitutions both at known antigenic region and at a Th cell recognition site. The purpose of this study was to see a *shift*, if any, in the memory repertoire. This model system was particularly useful because it is possibly closer to the human situation where individuals are exposed to recurrent virus infection.

Chapter-2

Materials and Methods

2. Materials and Methods

2.1. Mice

CBA/Ca mice were bred under specific-pathogen-free conditions at the National Institute for Medical Research (NIMR), and were infected intranasally with virus at 3-4 months of age.

Human Ig μ-chain transgenic mice were kindly provided by Dr. Simon Wagner (LMB Cambridge) and were immunised with virus >6 months of age.

CBA/Ca x BALB/c (F1) and nude mice were bred at the NIMR, and were used for production of ascitic fluid.

2.2. Viruses

All influenza viruses were grown in the allantoic cavity of 10-day-old embryonated hen eggs at 33°C, the allantoic fluid was harvested after 2 days and stored at -70°C (Beveridge, 1946 ; Hirst, 1962). Virus titres were determined in haemagglutination assays (Salk, 1944). Doubling dilutions of allantoic fluid were made in PBS (see appendix I) and 50μl of each dilution was mixed with 50μl of a 1% TRBC suspension. This was incubated at room temperature for 30 minutes. The highest dilution which showed haemagglutination activity was deemed to contain 1HAU/50μl thus allowing determination of HA titre of the allantoic fluid.

The X31 virus is a recombinant between A/Aichi/2/68 and A/PR/8/34 which expresses surface glycoproteins of the H3N2 subtype and PR8 internal proteins (Kilbourne, 1969).

Natural variants were mainly isolated from the major influenza outbreaks between 1968 annd 1984, provided by The World Influenza Centre at NIMR.

mAb-selected mutants were produced by growing X31 in the presence of neutralising antibody (HI positive) in eggs. Mutants were cloned by limiting dilution in ovo and the allantoic fluid was stored at -70°C (further details outlined in section 2.11).

2.3. Generation of B-cell hybridomas and definition of antibody binding specificities

2.3.1. Culture media

B cell hybridomas and myelomas were allowed to proliferate in RPMI 1640 medium (Flow Laboratories), supplemented with heat inactivated FCS (foetal calf serum; Flow Laboratories), 2mM Glutamine (Flow Laboratories), 5×10^{-5} M β-mercaptoethanol (Sigma), 100 U/ml Penicillin, 100 μg/ml Streptomycin (Flow Laboratories).

RPMI 1640 (Flow Laboratories) medium was converted to selective HAT (hypoxanthine-aminopterin-thymidine) medium by addition of hypoxanthine, Aminopterin and thymidine solution (all reagents from Flow Laboratories) at a final concentration of 100 μM, 0.4 μM, and 16 μM respectively. The HT (hypoxanthine-thymidine) medium was prepared in the same way, but without aminopterin.

2.4. Production of mAbs.

CBA/Ca mice were infected intranasally and /or immunised with 5 HAU of X31, allowed to recover for six to ten weeks. Following intraperitoneal boost with 2000 HAU three days prior to hybridoma fusion, half spleens were removed surgically under general anaesthesia (kindly performed by Ms V.Attenburrow) and used to generate B cell hybridomas. Splenic lymphocytes from individual donors were fused with the BALB/C derived myeloma line JK-Ag8 (P3-X63-Ag8653) (Kearney, *et al.*, 1979; Kohler and Milstein, 1975) following the Fazekas de St. Groth and Scheidegger (1980) protocol. After 8-10 weeks, mice were infected with 2000 HAU of a variant virus A43 (HA1 158, HA1 145 and del HA1 224-230), boosted intraperitoneally with 5000 HAU and the other half spleens used to generate B cell hybridomas.

Human Ig μ-chain transgenic mice were immunised intraperitoneally with inactivated X31 and whole spleen was used to generate B-cell hybridomas.

Equal number of washed spleen cells and myeloma cells were fused in 1ml of a 50% solution of PEG (polyethylene glycol mol. wt.1,300-1,500; Sigma) in RPMI 1640 and 50 μl of DMSO (dimethyl sulphoxide; BDH). After gentle shaking for 5 min. at 37° C, the cells were washed and resuspended in medium with 20% FCS and HAT. 50 μl aliquots of fused cells were added to 150 μl of PECs (peritoneal exudated cells) in selective medium, in flat-bottom-96-well-microtitre plates, and cultured at 37°C in 6% CO_2: 2×10^7 spleen cells and PECs from one mouse, were dispensed onto each plate. On day 5, 100 μl of medium was removed from the cultures and replaced with 100μl of HAT medium containing 20% FCS. On day 14 , this procedure was repeated but this time 100 μl HT medium was added. Subsequently, fresh standard culture medium containing 10% FCS was added when required.

Hybridomas were screened for anti-HA activity by haemagglutination inhibition assays, and mAb isotypes determined by ELISA (Ishiguro, et al., 1983). mAbs bound to X31 were detected using biotinylated goat anti-mouse Ig isotype specific antibodies (Southern Biotech) and Streptavidin-alkaline phosphatase conjugate (Southern Biotech) developed with p-nitrophenyl phosphate at 1mg/ml in diethanolamine buffer (see appendix I) and the absorbance at 405 nm was measured. For transgenic mice, anti-κ or anti-λ light chain antibodies (Sigma) were used.

2.5.Haemagglutination Inhibition (HI) Assay.

Culture supernatants were screened for HA-specific neutralising antibody in a haemagglutinin inhibition (HI) assay (WHO expert committee on influenza, 1953) from day 10. Two-fold serial dilutions in PBS of 50μl antibody samples were made in round bottom 96 well microtitre plates. Eight HAU of virus in 25μl

volumes was added to each well and the plates incubated at room temperature for 30 minutes. 25 μl of a 2% turkey RBC suspension was added to each well and incubated for a further 30 min at room temperature to allow agglutination. Positive hybrids were allowed to attain confluency and then transferred to a 24 well plate (Costar) containing PECs and 2 ml of standard medium supplemented with 10% FCS per well.

2.6. Cloning and expansion of HA specific hybridomas.

Once confluent in the Costar plates, HA specific hybridomas were cloned by limiting dilution. Briefly, flat bottom 96 well microtitre plates were seeded with 100μl of a PECs suspension in RPMI 1640 medium containing 20% FCS. The hybridoma cells were diluted to a final concentration of 3 cell/ml (equivalent to 0.3 cell /well) and then 100μl/well were added to the 96 well plates. After 10-14 days the wells were screened for HA specific antibodies in HI assays. The positive clones were expanded in 24 well plates and when confluent, transferred to 25 cm^3 flasks (Falcon). Large amounts of HA specific mAbs were generated by growing hybridomas as ascitic tumours in mice. Briefly, (CBA/Ca × BALB/c) F1 mice were given a single 0.5 ml intraperitoneal injection of Pristane (2,6,10,14 tetramethyl pentadecane; Koch Light) one week before the intraperitoneal injection of at least 1 ×10^6 hybridoma cells per mouse in 1.5 ml of PBS. Each mAb was used to select a neutralising escape mutant.

2.7. mAb isotype determination.

The mAb isotype determination was performed using standard indirect ELISA techniques (Ishiguro, et al., 1983). Briefly, plastic 96 well microELISA plates (Nunc) were coated with 1μg/well BHA or 100 HAU of whole virus in borate buffered saline saline pH 8.6 (BBS, see appendix I) for 2 hours at 37°C and washed three times with PBS-Tween 20 (0.05%; Sigma) allowing a 1 min. soak

between washes. 50µl hybridoma culture medium supernatant or ascitic fluid was added to each well, and a negative control of PBS-Tween set up. The plates were incubated at room temperature for 1 hour and then washed again (3×). mAbs bound to BHA were detected by adding 50µl/well 1:1000 dilution in PBS-Tween of biotinylated goat anti-mouse Ig isotype specific antibodies (Southern Biotech) for 2 hours at room temperature. The plates were washed as before and then 50µl/well of streptavidin-alkaline phosphatase conjugate (1:1000 dilution in PBS-Tween) (Southern Biotech) was added for 1 hour at room temperature. Plates were then developed with 50µl/well p-nitrophenyl phosphate (Sigma) in 1M diethanolamine buffer pH 9.8 (Sigma), the reaction stopped by addition of 50µl of 5 M NaOH (BDH) and the absorbance at 405nm was measured.

IgM mAbs were detected with alkaline phosphatase-conjugated goat antibodies specific for mouse κ or λ light chain (Sera-lab) used at a dilution of 1:1000 in PBS-Tween.

2.8. Virus specificity for neo-glycoproteins.

Substrates α, 2-3 sialyllactosaminyl-BSA (Dextra Laboratories) and α, 2-6 sialyllactosaminyl-BSA (Prepared in the laboratory by enzymatic modification of N-acetyllactosaminyl-BSA using α, 2-6 specific neuraminyl transferase and CMP-N-acetyllactosaminidate, (as recomended by the manufacturer Boehringer, Mannheim Biochemica) were coated on ELISA plates at 30 µg/ml respectively. The plates were incubated overnight at 4°C, washed ×4 and blocked with 2.5% BSA. The plates were washed as before and X31 and variant viruses were added at 50 and 100 HAU/100µl, and incubated for one hour at room temperature. The plates were washed and blocked again with BSA followed by addition of 50µl of rabbit anti-HA Ab at 1:3,200 dilution (in PBS-Tween) and incubated for an hour at room temperature. Plates were washed again as before and 50µl of Goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) was added at 1:1000 dilution

for an hour at room temperature. Plates were then developed with 50µl/well p-nitrophenyl phosphate (Sigma) in 1M diethanolamine buffer pH 9.8 (Sigma), the reaction stopped with 5 M NaOH (BDH) and the absorbance at 405nm was measured.

2.9. Neuraminidase treatment of IgM mAbs.

Neuraminidase purified from Vibrio cholerae (Boehringer Mannheim) used at 1unit/ml was mixed with IgM mAb containing ascitic fluid and incubated overnight at 37°C followed by a 2 hour incubation at 56°C to inactivate neuraminidase.

2.10. Neutralisation assay.

MDCK cells were grown in DMEM (Dulbecco Modified Eagle's medium: Gibco BRL) supplemented with heat inactivated FCS (foetal calf serum; Flow Laboratories), 100 U/ml Penicillin, 100 µg/ml Streptomycin (Flow Laboratories) in small petri plates (Nunc).

Virus-mAb was mixed and left for one hour at room temperature. Confluent MDCK cells were washed with DMEM, the virus-mAb mix was added and incubated for 30 minutes at 37°C. DMEM at 4°C (supplemented with glutamine, trypsin: 0.25%, non-essential amino acid and antibiotics) was mixed with hot agar (70°C) and added to the plate and incubated at 37°C for a further 48 hours. Plates were assayed for plaque forming units (PFU) following fixation with 0.25% (v/v) glutaraldehyde for 20-30 minutes after which plates were stained with 1% (w/v) crystal violet for 10 minutes. Colonies were counted manually following destaining.

2.11. Selection of mAb neutralisation escape mutant viruses.

Monoclonal antibody selected mutants were produced by growing X31 in the presence of neutralising antibody in eggs. Allantoic fluid, containing 2000 HAU/ 100µl of X31, was mixed with an equal volume of neat ascitic fluid and incubated at room temperature for 30 minutes. 500µl aliquots were injected into ten-day-old-embryonated hen eggs. Allantoic fluid was harvested on day three and tested for mutant virus by haemagglutination inhibition (HI) assay (World Health Organisation Expert Committee on influenza, 153). Escape mutants were cloned by limiting dilution.

2.12. Purification of viruses.

X31 and mAb selected variants were purified from allantoic fluid using the method described by Skehel and Schild, 1971. Briefly, the allantoic fluid was clarified by spinning at x 500 g for 20 min at 4°C (using a Beckman JS-4.2 swinging bucket rotor). The virus was then pelleted by spinning at x 23,000 g for two hours at 4°C (using a Beckman T19 fixed angle rotor). The pellet obtained was resuspended in 3-4 mls Tris/saline (see appendix I), homogenised or sonicated for 5 minutes in a water bath sonicator (Camlab Transonic T310). The resulting suspension was then loaded onto a continuous gradient of 15% - 40% sucrose w/v in Tris/saline and ultracentrifuged at x80,000g for 45 min at 4°C (using a Beckman SW28 swinging bucket rotor). The virus band, clearly visible about half way down the gradient was harvested, diluted to 30 ml in Tris/saline. The virus was pelleted by spinning at x 80,000g for 90 minutes 4°C (SW28 rotor) and resuspended in 0.5 ml Tris/saline. (Skehel and Schild, 1971).

2.13. Bromelain digestion of influenza virus to produce BHA.

X31 and variant virus haemagglutinins were cleaved by digestion with bromelain. 10mg of purified virus was mixed with 5mg of bromelain (Sigma) in 3mls of digestion buffer (Tris/saline with 50mM β-mercaptoethanol, 0.1% sodium

azide) and incubated at 37°C for 2 hours. The reaction mix was then spun at x110,000g for 30 minutes at 4°C (in a Beckman SW41 swinging-bucket rotor). The pellet was resuspended in 3mls of digestion buffer containing 5mg of bromelain and incubated at 37°C for 3 hours. The reaction mix was spun as before. The supernatant was collected and the pellet was resuspended in 3mls of digestion buffer containing 5mg bromelain and incubated at 37°C overnight. The reaction mix was spun as before and the supernatant collected. The supernatant was layered onto continuous gradients of 5-25% sucrose w/v (1.5ml of supernatant/ gradient) and spun at x150,000g for 15 hours at 4°C (SW41 rotor). The gradients were harvested by collecting 1ml fractions from the bottom of the tube using a peristaltic pump. The absorbance at 280nm was measured and those fractions which contained protein were pooled.

The BHA solution, generally in a volume of around 12mls, was concentrated down to a volume of 3 mls using an Amicon Ultrafiltration Stirred Cell System with a 10,000 mol.wt filter cut off. Protein concentration was determined by measuring the absorbance at 280nm and the purity of preparation was checked by gel electrophoresis (see section 2.14).

2.14. Gel Electrophoresis of Proteins.

The purity of BHA preparation was assessed by SDS-polyacrylamide gel electrophoresis. Samples were electrophoresed through a stacking gel containing 4% acrylamide, 0.25% Bisacrylamide, 0.1% SDS (all from BDH) in 60mM Tris-HCl pH 6.7 and a separating gel containing 12.5% acrylamide, 0.25% Bisacrylamide, 0.1% SDS in 0.4 M Tris-HCl pH 8.9. The electrophoresis buffer was Tris/Glycine buffer pH 8.3 (see appendix I).

10 μl of sample was mixed with 10 μl of either a reducing or non-reducing loading buffer (see appendix I), boiled for two minutes and loaded onto the gel. 200 volts was applied across the gel for 50 minutes. Gels were stained in 40% methanol, 10% acetic acid, 0.5% coomassie blue for 10-30 minutes and then de-stained in 40% methanol, 10% acetic acid.

2.15. Horse Serum Inhibition Assay.

Two-fold serial dilutions in PBS of horse serum were made in round bottom 96- well microtitre plates. Eight HAU of X31 or variant virus in 50µl volumes was added to each well and the plates incubated at room temperature for 1 hour. 50 µl of a 1% TRBC suspension was added to each well and incubated for a further 30 min at room temperature to allow agglutination.

2.16. RNA extraction from purified virus.

Viral RNA was extracted according to Hay et al., 1977. 2-10 mg/ml purified virus was solubilised in 60 mM sodium acetate pH 5.0 (BDH), 6 mM ethylene diamine tetracetic acid pH 7.0 (EDTA: BDH) and 1% sodium dodecyl sulphate (SDS: Sigma). The RNA was extracted 2-3 times with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). RNA was precipitated from the aqueous phase with 20 mM NaCl (BDH) and twice the volume of absolute ethanol on dry ice for 1-2 hours. The pellet was collected by centrifuging at 13,000g for 10 minutes at 4°C, dried, redissolved in sterile double distilled water and the concentration was calculated from the A_{260} value and the $A_{260} : A_{280}$ ratio was determined. RNA samples were stored at -20°C.

2.17. Viral RNA extraction from allantoic fluid.

200 ml of the allantoic fluid was mixed with 200 µl TES buffer (see appendix I). RNA was extracted 2-3 times with an equal volume of phenol/chloroform, aqueous phase recovered and the RNA precipitated with 1/10th of the volume of 2 M NaCl and double the volume of chilled absolute ethanol on dry ice for 1-2 hours. The pellet was collected by centrifuging at 13000

g for 10 min, 4°C, dried, dissolved in sterile double distilled water and used for RT-PCR amplification.

2.18. cDNA synthesis from viral RNA.

cDNA was synthesised from viral RNA in a 20 µl volume of buffer containing 100 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 800 mM 200 mM KCl, with 40U of AMV Reverse Transcriptase (Boehringer Mannheim), 25U RNAsin (Amersham), 0.67 mM FHA (see 2.21) and 40mM dNTP. This was incubated at 42°C for 1 hr. Samples were then boiled at 100°C for 3 minutes, then quenched immediately on dry ice.

Samples were then thawed and spun for 10 min in a microfuge. The cDNA containing supernatant was used in PCR. Each reaction was set up in 100 µl volume containing 10 mM Tris pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 2.5% glycerol v/v, primers FHA and RHA2 (see 2.21) at 0.5 µM, each dNTP at a concentration of 250 µM, 2.5 U Taq polymerase (Pharmacia Biotechnology) and 20 µl of cDNA template. Each tube was then overlaid with a drop of mineral oil (Sigma). Once amplified, PCR products were visualised by running a 5 µl sample together with 1 µl sucrose gel dye (45% sucrose, 0.25% bromophenol blue) on a 1% agarose gel in 1 x TBE (100 mM Tris-base, 100 mM boric acid, 5 mM EDTA pH 8, 0.5 mg/ml ethidium bromide) at 100V. The amplification cycle was 1 x (95°/3 min, 50°/1 min,70° /1.5 min), 40 x (95° /1 min, 50°/ 1min, 70° /1.5 min), 1x (70°/5min).

2.19. Purification of PCR products for sequencing.

A 40 µl sample of the amplified product mixed with 5 µl sucrose dye was loaded equally into two adjacent wells of a 1% low melting point agarose (FMC Sea Plaque: Sigma) gel in TBE (see appendix I) with ethidium bromide. Molecular

weight marker was also loaded onto the gel which was run at 100V. The gel was then viewed on a low intensity UV light box and bands of correct size cut and placed in eppendorf tubes. DNA was recovered from gel slices by melting the agarose at 75°C for 30 minutes followed by purification using Wizard PCR Prep (Promega) following the manufacturer's instructions. A 2 µl sample of the purified DNA was run on a 1% agarose gel in TBE together with a known concentration of DNA molecular weight marker to estimate the concentration of DNA in the sample for sequencing.

2.20. Direct sequencing of PCR products.

the PCR product was determined using a modified chain terminating method (Sanger, et al., 1977). Purified PCR product was mixed with primer to a template: primer molar ratio of between 1:35 and 1:70 in 3 µl. This was added to 3 µl of annealing mix (125 mM Tris-HCL pH 7.5, 50mM MgCl$_2$, 100mM NaCl, 20% DMSO). Different primers used were FHA, DP, CCP2, CCP4, RHA1 and RHA2 (see 2.21).

Samples were incubated at 100°C for 3 minutes, immediately quenched on dry ice, thawed after 5 min, briefly centrifuged and then 4 µl of labelling mix (25 mM DTT, 2.6 u sequenase and 10 µCi ^{35}S-dATP) was added to each reaction tube. 2.5 µl of template/ primer mix was added to 2µl of each of the 4 dNTP/ ddNTP mixes. These mixes contained dGTP/ CTP/ TTP at 80µM and one ddNTP at either 8µM (ddG/C/T) or 0.16µM (ddATP) in 20% DMSO. This was incubated at 37°C for 5 min, followed by 2 µl of chase mix (0.25 µM of each dNTP, 50 mM NaCl, 10% DMSO) and then incubated again for further five minutes before the reaction was stopped by addition of 6 µl per well of formamide dye. This reaction mix was boiled for 3 minutes and run immediately on denaturing 8% polyacrylamide gel containing 8 M Urea in TBE. Gels were dried for 1½ hours at 85°C under vacuum on a gel drier (Biorad model 583) before autoradiography

(Kodak film) for 24-72 hours at room temperature. Autoradiographs were read and sequences recorded.

2.21. Primers used in amplification and sequencing of the HA1 gene of influenza virus.

FHA	TGAAGACCATCATTGCTTTGAGC
DP	TGCTACTGAGCT
CCP2	GAGACATGGGACCTTTTCTT
CCP3	TCACTTGGACTGGGGTCACT
CCP4	CAGTAGACTGAACTGGTTGA
RHA1	TCGAAGGTAAACCTCACTACGT
RHA2	AGATAGTAAGGGAGGGTTGGTAA

CHAPTER-3

The structural analysis of the neutralising antibody repertoire of transgenic mice expressing a human IgH mini-gene.

3.1. Introduction

The recognition sites for neutralising antibodies have been previously located to five regions (A→E) on the membrane distal ectodomain of the HA1 subunit (Caton, *et al.*, 1982; Gerhard, *et al.*, 1981; Lubeck and Gerhard, 1981; Underwood, 1982; Wiley, *et al.*, 1981). All of these exposed regions, proximal to the receptor-binding pocket are *potential* recognition sites for neutralising Abs. However, recent studies from this laboratory have shown the secondary antibody response to be unexpectedly restricted in specificity following *natural infection* with X31. It has been shown that there is striking immunodominance in the neutralising antibody response in different strains of mice. As deduced by sequencing the HA genes, mAb selected laboratory mutants were found to differ from wild type X31 virus by a single amino acid change, HA1 158 G→E in the H-2^K haplotype, (Smith, *et al.*, 1991; Temoltzin-Palacois and Thomas, 1994) HA1 198 A→E in the H-2^d haplotype and there was co-dominance in MHC congenic BALB/k mice with a single residue change at either HA1 158 or HA1 198, depending on the donor origin of the selecting mAb (Patera, *et al.*, 1995). This contrasts with the diversity of antibody specificities seen following i.p. immunisation of BALB/c mice with inactivated virus (e.g. HA1 63, 135, 143, 144, 145, 156, 189, 198, 199, 205).

There is considerable knowledge of antibody responses in CBA/ Ca, BALB/c and BALB/ k mice to virus infection as well as heavy and light chain gene usage. Patera et al. (1995) have shown recently that, despite immunodominance of the above two residues, there was extensive progenitor B cell diversity contributing to the memory response: the individual's response to a single antigenic site was derived from a minimum of 3-6 progenitor cells. I wished to extend these findings to an analysis of the *memory repertoire* of transgenic mice expressing human μ chains in the absence of endogenous murine μ chains, following immunisation with X31 virus. These mice were made by crossing transgenic mice carrying a human Ig H chain gene with mice that had been rendered deficient in endogenous immunoglobulin production by

gene targeting (Wagner, *et al.*,1994). The DNA construct used for integration is shown in figure 6 (Bruggemann, *et al.*, 1991).

This transgenic model provides a unique opportunity to investigate the neutralising antibody response under conditions in which (a) there is no class switching from IgM to IgG, and affinity maturation due to somatic hypermutation and (b) highly restricted V_H region gene usage.

I wished to determine whether a low affinity IgM antibody response would be similarly focused on a limited region(s) of the HA1 subunit.

Fig 6: Structure of human Ig H cosmid.

Cos 25 contains the subcloned V_H 26, D segments (D21/ 10, D3, D21/ 9, DQ 52), the functional J segments, the intron enhancer (E) the μ constant region of the human IgH gene, and an additional mouse V_H 186 gene with disruption of mouse membrane μ. (C μ) and the membrane μ (M μ).

HuIgH

HuE MoE

V_H26 V_H186 Ds   DQ52 J1-6   Cμ   Mμ

1 kb
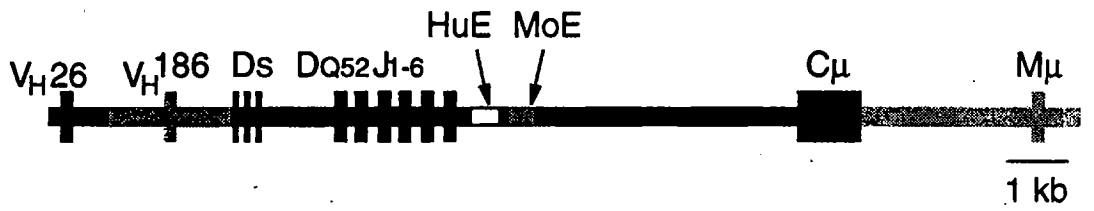
3.2. RESULTS

3.2.1. Generation of HA specific B-cell hybridomas from HuIg-μ chain transgenic mice.

In an investigation of the neutralising antibody response to HA in transgenic mice (Tg) expressing human Ig heavy chains, the following strategy was employed (Fig 7): Tg mice were immunised intraperitoneally with 1000 units of UV inactivated X31 virus and boosted 3 days before spleens were taken for B-cell hybridoma production. B-cell hybridomas secreting HA-specific neutralising antibodies, were screened for by inhibition of viral haemagglutination (HI) of turkey erythrocytes.

Thirty out of sixty hybridomas (the TgM series) were cloned by limiting dilution. These antibodies were cloned in 96 well microtitre plates at about 0.3 cell/ well and considered clonal if ten to twelve wells/plate were HI positive. A total of four HA-specific hybridomas were obtained from one donor and high titer mAb-bearing ascitic fluid was obtained by further expansion in ηu/ ηu mice (table 1). Equivalent HI titers were obtained for each of the selected mAb.

3.2.2. mAb recognition specificity for variant viruses.

In an initial attempt to determine their fine specificity, mAbs were tested by HI assay against a panel of laboratory variant viruses with known single amino acid substitutions within the HA1 subunit (table 2). The results, however, did not provide a definitive assignment of amino acid residues in the HA1 subunit that constituted part of the antibody recognition site. For instance mAbs 7.1 and 21.2 recognised all variants in the HI assays with the singular exception of the mutant M1-9 (HA1 226 L→Q, 135 G→V). However, the variants containing the corresponding single substitutions HA1 226 L→Q or HA1 135 G→R/ D were recognised. Hence, the structural basis for recognition specificity could not be

Fig 7: Schematic representation to show generation of mAbs

X31
infection

H-2^k/ H-2^d

boost

Splenocytes + myeloma cells

protective mAbs

Ascites + X31

neutralising escape
mutants

HA gene sequencing

Table 1:   HI reactivity of anti-HA mAb containing ascitic fluid against
            X31.
            HI titre expressed as the reciprocal of the dilutions required
            to inhibit agglutination of turkey erythrocytes by 100%.

mAb (ascites)	HI titers (X31)
1.1	51200
4.1	12800
7.1	25600
21.2	51200

Table 2:   Values indicate HI titres for mAb-containing ascitic fluid, in
the agglutination of turkey erythrocytes by X31 or its laboratory variants.

        HI titre expressed as the reciprocal of the dilutions required to
inhibit agglutination of turkey erythrocytes by 100%.

TABLE 2. HI specificities of MAbs for laboratory variants of X31

MAb	X31	Site E		Site A						Site B		Site C
		62 (I→R*)	63 (D→N)	135 (G→R)	140 (K→L)	142 (G→R)	145 (S→N)	158 (G→E)	189 (Q→H)	196 (V→N)	198 (A→E)	273 (P→L)
7.1	12,800	3,200	3,200	1,600	12,000	6,400	1,600	25,600	6,400	12,800	6,400	3,200
21.2	51,200	51,200	12,800	6,400	6,400	12,800	3,200	12,800	12,800	25,600	12,800	12,800
1.1	25,600	6,400	6,400	<100	12,800	6,400	1,600	12,800	6,400	6,400	6,400	6,400
4.1	6,400	6,400	3,200	<100	3,200	3,200	1,600	3,200	3,200	6,400	3,200	3,200

* HI titer for MAb-containing ascites fluid in the agglutination of turkey erythrocytes by X31 or its laboratory variants.

Table 3 :   Haemagglutinin inhibition specificity of TgM-mAbs for natural isolates of the H1, H2 or H3 subtype.

HI titre expressed as the reciprocal of the dilutions required to inhibit agglutination of turkey erythrocytes by 100%.

NR: not recognised.

mAbs	ENG/69 (H3)	VIC/75 (H3)	PC/73 (H3)	HK/71 (H3)	TEX/77 (H3)	PR/34 (H1)	JAP/57 (H2)
7.1	100	800	800	200	400	NR	NR
21.2	6400	6400	12800	3200	100	NR	NR
1.1	1600	400	400	400	100	NR	NR
4.1	3200	200	NR	400	NR	NR	NR

Table 4:   Haemagglutinin inhibition specificity of TgM-mAbs for natural isolates of the H1 subtype.

HI titre expressed as the reciprocal of the dilutions required to inhibit agglutination of turkey erythrocytes by 100%.

< HI titre below 100.

TgM abs	Tex/91	OMS/93	Twm/1/87	Sch/4/88	Vic/88	X31
7.1	<	<	<	<	<	25600
21.2	<	<	<	<	<	51200
1.1	<	<	<	<	<	25600
4.1	<	<	<	<	<	6400

Table 5:   Haemagglutinin inhibition specificity of TgM-mAbs for natural isolates of the H2 subtype.

HI titre expressed as the reciprocal of the dilutions required to inhibit agglutination of turkey erythrocytes by 100%.

< HI titre below 100.

B cell hybridoma clone (IgM)	Eng/10/67	Jap/3/57	F1/1/68	X31
7.1	200	<	<	25600
21.2	<	<	<	51200
1.1	<	<	<	25600
4.1	<	<	<	6400

established. In contrast, the remaining mAbs 1.1 and 4.1 *failed* to recognise HA1 135 G→R. There was some considerable degree of variation in HI reactivity for the panel of variant viruses as illustrated by heteroclitic reactivity of mAb 7.1 for 158 or reduced reactivity for 135 or 145. The reactivity of mAbs 1.1 or 4.1 was sensitive to HA1 135 G→R change.

Since each of these mAbs was cross-reactive in the above HI assay, there was some concern as to whether mAbs were indeed specific for X31. The serological assays against a panel of H1, H2 and H3 subtype viruses (table 3, 4, 5) do indicate fine specificity since none of the mAbs recognised either H1, or H2 subtype viruses.

3.2.3. Selection and sequence analysis of X31 laboratory variants.

To obtain a definitive assignment of neutralising antibody binding sites, the above mAbs were used to select laboratory mutants of X31. The HA gene of each cloned laboratory mutant was sequenced and the amino acid substitution was deduced from the nucleotide sequence data. Four mutants were selected and characterised using TgM antibodies and named the TgMm series (table 6). Three out of four mutants had amino acid substitutions at two different positions on the HA1 subunit. A common change, residue HA1 135 G→R was present in majority of the mutants and the other changes were either proximal to, or within the receptor binding site as shown in table 6.

3.2.4. Novel feature of TgM mutants.

One of the interesting features of the TgM mutants was the occurrence of double mutations: one of the amino acid substitutions was present in a conserved residue that constitutes part of receptor binding pocket, and the other amino acid substitution in a region of known antigenic change: Mutant 7.1, selected by mAb TgM-7.1, had substitutions

Table 6:   Recognition specificities of mAb as deduced by sequencing
of the HA genes of mAb- selected X31 mutants.

B-cell hybridoma	Variant virus (TgM series)	HA1 change.	Nucleotide substitution in HA gene.
7.1	m7.1	145 S→N 226 L→P	511 G→A 754 T→C
21.2	m21.2	135 G→R 225 G→D	480 G→A 751 G→A
1.1	m1.1	135 G→R 158 G→E	480 G→A 550 G→A
4.1	m4.1	135 G→R	480 G→A

at positions HA1 226 L→P, and 145 S→N. HA1 226 has been shown to be crucial in host-range restriction and thus change at this position alters the receptor binding characteristics of the virus. The other two antibodies 1.1 and 21.2 also selected double mutants. Antibody 21.2 selected a variant with a substitution at HA1 225 G→D and a further amino acid change at HA1 135 G→R. HA1 225 is also a conserved residue located within the receptor-binding pocket. Antibody 1.1 selected a variant with changes at HA1 135 G→R and HA1 158 G→E. Residue 158 has been implicated in receptor binding specificity and both HA1 145, and HA1 158 occupy positions in close proximity to the receptor binding site (Underwood, *et al.*, 1987).

3.2.5. HI reactivity of TgM mAbs for their own mutants.

Table 7 shows the reactivity of the TgM mAbs with mutants that they had selected as well as relevant single mutants. mAb 7.1 and 21.2 do not recognise their "own" mutants HA1 145 S→N, 226 L→P and HA1 135 G→R, 225 G→D respectively. However, variants with single residue changes at these positions, 135 G→R or 226 L→Q or 145 S→N or 158 G→E were all recognised. Similarly, mAb 1.1 and 4.1 failed to recognise their selected variants HA1 135 G→R, 158 G→E and HA1 135 G→R respectively but were found to be sensitive to changes in m-21.2. The HA1 226 L→Q variant virus was used in all above assays since laboratory variants with single residue change at 226 L→P or 225 G→D have not been reported. It was not possible to establish whether failure to recognise the selected virus required either (a) amino acid changes at both positions or (b) the same critical residue change at HA1 226 L→P or HA1 225 G→D. Also different amino acid substitutions at the same position are known to affect antibody recognition and specificity.

3.2.6. mAb reactivity for variant viruses in ELISA.

Since all the mutations had occurred either proximal to or within the receptor binding pocket, and might affect receptor binding, it was necessary to

Table 7:   HI reactivity of TgM antibodies
with TgM mutants compared to X31.

< :   < 100

        HI titre expressed as the reciprocal of the dilutions required to
inhibit agglutination of turkey erythrocytes by 100%.

TgM abs	226, 145 (m-7.1)	225, 135 (m-21.2)	135, 158 (m-1.1)	135 (m-4.1)	X31
1.1	12800	<	<	<	6400
4.1	12800	<	<	<	6400
7.1	<	<	3200	6400	12800
21.2	<	<	6400	12800	12800

Fig 8: mAb recognition specificity for variant viruses by ELISA.

(a) Reactivity of mAb 21.2 (●), or mAb 7.1 (▲) or mAb 4.1 (■) with variant virus m-21.2.

(b) Reactivity of mAb 1.1 with variant virus m-1.1(●), or m-7.1 (▲) or m-4.1(■).

Procedures as described in Methods section 2.7 for ELISA.

a)

b)

Absorbance at 405nm

mAb dilution
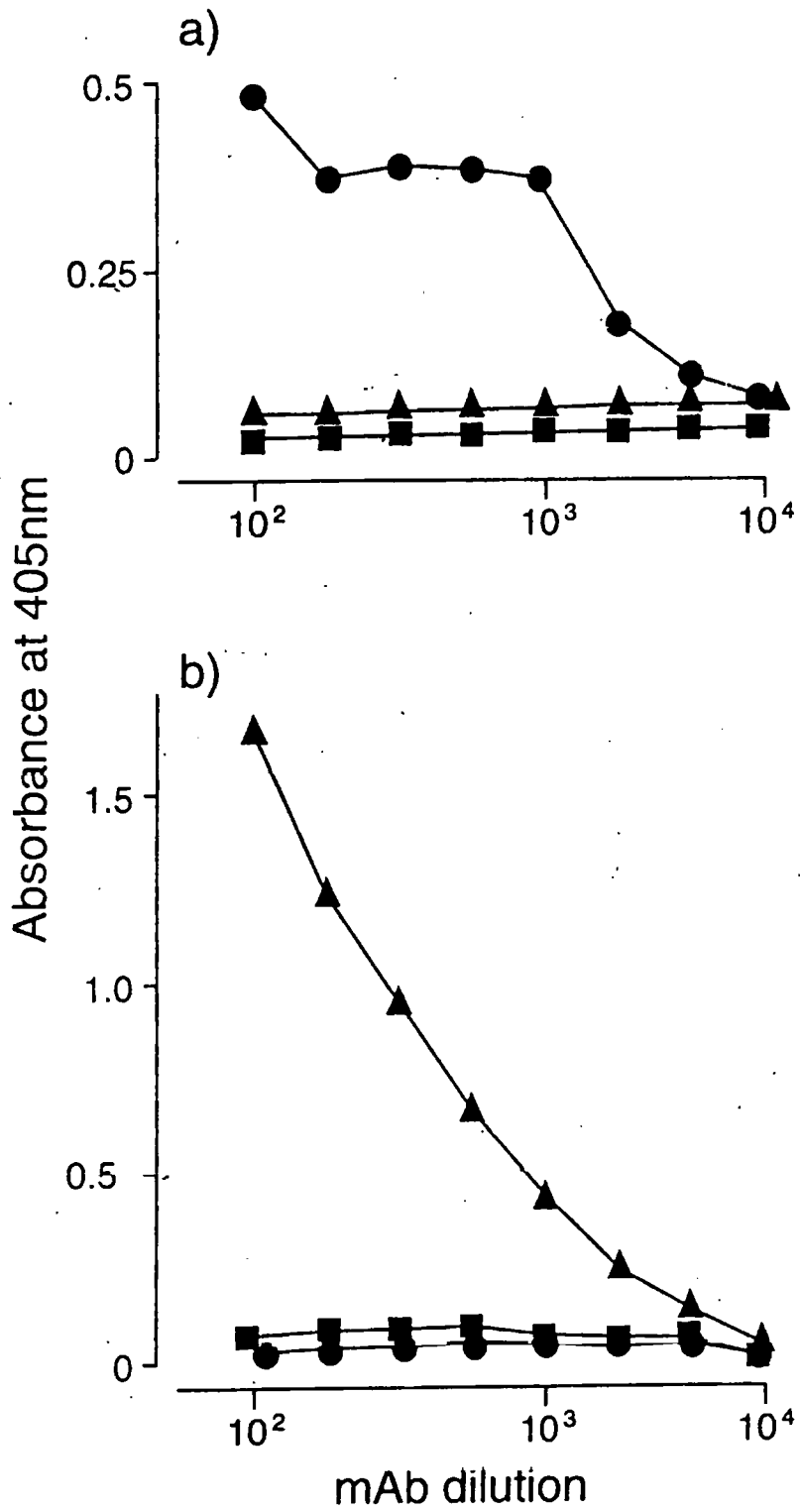
Fig 9: mAb 7.1 recognition of variant viruses by ELISA.

(□) 145 S→N; (Δ) X31; (O) 226 L→Q; (●)m-7.1 (HA1 145 S→N, 226 L→P).
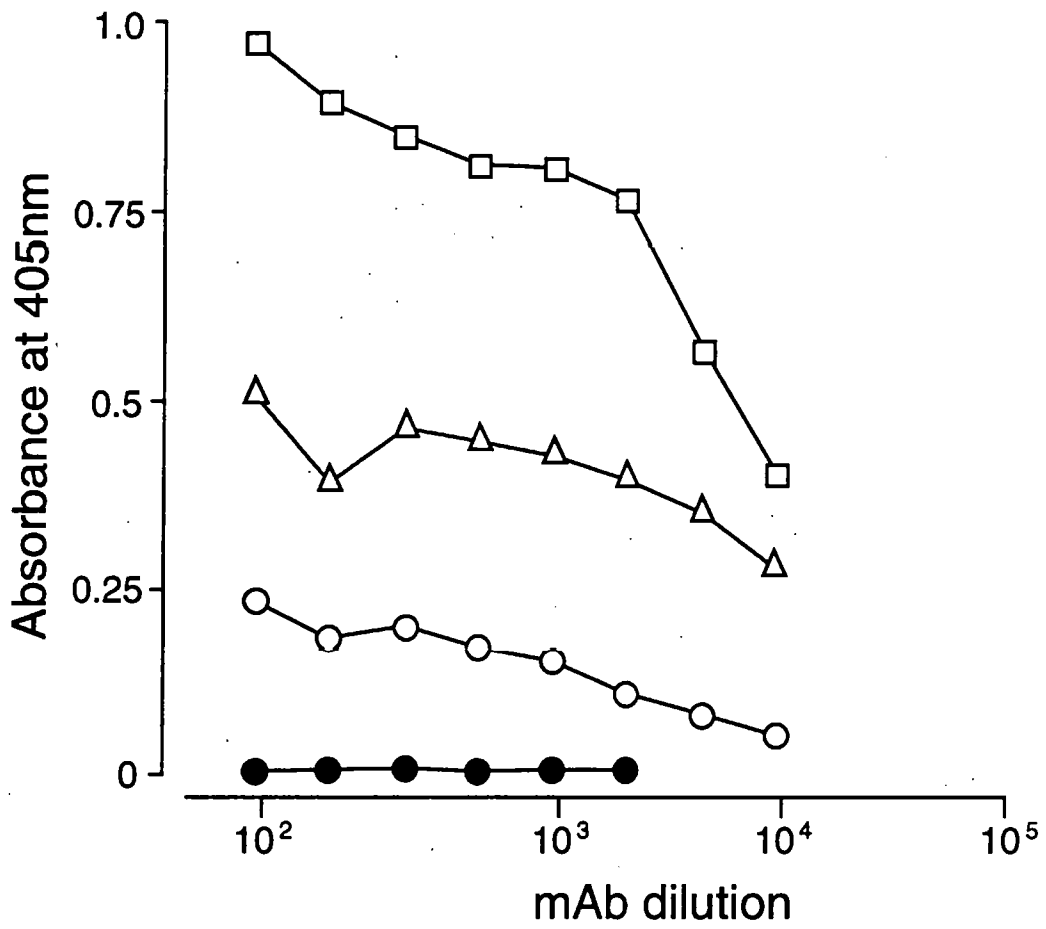
Fig 10: mAb 21.2 recognition of variant viruses by ELISA.

(●) X31; (□) m-7.1 (HA1 145 S→N, 226 L→P); (Δ) m-1.1 (HA1 135 G→R, 158G→E);

(▲) m-4.1 (HA1 135 G→R);

(■) X31-HS (HA1 226 L→Q).
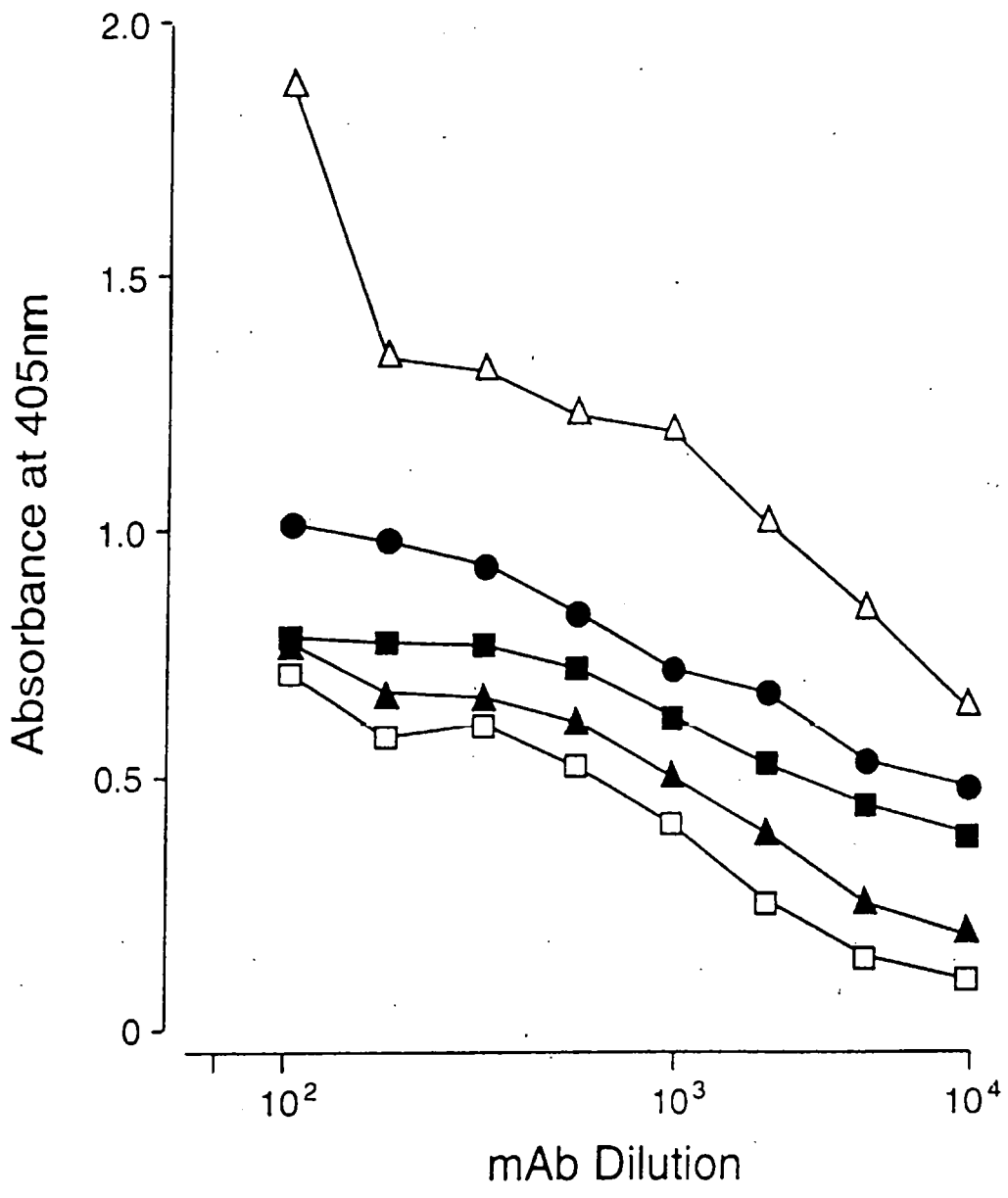
establish whether the laboratory variant viruses containing these substitutions were antigenic or receptor binding-variants. Each mAb was tested by ELISA assay for its ability to bind to the mutant virus which it had selected. Antibody 7.1, that had selected a variant HA1 145 226, recognised and bound to variant viruses with single amino acid substitution either at position 145 S→N or HA1 226 L→Q but failed to recognise its selected variant. A heteroclitic response was seen to variant 145 S→N as compared to X31 (Fig 9).

Antibody 1.1, that had selected HA1 135 G→R, 158 G→E also failed to bind to its selected mutant. This effect might be due to the substitution at position 135 G→R which is not recognised by the antibody either in ELISA as shown in fig 8b or in HI (table 7). These viruses therefore qualify as antigenic variants.

3.2.7. mAb 21.2 still binds to its variant (HA1 135, 225) in ELISA.

mAb 21.2, that had selected a variant with changes at positions HA1 135 G→R and HA1 225 G→D, still recognised its selected mutant in ELISA. And although antibody binding was reduced (as compared to X31; fig 10), it qualifies as a receptor-binding rather than antigenic variant. mAb 21.2 also bound well to HA1 135 G→R or HA1 226 L→Q.

It should be emphasised that neutralising antibodies do not usually recognise conserved regions of HA1 resulting in the selection of variant viruses with changes in the receptor-binding pocket.

3.2.8. Is terminal Sialic acid, a selective determinant ?

IgM antibodies are glycoproteins containing terminal sialic acid and all the mutants selected had substitutions either in the receptor binding pocket, or of residues proximal to the pocket. The question therefore arose as to whether terminal sialic acid (SA), present on the mAbs, was playing a role in the selection process. For this reason, mAb-containing ascitic fluid were incubated

Fig 11:   PNA (peanut agglutinin) binding to NANAse treated IgM
mAb (O) or untreated mAb.(□) in ELISA.

Wells coated with   mAbs (used at ~ 400 ng/ ml) were incubated with
biotin-conjugated PNA, followed after washing by alkaline phosphatase
conjugated Streptavidin, followed by substrate addition, as described in
Methods, section 2.7.
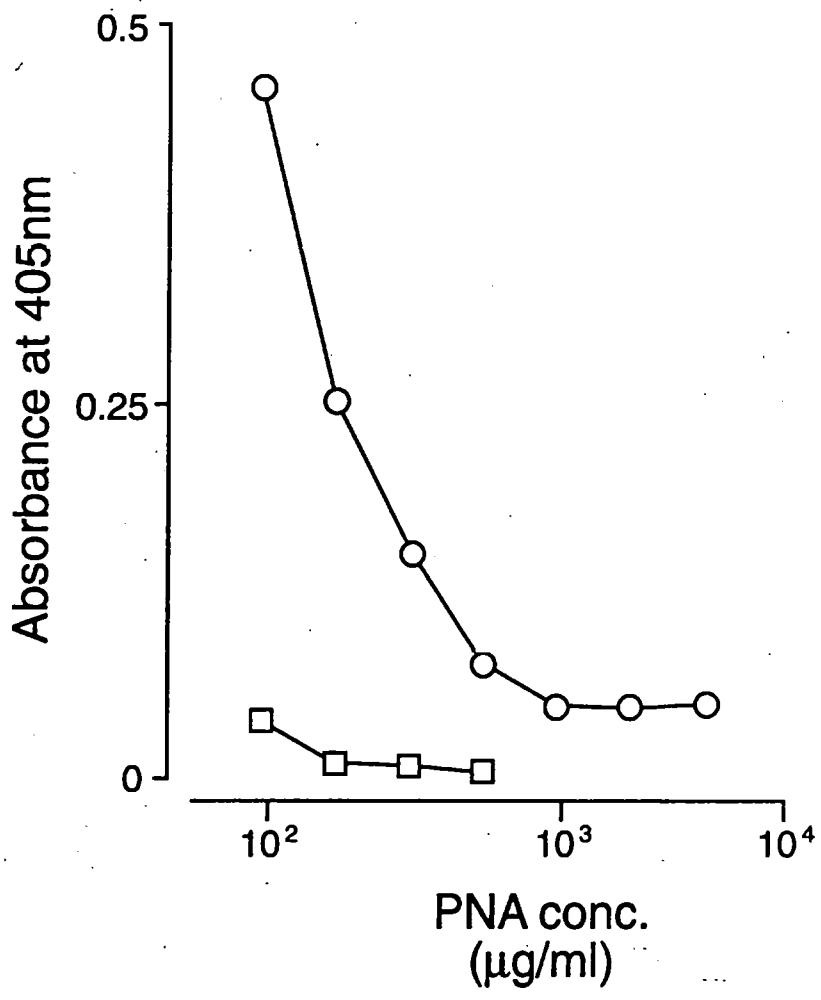
overnight with neuraminidase to remove SA. To monitor the removal of SA, binding assays were performed with two different lectins, as shown (fig 11). Peanut agglutinin (PNA) binds to terminal galactose residues of glycoproteins whereas Elderberry bark lectin has specificity for terminal SA. Following enzymatic digestion, the binding of Elderberry bark lectin to neuraminidase (NANA) treated antibodies was reduced to base line, with a reciprocal increase in binding of PNA, thereby confirming the efficacy of NANAse treatment.

3.2.9. Selection of mutants with NANAse treated mAbs.

mAbs, 7.1, 21.2 and 1.1 that had previously selected HA1 145 S→N, 226 L→P; HA1 135 G→R, 225 G→D, and HA1 135 G→R , 158 G→E respectively were used to select mutants of X31 after treatment with NANAse. Several rounds of selection were done with different dilutions of both mAb and virus before mutants were obtained: table 8 shows the mutants that I have characterised: mAb 21.2 was again found to select HA1 135 and 225, mAb 7.1 selected variant 135/ 225 or 145/ 226 in two different selections.

It is therefore reasonable to conclude that SA was not responsible for the selection and this is consistent with the failure of the mAbs to bind viruses of different subtypes.

3.2.10. Neutralisation assays with NANAse-treated and untreated TgM antibodies.

HI is usually considered a reliable index of virus neutralisation: antibody inhibits haemagglutination by blocking the receptor binding site. I wished to confirm that the antibodies not only prevented attachment of virus host cell receptors but also neutralised virus infectivity. Briefly, X31, or the TgM mutants or other natural virus isolates were incubated with different dilutions of mAbs for an hour, layered on trypsinised MDCK cells

and incubated for three days at 37° C. All mAbs failed to neutralise their own mutants as well as the natural isolates as was seen by the appearance of *plaques*. X31, however, was recognised by both treated and untreated mAbs and hence no colonies were observed.

Table 8 :   Recognition specificities of NANAse treated mAbs as deduced by sequencing the HA genes of mAb selected X31 mutants.

B cell hybridoma	variant virus (TgM series)	HA1 change	Nucleotide number in HA gene.
7.1NA	7.12	135 G→R	480 G→A
		225 G→D	751 G→A
7.1 NA	m7.1N	145 S→N	511 G→A
		226 L→P	754 T→C
21.2NA	m21.2 N	135 G→R	480 G→A
		225 G→D	751 G→A
1.1NA	m1.11	145 S→R	511 C→A
1.1NA	m1.12	145 S→R	511 C→A
		158 G→E	550 G→A

3.2.11. Summary

A panel of neutralising IgM mAbs was established from transgenic mice, expressing a human Ig H minigene, following immunisation with X31 virus. Their recognition specificities for HA were determined by the selection and sequencing of X31 laboratory variants. The majority of variant viruses differed from wild type by two amino acid residue changes, including substitutions in conserved residues that constitute part of the receptor binding pocket.

First, consider the receptor specificity of mAb 7.1 that initially selected a laboratory variant (HA1 145 S→N, 226 L→P). These residue changes represent small polar (S) to small polar (N), or large non-polar (L) to small non-polar (P) changes within the HA1 subunit. HA1 145 is a known antigenic site that has featured in the residue changes of both natural variant viruses (S→N; VIC/75 or TEX/ 77) and laboratory variants (S→N, S→R, S→K). HA1 226, however, has been a conserved residue for all of H3 natural isolates obtained between 1969 and 1990 and has not been selected for, hitherto, by neutralising mAbs. It is part of the receptor binding pocket and variant viruses at this position can be selected for by glycoprotein inhibitors. Following NANAse treatment of mAb 7.1, a further laboratory variant (7.1N) was selected containing residue changes 135 G→R (small polar to large polar) and 225 G→D (small polar to small polar). Here again HA1 135 has been demonstrated to be an antigenic site and laboratory variants of X31, containing this single residue change, have been selected for by neutralising mAbs of the IgG class. However, HA1 225 is a highly conserved residue in H3 subtype viruses and, once again constitutes part of the receptor binding pocket.

It should be emphasised here that a majority of previously reported laboratory variants differ from the wild type by a single residue change within one of the five major antigenic sites.

CHAPTER-4

Receptor-binding characteristics of mAb-selected variants

4.1. Introduction

Haemagglutinin is responsible for the attachment of the virus to cell surface glycoconjugates by binding to terminal sialic acid (SA) residues (Wiley and Skehel, 1987). Binding to SA involves a shallow pocket of *conserved* amino acid residues near the membrane-distal tip of each subunit as defined by crystallographic studies of oligosaccharide-HA complexes (Weis, *et al.*, 1988). Sequence analyses of the receptor variants e.g. X31-HS, selected with non-immune horse serum, indicated that differences in receptor specificity could be accounted for by a single amino acid residue change HA1 226 L→Q, which is located in the receptor-binding pocket (Rogers, *et al.*, 1983a; Rogers, *et al.*, 1983b). While X31 preferentially binds to the Neu Ac α, 2→6 Gal linkage and shows sensitivity to inhibition by γ-inhibitors present in non-immune horse serum, receptor-binding variant viruses show increased affinity for oligosaccharides containing the α, 2→3 Gal linkage and are resistant to inhibition by horse serum.

Neutralising mAbs do not usually select for mutations in conserved residues that constitute part of the receptor binding site. In the present investigation, however, a majority of mAbs selected variant viruses that had amino acid substitutions within or proximal to the receptor-binding pocket. The question therefore arose as to whether these variant viruses also exhibited altered receptor-binding specificity.

4.2. Results

4.2.1. Horse-serum inhibition assay.

In an initial attempt to determine the receptor-binding specificity of the variant viruses, horse serum inhibition assays were performed with the TgM mutants. X31 and two laboratory variant viruses X31-HS (HA1 226 L→Q), and M1-9 (HA1 226 L→Q, 135 G→V) were used as controls. The results in table 9 show that variant m4.1 (HA1 135 G→R) behaved as wild type and was sensitive to inhibition by horse serum. X31-HS, M1-9 and TgM mutant m7.1 (HA1 226 L→P, 145 S→N) showed complete resistance to inhibition whereas m1.1 (HA1 135 G→R, 158 G→E) and m21.2 (HA1 135 G→R, 225 G→D) showed partial resistance to inhibition by horse serum.

4.2.2. Binding Specificity for α,2→3 or α,2→6 Sialyllactosaminyl-BSA.

ELISA assays were performed with neoglycoconjugates and the specificity of the TgM mutants was compared with binding of X31 or X31-HS to α,2→3 or α,2→6 Sialyllactosaminyl-BSA. Bound virus was detected with biotinylated rabbit and/ or goat α-HA Ab and streptavidin-alkaline phosphatase conjugate.

Figure 12 indicates that, X31 binds more effectively to the α,2→6 substrate as compared to X31-HS for which there were base line values. However, X31 and X31-HS bound to the α,2→3 substrate equally well. With the exception of m21.2 (HA1 135 G→R, 225 G→D) showing preferential binding to α,2→6 Sialyllactosaminyl-BSA, the remaining TgM variant viruses m7.1 (226 L→P, 145 S→N) and m1.1 (HA1 135 G→R, 158 G→E) showed no significant difference in binding to either α,2→3 or α,2→6

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Table 9:   Horse serum inhibition assay with

TgM mutants,

X31,

X31-HS (226 L→Q)

M1-9 (226 L→Q, 135 G→V)

< = <20

Values indicate the reciprocal of horse serum dilution required to inhibit virus haemagglutination, as described in Methods, section 2.15.

Virus	Horse Serum
X31	1280
226, 145	<
135, 225	<
135, 158	160
135	1280
M1-9	<
X31-HS	<

Fig 12:   Receptor-binding specificity of X31 and its variants for sialyl α-2,6 (a) or sialyl α-2,3 (b) N-acetyllactosaminyl-BSA in ELISA.

● X31; ■ m-7.1 (145 S→N, 226 L→P); ▲ m-21.2 (135 G→R, 225 G→D); ▼ X31-HS (226 L→Q).

The procedures employed in this assay are described in detail, in Methods section 2.8.

(a)

(b)

Absorbance at 405nm

Substrate concentration (μg/ml)
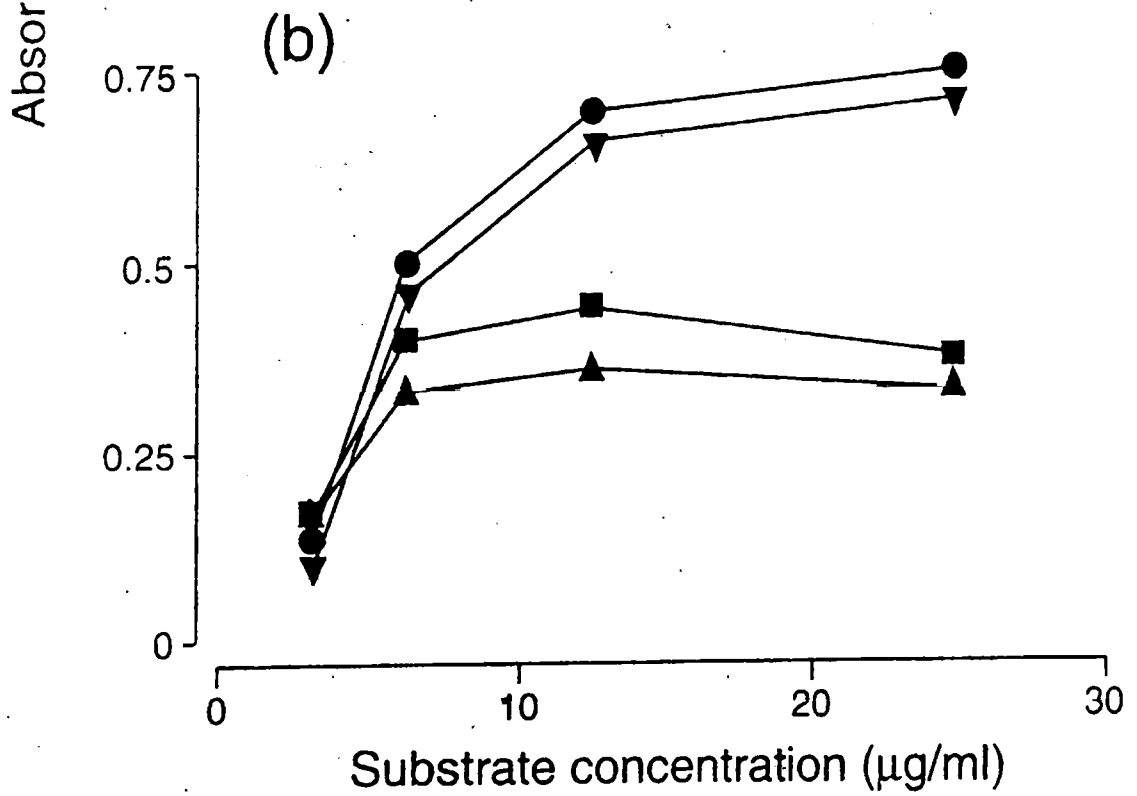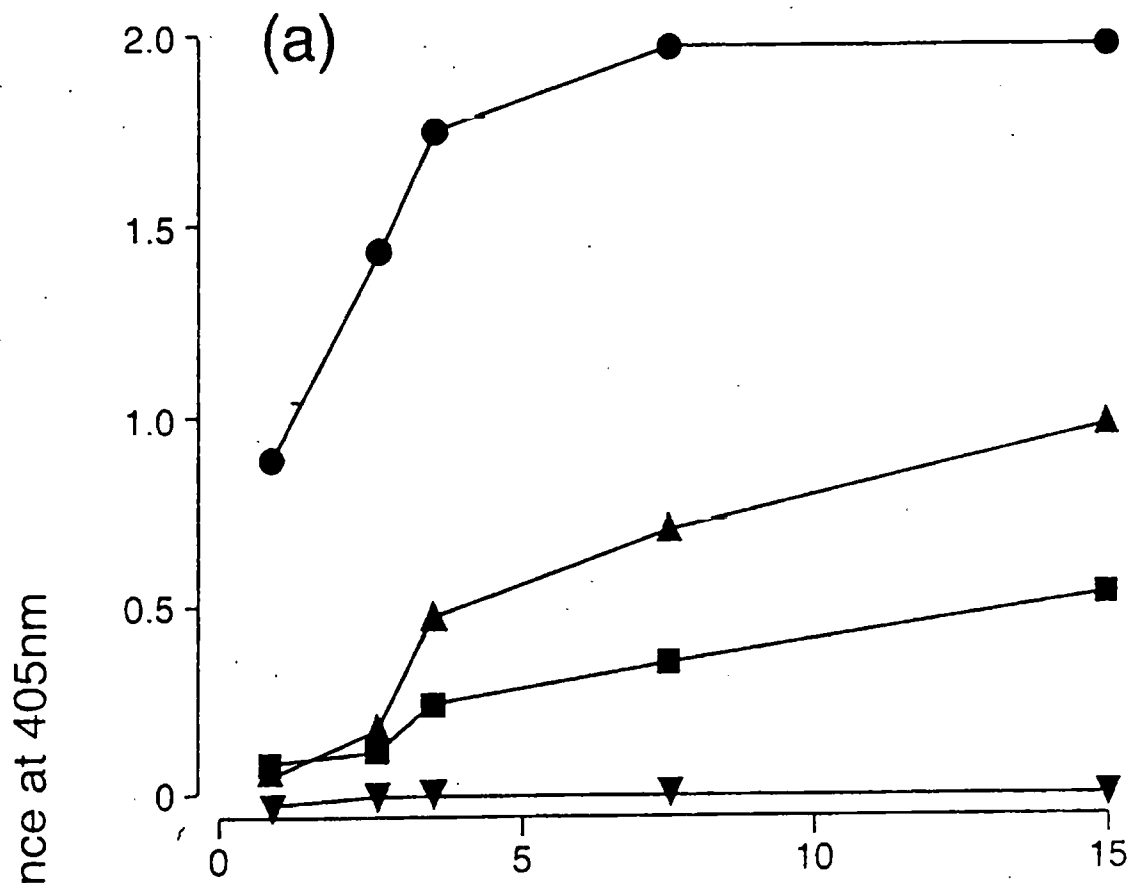
4.2.3. Summary

The receptor-binding specificities of the laboratory variants isolated in the study were investigated in parallel with X31-HS (HA1 226 L→Q), a variant selected by horse serum treatment. Variant m-7.1 (HA1 145, 226) and m-21.2 (HA1 135, 225) exhibited a similar specificity, to X31-HS, in their resistance to horse serum inhibition of agglutination, whereas partial resistance was seen for m-1.1 (HA1 135, 158), thereby confirming altered receptor function.

Then, I extended these studies to binding assays of variant viruses with ELISA plates coated with neoglycoconjugates either α, 2-3 (0-30 $\mu g/ml$) or α, 2-6 (0-15 $\mu g/ml$) sialyllactosaminyl-BSA. Clear cut differences were found in the binding specificities for X31-HS, with base-line values using the α, 2-6 substrate and wild type values for the α, 2-3 substrate. X31 bound optimally to both substrates at approximately equivalent substrate concentrations (Fig 12; 10-15 $\mu g/ml$) suggesting that the binding assay was not sufficiently discriminating, although there was a two-fold difference in absorbance (for bound virus) between the two substrates.

In comparison with X31, variants m-7.1 and m-21.2 bound at significantly reduced levels to both α, 2-3 and α, 2-6 substrates. This reduction was most pronounced for m-7.1 (HA1 145, 226) in its binding to the α, 2-6 neoglycoprotein; and this is consistent with the known critical role of HA1 226 in conferring receptor-substrate specificity.

In previous reports, receptor-binding variants of influenza were still recognised by their selecting mAb in ELISA. Variant 21.2 exhibits such a phenotype and therefore qualifies as a receptor binding variant. Paradoxically, whereas m-7.1 was not recognised by its selecting mAb (in

either HI or ELISA), it was negative by HI but positive in ELISA with mAb 21.2- (a receptor-binding variant phenotype).

Altered receptor-binding specificity was also indicated by the horse serum inhibition assays and mAb-selected variant viruses were resistant to inhibition with the exception of variant, HA1 135 G→R.

In the binding assay with α,2-3 or α,2-6 sialylglycoconjugates, binding of all variants was much reduced compared to X31. Thus, these assays indicate altered receptor binding specificity of the selected variant viruses.

CHAPTER-5

The neutralising antibody response to a variant viral antigen in the face of concomitant immunity

5.1. Introduction

The recurrence of influenza virus infection is attributed primarily to changes occurring in the antigenic structure of the virus surface glycoproteins, especially of the haemagglutinin (HA) molecule (Gerhard, *et al.*, 1981). The human population is subject to *sequential* challenge with new variants of influenza virus and therefore mounts an immune response to limit infection. Haemagglutinin specific antibodies neutralise virus infectivity and as a consequence, provide selective pressure for the HA molecule to mutate (Wiley and Skehel, 1987).Thus the efficacy of an influenza virus vaccine is short lived.

Antigenic drift is a dynamic process and represents recurrent interplay of the human B cell repertoire and its diverse MHC genes, Ig genes and TcR genes with the newly emergent epidemic strain of influenza virus. Although a structural analysis of laboratory variants, selected with murine mAbs has provided considerable insight on the molecular basis for antigenic change, there is a major caveat in extrapolating these findings to the human repertoire. In the murine model, a virgin B cell is recruited into memory by a single challenge (and boost) with a reference laboratory isolate, whereas the human immune system is subject to recurrent infection and antigenic challenge.

Following infection with influenza virus, and subsequent re-infection with a variant strain, the individual is likely to mount both a secondary (cross-reactive) antibody response and a primary response to altered antigenic sites. The immunodominance that is evident following primary infection of inbred mice, *may (or may not)* have a profound influence on the neutralising antibody repertoire to a variant virus challenge.

To this end, I have attempted to establish a model system to investigate the neutralising antibody repertoire of CBA/ Ca mice to a *recurrent* infection

with an influenza variant virus - a system that might be homologous to the human situation.

First, CBA/Ca donors were infected with X31 virus and after *hemisplenectomy*, the neutralising antibody repertoire was analysed by production of mAbs from the first half spleen. The same donors were then reinfected with a laboratory variant, with amino acid substitutions in regions that were known to be recognition sites for neutralising antibody and / or class II restricted T cells, and a second batch of neutralising mAbs established from the other half spleen. This approach allowed me to study the neutralising antibody repertoire to a variant virus in the face of concomitant immunity.

5.2. Results

5.2.1. Generation of HA specific B-cell hybridomas from CBA/ Ca mice after infection with X31 virus.

A brief summary of the procedures employed in the present investigation is as follows:

Mice were infected with X31 and, following recovery, half spleens were surgically removed to generate HA-specific B-cell hybridomas. The same mice were rechallenged with a variant virus A43 (Daniels et al., 1987) containing amino acid substitutions in regions recognised by both antibodies (HA1 158, 145), and T cells (del HA1 224-230) from CBA/ Ca mice. The remaining half spleens were used to generate a second batch of B cell hybridomas. The strategy is shown in fig 13.

A total of twenty neutralising mAbs (SCA series) from four individual donors were generated from the first half spleen. A majority of the mAbs were generated from donor 4 (8/20) and donor 3 (7/20). As expected of a TH1 type response to virus infection, the predominant Ig isotype among these mAbs was IgG2a (14/ 20) , or IgA (3/20) as shown in the table 10.

5.2.2. Selection and sequence analysis of X31 variant viruses.

Four variants viruses were characterised and are referred to as the SCAM-3 series. As shown in the table 11 , two of four mutants, (m3-H7 and m3-G5) had the same amino acid substitution at position HA1 158 G→E which was previously shown to be an immunodominant site. m3-D5.1 had an amino acid substitution at position HA1 218 G→E, and m3-G11 had a change at position HA1 188 N→D.

Fig 13:   Protocol employed in hemisplenectomy.

(Mice were infected with X31 and half spleens were surgically removed to generate mAbs. Following re-infection with A43 (145, 158, del 224-230), a second batch of mAbs were generated).

Intranasal infection
with X31

H - 2^k

↓ Boost

Hemisplenectomy

↓

mAb from first half of spleen

↓

Escape mutants (A)

↓

HA gene sequencing

Rechallenged with A43

↓ Boost

Second half of spleen

↓

mAb from second half of spleen

↓

Escape mutants (B)

↓

HA gene sequencing

SCA - 1		SCA - 3		SCA - 4		SCA - 5	
B-cell hybridoma clone	mAb	B cell hybridoma clone	mAb isotype	B-cell hybridoma clone	mAb isotype	B-cell hybridoma clone	mAb isotype
D9	γ2a	D5	γ2a	H2	γ2a	F4	γ2a
C10	γ2a	F9	α	G11	γ2a	G8	γ2a
H2	γ2a	F11	α	D9	γ2a		
		H7	γ2a	B10	γ1		
		G3	γ2a	G5	γ2a		
		G11	α	G12	γ2a		
		G5	γ2b	I1	γ2a		

Table 11:   Recognition specificities of mAbs from first half spleen as deduced   by sequencing of the HA gene of X31 laboratory variants (SCAM).

B-cell hybridoma	Variant virus (SCAM series)	HA1 change.	Nucleotide substitution in HA gene
3-D5.1 (γ2a)	m-3 D5.1	218 G→E	730 G→A
3-H7 (γ2a)	m-3 H7	158 G→E	550 G→A
3-G5 (γ2b)	m-3 G5	158 G→E	550 G→A
3-G11 (α)	m-3 G11	188 N→D	640 A→G

5.2.3. mAb reactivity for variant viruses by haemagglutination inhibition assay.

One of the main reasons for choosing the A43 variant for further infection was that it contained the HA1 158 change, and a deletion of HA1 224-230 which is one of the predominant T-cell recognition sites for CBA/ Ca mice.

Table 12 indicates that a majority of mAbs (12/ 19) failed to recognise A43 virus (HA1 145 S→I, 158 G→E and del 224-230). 8/19 mAbs did not recognise HA1 158 G→E and 4/ 17 failed to recognise A91 virus. Thus A43 seemed to be a good candidate for re-infection.

5.2.4. Generation of B cell hybridomas after further infection with A43 virus.

Mice that had undergone hemisplenectomy were re-infected with A43 virus. Fusions were performed three days after the boost. Tissue culture supernatants of hybridomas were tested by HI assay and ELISA against both X31 and A43. The majority of the wells contained antibody activity in ELISA but were HI negative. Two wells showed low but significant activity in HI (with both X31 and A43) and were successfully cloned and expanded as ascities and named SCB-3 G1 and SCB-3 G8. Despite the low HI activity of hybridoma supernatant, respectable HI titres were observed for mAb containing ascitic fluid (table 13), but with preferential reactivity for the wild type, X31 virus.

mAbs were also tested by HI assay against variant viruses. Both mAbs recognised A91 (del HA1 224 - 230) and HA1 158 G→E equally well (either culture supernatant or ascitic fluid).

Table   12:   Haemagglutination inhibition assay (HI) with mAbs

generated after X31 infection.

Variant A43:   HA1 158 G→E , HA1 145 S→I,

del HA1 224 -230.

Variant A91:   del HA1 224 - 230.

mAbs	HA1 158	A43	A91	X31
1-D9	+	-	+	+
1-C10	+	-	-	+
3-D5	+	-	-	+
3-F9	+	+	+	+
3-F11	-	+/-	-	+
3-H7	-	-	+	+
3-G3	-	-	-	+
3-G11	+	+	+	+
3-G5	-	+	+	+
4-G11	+	-	+	+
4-G5	+	-	+	+
4-H2	+	+	+	+
4-B10	+	+	+	+
4-D9	-	-	ND	+
4-H1	-	-	ND	+
5-G8	-	-	+	+
5-F4	-	-	+	+

Table 13:   HI titres of mAbs SCB-3G8 and SCB-3G1 from ascites
              fluid.

        HI titre expressed as the reciprocal of the dilutions required to
inhibit agglutination of turkey erythrocytes by 100%.

mAb Ascites	A43	X31
SCB-3 G8	800	12800
SCB-3 G1	800	12800

5.2.5. Selection and sequence analysis of variants of A43 with mAbs from the second half spleen.

(a). Variant selection with mAb SCB-3 G1.

mAb B-3 G1 was used to select a variant of A43 and sequence analysis of its HA gene showed amino acid substitutions at two more residues: HA1 145 I→S, 189 Q→K (Table 14). As already mentioned, A43 differs from X31 at HA1 145 S→I and the selection with mAb B-3G1 had resulted in a *reversion* to wild type at this position.

(b). Variant selection with mAb SCB-3 G8.

mAb SCB-3 G8 selected an A43 variant with two further amino acid substitutions, HA1 196 V→A, 145 I→S (table 14). Interestingly, *both* mAbs from the second half spleen selected variant viruses that had reverted to wild type at HA1 145.

5.2.6. Selection and sequence analysis of X31 variants with mAbs from second half spleen.

(a) Variant selection with mAb SCB-3 G1.

mAb B-3 G1, generated after further infection with A43, still recognised X31 in HI tests and it was of interest to obtain a definitive assignment of recognition specificity. Variants of X31 were selected, and their genes sequenced. Table 15 shows that variant m3-G1.1 has amino acid

substitutions at three different positions, HA1 193 S→N, 198 A→E, 226 L→R.

The amino acid substitution at HA1 226 was of some interest, in view of our earlier findings with human transgenic µ chain mAbs, and the role of this residue in receptor binding specificity. Thus, to *confirm* recognition specificity, mAb 3-G1 was used to select further laboratory variants of X31. Further selection produced a variant (m3-G1-C) with amino acid substitutions at HA1 198 A→E, 223 V→I. Again, the precise specificity of this mAb could not be deduced since the variant had two amino acid substitutions, with HA1 198 A→E being common change to both mutants. Therefore, the mAb was used for a third selection and sequence analysis of the HA gene showed a single amino acid substitution at position HA1 198 A→E.

Thus, following selection with mAb B-3 G1, two of three variants had more than one amino acid substitution in HA1.

(b) Variant selection with mAb SCB-3 G8.

The variant virus selected with mAb SCB-3 G8, had a single amino acid substitution at position HA1 198 A→E. Thus, mAbs generated after sequential infection with A43 seemed to be specific for HA1 198, but what is the possible significance (if any) of concomitant changes at HA1 193 and 226, or 223? I will later speculate that there are distinct similarities to the recognition specificties shown by low affinity mAbs from human transgenic mice.

Table 14 : Recognition specificities of mAbs generated from the second half of the spleen deduced by sequencing of the HA gene of A43 variants.

Variant A43: HA1 158, 145, del 224-230

B-cell hybridoma (isotype)	Variant virus	HA1 change.	Nucleotide substitution in HA gene.
SCB-3 G1 (γ2a)	m-G1.43	145 I→S	511 A→G
		189 Q→K	642 C→A
SCB-3 G8 (γ2a)	m-G8.3	145 I→S	511 A→G
		196 V→A	663 T→C

Table 15:   Recognition specificities of mAbs generated from the second half of the spleen, deduced by sequencing of the HA gene of X31 laboratory variants.

*mAb SCB-3 G1 was used three times for the separate selection of escape mutants.

B-cell hybridoma	variant virus	HA1 change	Nucleotide substitution in HA
SCB-3 G1 (γ2a)	m 3-G1.1	193 S→N	654 G→A
		198 A→E	670 C→A
		226 L→R	754 T→G
	m 3-G1-C	198 A→E	670 C→A
		223 V→I	742 G→A
	m 3-G1.3	198 A→E	670 C→A
SCB-3 G8 (γ2a)	m 3-G8.4	198 A→E	670 C→A

5.2.7. Summary.

In this section, a comparison was made of the neutralising antibody repertoire of individual CBA/Ca mice to *natural infection* with X31 (by hemisplenectomy and hybridoma production) followed by re-challenge with a variant virus, A43 that differed form wild type at HA1 145, 158, del 224-230), and further hybridoma isolation. The recognition specificities of neutralising mAbs, obtained from the first and second hybridoma fusion were determined by selection of variants for both X31 and A43. A majority of mAbs, isolated following X31 infection, failed to recognise variants HA1 158 or A43, and selected X31 variants with single residue changes at either HA1 158 or HA1 218.

In contrast, mAbs obtained after re-challenge with A43 still recognised wild type and selected X31 variants with residue changes at HA1 193 S→N, 198 A→E, 226 L→R or HA1 198 A→E, 223 V→I or HA1 198 A→E. Interestingly, the same mAbs selected A43 variants with changes at HA1 145 I→S, 189 Q→K or HA1 145 I→S, 196 V→A. There are two significant points to note from these findings. First, the prevalent selection of variant viruses with multiple substitutions using mAbs established from the second half spleen after re-infection with A43. Secondly, the residue changes identified in X31 variants, selected by the "second-set" mAbs, have never been reported (in this laboratory) for mAbs established from CBA/Ca mice following primary infection with X31.

I conclude that a different neutralising Ab repertoire may be recruited following recurrent infection with influenza variant viruses, and thus may be of some relevance to the human situation, in which there is selective pressure for antigenic variation from herd immunity, and multiple exposure to infectious virus.

CHAPTER-6

Novel specificity of a neutralising mAb from a BALB/c donor, elicited by natural infection

6.1. Introduction

Neutralising mAbs of the IgG subtype, are of moderate affinity and do not usually select for mutations within the conserved residues of the HA1 subunit. The X31 variants selected for by mAbs from HuIg μ chain transgenic mice are an obvious exception and may be a consequence of low affinity IgM responses. Moreover, a mAb of the IgG2b subtype established from a BALB/c donor following X31 infection had selected a variant virus HA1 226 L→Q, 135 G→V (Dr. Claire Smith, unpublished finding). The selecting antibody failed to recognise its variant in both HI and ELISA qualifying it as an antigenic variant. HA1 226 L→Q has featured recently in H3 isolates (e.g. A/ BEIJING/ 93), but there has been no evidence to indicate antibody recognition of conserved residues within the receptor-binding pocket.

The purpose of this study was to investigate the recognition specificity by further selection and sequencing of variant viruses produced by this particular antibody and to investigate if there was any affect on receptor-binding specificity.

/

6.2. Results

6.2.1. Haemagglutination-Inhibition (HI) assay.

mAb B1-9.1 (Dr. C.A. Smith; unpublished results) was tested by HI assay against a panel of laboratory variant viruses with known single or double amino acid substitutions within the HA1 subunit (table 16). The results show that mAb B1-9.1 did not recognise its selected mutant HA1 226 L→Q, 135 G→V, or HA1 135 G→R, 158 G→E. The same mAb, however, recognised HA1 135 G→R, that was included in the assay (due to the unavailability of HA1 135 G→I). The remainder of the laboratory variants were recognised as well as X31.

6.2.2. mAb reactivity for laboratory variant viruses in ELISA.

Since mAb B1-9.1 had selected a variant virus with an amino acid substitution in a conserved residue of the receptor-binding pocket (HA1 226 L→Q), it was important to determine whether it recognised its selected variant in ELISA (Fig 14). The mAb failed to bind to the variant, thereby qualifying it as an antigenic mutant. HA1 135 G→R, 158 G→E was also not recognised, however, the mAb bound well to HA1 226 L→Q, HA1 135 G→R, and HA1 158 G→E (somewhat reduced titre for 158 G→E).

6.2.3. Further selection of X31 variant with mAb B1-9.1.

To obtain a definitive assignment of recognition specificity, B1-9.1 was used to select further X31 variants. Six mutants were characterised and named CCM1 series (table 17). All six variant viruses had the *same* two changes, HA1 135 G→R, 158 G→E. The amino acid change at HA1 135, however, differed from that obtained following the initial selection (HA1 135 G→V).

Table 16:   Haemagglutination inhibition assay (HI) with mAb B1-9.1
and laboratory variant viruses.

HI titre expressed as the reciprocal of the dilutions required to
inhibit agglutination of turkey erythrocytes by 100%.

< less than 100.

J

Variant	Inhibition
X31	102400
226 L→Q	204800
135 G→V, 226 L→Q,	<
135 G→R	204800
158 G→E	51200
135 G→R, 158 G→E	<
145 S→N, 226 L→P,	204800
135 G→R, 225 G→D,	204800
145 S→N	204800

Fig 14:   mAb B1-9.1 recognition specificity for variant viruses by
ELISA.

Reactivity with X31 (□); or 158 (●); or 226, 135 (∇); or 226 (O); or 135
(Δ).

The ELISA procedures are described in Methods section 2.7.
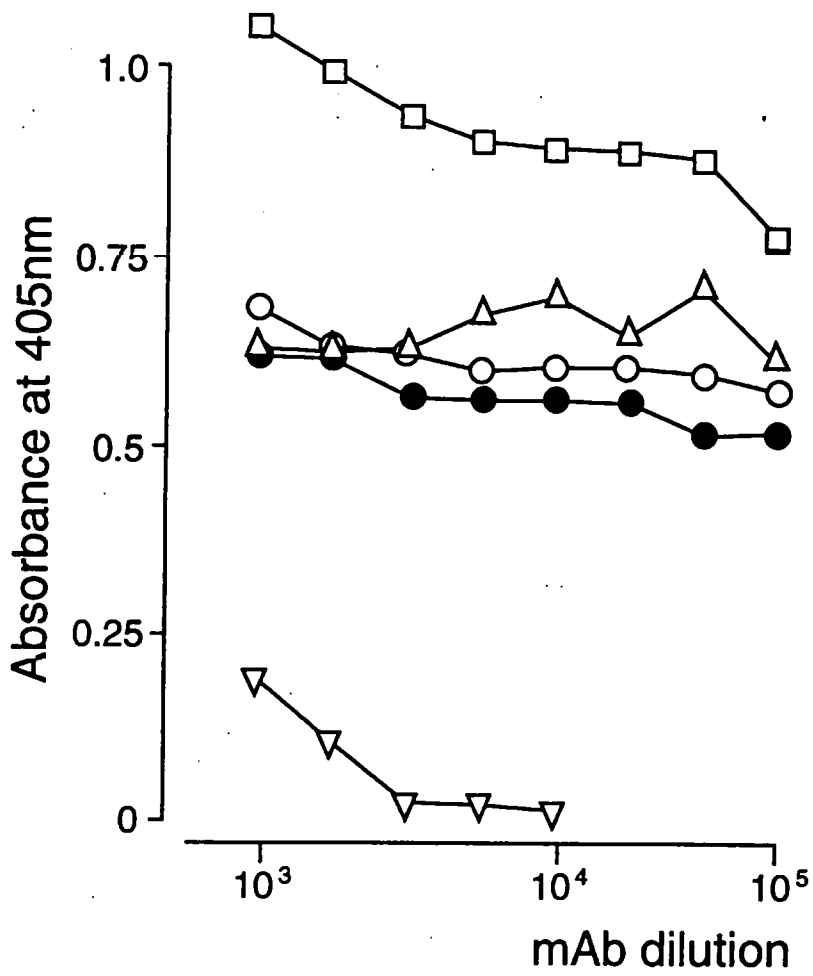
Table 17:   Recognition specificity of mAb B1-9.1.

All variant viruses were selected at *different* times and are therefore not

sibling isolates.

B-cell hybridoma (isotype)	variant virus (M1-9 series)	HA1 change	Nucleotide substitution in HA.
B1-9 (γ2b)	m1-9	226 L→Q,	754 T→A
		135 G→V	481G→T
	m1-9.5	135 G→R	480 G→A
		158 G→E	550 G→A
	m1-9.6	135 G→R	480 G→A
		158 G→E	550 G→A
	m1-9.7	135 G→R	480 G→A
		158 G→E	550 G→A
	m1-9.8	135 G→R	480 G→A
		158 G→E	550 G→A
	m1-9.9	135 G→R,	480 G→A
		158 G→E	550 G→A
	m1-9.10	135 G→R	480 G→A
		158 G→E	550 G→A

6.2.4. Selection and sequence analysis of further variants of either HA1 135 G→R or HA1 158 G →E.

The HI and ELISA results had shown that B1-9.1 recognised both HA1 135 G→R and HA1 158 G→E variants suggesting that amino acid substitutions at both 135 G→R and 158 G→E were necessary to abrogate antibody recognition.

Thus, mAb B1-9.1 was used to select variants of HA1 135 G→R. Three mutants were sequenced and named the PIB series. All three variant viruses had substitutions at three residues, HA1 135 G→R, 158 G→E and 251 L→I (table 18).

A further variant of HA1 158 G→E was also selected and sequenced and had changes at HA1 158 G→E, 146 G→D (table 19).

6.2.5. Horse Serum inhibition assay.

Horse serum inhibition assays were performed with B1-9.1 selected variants to determine their receptor-binding specificity. As indicated in table 20, X31 and HA1 135 (used as controls), were sensitive to inhibition by horse serum. In contrast, HA1 135, 158, HA1 146, 158, and HA1 135, 226, all behaved as the receptor-binding variant (HA1 226) and showed resistance to inhibition, thus, indicating altered receptor-binding phenotype.

Table 18:   Selection and sequencing of variants of HA1 135 (G→R)

using mAb B1-9.1.   The further residue changes (HA1 158, 251) are

indicated.

B-cell hybridoma	Variant virus	HA1 change	Nucleotide substitution in HA gene
B1-9 (γ2b)	PIB-2.11	135 G→R	480 G→A
		158 G→E	550 G→A
		251 L→I	828 C→A
	PIB-2.22	135 G→R	480 G→A
		158 G→E	550 G→A
		251 L→I	828 C→A
	PIB-2.3	135 G→R	480 G→A
		158 G→E	550 G→A
		251 L→I	828 C→A

Table 19:   Amino acid residue change following mAb-9.1 selection of

X31 variant (HA1 158).

B-cell hybridoma	Variant virus	HA1 change	Nucleotide substitution in HA
B1-9 (γ2b)	PIB	146 G→D 158 G→E	514 G→A 550 G→A

Table 20: Horse serum inhibition assay with B1-9.1 selected variants of X31 or HA1 135 (G→R), or X31-HS (226 L→Q). Values represent the reciprocal of horse serum dilution required to inhibit haemagglutination (Materials section 2.15).

< less than 20.

Virus	Horse Serum
X31	1280
226	<
135, 158	80
146, 158	<
135	1280
226, 145	<
135, 225	160
135	1280
145	20
226, 135	<

6.2.6. Summary

A neutralising mAb of the IgG2b isotype (B1-9.1) isolated and characterised by other members of this laboratory had selected an X31 variant HA1 135 G→V, 226 L→Q. In view of my findings for mAbs from HuIg transgenic mice, which had selected variant viruses with similar residue changes within the receptor binding pocket, I wished to determine whether this was a consistent phenotype for variants selected with this mAb.

A further series of six variant virus selections was made with mAb B1-9.1. All variant viruses contained the same residue changes HA1 135 G→R, 158 G→E. However, the selecting mAb B1-9.1 still recognised X31 variants containing single residue changes at HA1 135 or 158, indicating a requirement for mutations at two distinct sites to abrogate mAb neutralisation. Advantage was taken of this finding to select further variants of HA1 135 G→R or HA1 158 G→E:

mAb B1-9.1 selected three further variants of HA1 135 G→R which were cloned and their HA genes sequenced. Each variant virus contained the same substitutions at HA1 135 G→R, 158 G→E, 251 L→I.

mAb B1-9.1 selected a further variant of HA1 158 G→E which had an additional residue change at HA1 146 G→D.

Resistance to horse serum inhibition in HI assays confirmed that variant viruses HA1 135, 226 or HA1 135, 158 or HA1 146, 158, which had been selected for by mAb B1-9.1, had altered receptor-binding specificity.

7. Discussion

The initial aim of the thesis was to investigate the influence of affinity on the recognition specificity of the neutralising antibody repertoire for influenza virus HA using a novel murine transgenic model in which IgM to IgG class switching and concomitant somatic hypermutation/ affinity maturation were absent. Use was made of transgenic mice containing a human Ig H minigene, and disrupted murine Cµ to generate neutralising IgM mAbs which were used to select laboratory variants of X31. Sequencing of the HA genes of variant viruses indicated that several amino acid substitutions were within conserved residues that constitute part of the receptor binding pocket and which affected receptor binding specificity. With hindsight, the initial proposal by Fazekas de St Groth, in the 1970's, that low affinity Abs preferentially select adsorptive (i.e. receptor binding) mutants is now seen to be remarkably prophetic, and based on work with polyclonal Abs, in the absence of structural information on the HA molecule. Given the most recent and extensive structural information available for influenza virus HA, obtained from crystallographic studies, and the sequence analysis of the HA genes of the variant viruses, it is now possible to correlate changes in receptor binding specificity with 3-D structure.

The conclusion that I wish to propose in this thesis, is that Abs of low affinity may preferentially select for receptor binding variants; and a similar situation may pertain for in vitro situations in which laboratory variants are selected using sub-neutralising levels of antibody. Such bias in variant selection, in the absence of an optimal level of high affinity Abs may be of some relevance to herd immunity and immune evasion by influenza viruses.

Our understanding of influenza virus HA antigenicity has been significantly advanced on two fronts by (a) crystallographic studies on the structure of B-HA and (b) the use of murine mAbs to select, in vitro, antigenic

variants. From the immunologist's standpoint, however there are two important caveats.

First, mAbs are usually established following virus (or viral protein) immunisation of a naive donor (not previously exposed to multiple infections with influenza virus - as is the case in humans). Secondly, the majority of mAbs are of the IgG class, of moderate affinity, and produced several weeks or months after primary immunisation - thereby ensuring extensive B-memory cell development.

The human Ig H transgenic mouse provides a novel model system to investigate the influence of antibody affinity on recognition specificity, in the absence of class switching from IgM to IgG. In a wild type mouse, immunisation or infection with influenza virus results in class switching by day 6; and there is no experimental procedure to regulate (or restrict) affinity maturation.

The second part of the thesis was concerned with the influence of concomitant immunity on the neutralising antibody response to a variant virus, in an attempt to address the question of multiple exposure to infectious virus, as is the case in the human situation. In both model systems (transgenic and hemisplenectomy) I have found that mAbs preferentially select for variant viruses containing multiple residue changes, including substitutions within conserved residues of the receptor binding pocket.

The advent of mAb technology, for the selection and subsequent sequencing of laboratory variant viruses allowed a definitive assignment of neutralising antibody recognition sites to the 3D structure of Bromelain cleaved HA, as deduced from crystallographic studies. In most instances, laboratory variants had been selected for by mAbs of the IgG class (and of moderate to high affinity due to maturation of B cell memory in the interval following immunisation, and subsequent boosting). Such laboratory variants, in most instances differ from wild type by a single amino acid substitution in one of the five major antigenic regions known to change in natural H3 isolates.

The model system that I have investigated differs from such an approach in one important respect- affinity maturation may have been compromised. As a result a series of novel laboratory variants have been characterised that differ from wild type virus by two or more amino acid substitutions in the HA1 subunit, including residue changes in conserved positions corresponding to the receptor-binding pocket.

It is generally considered that conservation of residues within the receptor binding site are a consequence of function: structural alterations within the receptor binding site affect specificity and therefore abrogate virus infectivity. Moreover, it has been argued e.g. for the rhinovirus receptor site, that the physical dimensions of the pocket (or canyon) might prevent accessibility to neutralising antibody- the so called "canyon hypothesis"(Rossmann, et al., 1985)

However, recent crystallographic information for both influenza B-HA complexed to Fab fragments of a neutralising mAb (specific for HA1 157) (Bizebard et al, 1995) and human rhinovirus HR-14 capsid Fab complexes (Smith et al, 1996) indicate that there can be a close contact between the CDR-3 loop of the Fab chain and the conserved residues of the pocket. Indeed, in B-HA complexed to Fab, the CD3 loop resembles a finger projecting directly into the pocket and contacting many of the conserved residues. One may conclude that conservation of binding specificity and function, rather than accessibility to antibody is the primary determinant ensuring that these critical residues are not subject to immune selection.

Although this thesis was initiated from an immunological perspective (of the laboratory), and I do not consider myself sufficiently knowledgeable to offer a critical appraisal of the structural implications of document findings, due consideration will be given to the molecular structure of HA as it relates to receptor structure and function, and the residue changes reported for variant viruses in this thesis.

To reiterate, the hallmark of the immune response is its specificity. The germline encodes a large but selected repertoire of antibody binding sites which is constantly displayed in a population of B lymphocytes with a rapid turnover. The large number of possible V-D-J segment combinations and the junctional imprecision of the rearrangement process give an individual mouse the ability to create from 10^6 to 10^{11} different antibody structures (Gearhart and Bogenhagen, 1983; Tonegawa, 1983). Because each B cell uses its unique Ig structure as its surface receptor for antigen, and because uptake of antigen by B cells is an initial step in T cell-dependent B cell responses, immunisation with an antigen results in selective stimulation of only a small fraction of B cells from a large pool of available specificities.

Specificity, however, is directly correlated with the affinity of the antigen-antibody interaction. In a normal individual, the initial antibody response is primarily of the IgM class which is rapid and tails off quickly. IgM antibodies generally express germ-line determined variable regions that have not yet been modified by somatic mutation and so they tend to bind antigen with low affinity. The low frequency of substitutions in IgM antibodies suggests that the mutational mechanisms do not occur in pre-B cells during the joining of V, D and J gene segments. By day 4-6, the frequency of antigen specific B cells increases about 1000-fold and they undergo class-switching from IgM to IgG and IgA. The antibodies produced at this stage react more efficiently, forming stable complexes with the antigen.

A structural basis for these changes emerged from studies using hapten-protein conjugates as a model system. Haptens such as 2,4-dinitrophenyl (DNP), 2-phenyl oxazolone (phOx), or phosphorylcholine elicit a hapten specific immune response when conjugated to a large carrier molecule. For example, DNP conjugated to bovine serum albumin (BSA) or ovalbumin enables the immune system to elicit a hapten specific secondary response on subsequent challenge with the hapten, on an unrelated carrier. It was noticed that antibodies isolated from serum 1-2 weeks after immunisation, bound ε-

DNP-lysine with low intrinsic affinities (10^5-10^6 M^{-1}) and antibodies isolated after a few months bound this ligand with affinities around 10^7-10^8 M^{-1} (Eisen and Siskind, 1964). After many months, when serum antibodies had fallen to near background levels, another exposure to the antigen (booster immunisation) resulted in prompt appearance of the antibodies with high affinity (Siskind and Benaceraff, 1969).

With the advent of mAb hybridoma technology, Milstein, Rajewsky and colleagues were able to establish the molecular basis for affinity maturation and directed their studies towards the more homogenous responses against haptens. Extensive sequence analysis of the heavy and light-chain mRNA of hybridomas immunised with a specific hapten has helped us to understand the interplay between genetic and selective events during the onset and maturation of the immune response.

Kaartinen et al., 1983, determined the sequence of both heavy and light chains of 15 mAbs obtained 7 days after immunisation, against the hapten phOx, coupled to chicken serum albumin (ph-CSA). The response was particularly restricted to IgG1 class with an affinity of 10^{-7} Mol^{-1}. Most of the anti-oxazolone antibodies had the same V_H and V_k combination encoded by a pair of germline genes, V_H-Ox1 and V_k-Ox1 respectively: 11 out of 15 hybridomas (73%) expressed V_H-Ox1 and V_k-Ox1. Minor differences in the sequences were concentrated in the D region and at the D-J_H and V_k-J_k joining boundaries (Kaartinen, et al., 1983). One week later (14 days after the primary injection) most antibodies still expressed similar, but no longer identical sequences: 6 out of 11 (54%) expressed that combination, suggesting a high degree of somatic mutation. After secondary immunisation, only 4 out of 23 lines were related to the V_H-Ox1 with V_k-Ox1 combination, indicating a shift to a new germline H-L chain combination and a V_H subgroup emerged which had not been detected at earlier stages of response (Berek, et al., 1985). Thus, although somatic mutation is a major factor in maturation of the immune response to phOx, the shift towards alternative germline gene expression is

equally important in further improvements in antibody affinity at later stages of the response.

The observations made on the maturation of the immune response to phOx are generally consistent with those made in other hapten systems. Thus, primary antibodies to nitrophenylacetate have λ chains and are largely unmutated, whereas hyperimmune antibodies have κ chains and are somatically mutated, and display increased affinity for the hapten (Bothwell, *et al.*, 1981).

A detail analysis of genes and proteins that participate in the murine immune response to phosphorylcholine was also documented by Malipiero et al., 1987. 37 out of 38 antibodies were encoded by 1 V_H gene of the S107 subfamily, and 3 V_k genes, V_kT15 of the V_k 22 subfamily, V_k M3 from the V_k 8 subfamily, and V_k 167 from the V_k 24 subfamily (Malipiero, *et al.*, 1987). No mutations were detected in those genes until the second week after immunisation. Sequence analysis of anti-PC antibodies showed a striking correlation between the presence of mutation and antibodies that had undergone class switching. No mutation was found in 18 heavy and light chains from IgM antibodies, whereas mutation was evident in 7 out of 13 chains in IgG3, 13 out of 24 chains from IgG1, and 8 out of 18 chains from IgA antibodies. *Most of the mutated antibodies had higher affinity for antigen than their germline counterparts, which suggests that the major role of somatic mutation is to increase affinity rather than to create new specificities.*

Antibody responses to proteins, and viruses, however, are different from anti-hapten responses, probably because of the antigenic complexity of proteins. Thus, in contrast to the progressive changes seen with the anti-hapten antibodies, a relatively rapid increase in the average antibody affinity was observed against hen egg lysozyme (HEL) (Newman, *et al.*, 1992). A majority of the antibody response was either IgG1 or IgG2a while IgM antibodies were rare even in the primary response. The overall affinity did not increase further during the course of the response which suggested that those mAbs represented a population of Ag-selected antibodies that had already undergone

somatic mutation during initial antigenic stimulation. Does this mechanism also apply in virus infection where high affinity neutralising antibodies are essential for protection?

Recent studies by Zinkernagel and colleagues, using vesicular stomatitis virus (VSV) in a murine model system reported rapid onset of high affinity serum IgG responses and "on rate" kinetics coincident with viral clearance (Roost, *et al.*, 1995). IgG antibodies were evident 6 days after the infection and the values did not increase in response to repeated booster injections spread over 150 days. Memory levels of neutralising anti-VSV IgG titres (between 1:80,000-1:320,000), remained stable within the range of 2-4 for >6 months, and did not increase by more than a factor of 4-8 after booster injections compared with the response after a single infection. Thus, the quality of the response did not improve further with time, or re-exposure. The direct implication is that *binding a target antigen rapidly may be as important as the thermodynamic stability of the resulting antibody-antigen complexes in limiting pathogen growth.*

It can be speculated that since VSV is a replicating virus with repetitive copies of its antigenic glycoprotein, it accelerates the tempo of affinity maturation, generating high affinity B cells by day 6. However, high affinity antibodies were also obtained by day 7 after immunisation with lysozyme which is a non-replicating protein antigen (Newman, *et al.*, 1992). Thus, there must be some other reasons for such a response. It could be the type of virus that has caused infection or more importantly, the route of infection. We do know from previous studies that infection with live virus elicits a memory repertoire different to the response following immunisation or vaccination with attenuated or dead virus (both with respect to longevity and specificity).

Thus, previous studies in this laboratory have shown, that the immune response to influenza HA following *natural infection* elicits an extremely narrow window of specificity, and immunodominance is observed in different strains of mice (Patera, *et al.*, 1995; Smith, *et al.*, 1991). Immunodominance

could not be attributed to V_H or V_L gene usage since the individual's response to a single antigenic site was derived from a minimum of 3-6 progenitor cells (Patera, *et al.*, 1995).

These studies contrast with several other reports where a highly diverse response against HA has been documented (Caton, *et al.*, 1986; Clarke, *et al.*, 1985; McKean, *et al.*, 1984; Staudt and Gerhard, 1983). It is important to note that such diversity was achieved following *immunisation* with the virus. For example, analysis of secondary haemagglutinin site (HA-Sb) specific mAbs isolated from mice on day 24 were highly diverse in contrast to primary antibodies that were structurally and functionally similar. This increased diversity in the secondary response was caused by the use of a larger repertoire of V_H and V_k genes and to somatic mutation. The effect of somatic mutation on specificity was such that even though secondary responses were oligoclonal, virtually every antibody had a unique antigen binding site and fine specificity (Clarke, *et al.*, 1985).

Balkovic et al., 1987 found that immunisation with inactivated influenza virus or viral protein elicited an IgG response with a subclass distribution similar to that of anti-protein antibodies, that is, predominantly $\gamma 1$. Infection of CBA/Ca or C3H/HeN mice with virulent or non-virulent influenza virus elicited high $\gamma 2a$, low $\gamma 1/\gamma 2b$ and very low $\gamma 3$ serum antibody levels typical of antiviral responses. Surprisingly, immunisation with inactivated virus also gave high $\gamma 2a$, moderate $\gamma 2b$ and very low $\gamma 1/\gamma 3$ profiles, whereas immunisation with HA/NA protein gave a typical protein isotype profile of high $\gamma 1$, low $\gamma 2a$ and very low $\gamma 2b/\gamma 3$ serum antibody (Balkovic, *et al.*, 1987).

Hocart et al., 1989, found the antibody responses to H3 subtype virus to be predominantly of the $\gamma 2a$ subclass irrespective of the route of inoculation, but the magnitude of response varied with the route and schedule of inoculation and dose (Hocart, *et al.*, 1989). The response to intranasally administered HA is much higher than i.p., suggesting the i.n. route can give better protection from influenza virus than the i.p. route due to the

amplification of antigen during virus growth or induction of secretory IgA (Coutelier, *et al.*, 1987). Inraperitoneal and i.v. immunisation elicited predominantly γ2a antibodies. No γ3 antibodies were detected in the primary response to i.v. virus. The primary response to i.p. HA on day 3 consisted of γ2a and γ2b antibodies only. By day 7, γ2a, γ2b, γ1 and γ3 were all equally represented and by day 14, following a secondary boost, γ1 antibodies exceeded γ2a, and γ2b and γ3 were absent from serum antibody. γ3 was also absent from the early primary response to i.v. HA, all isotypes were detectable by day 7 but γ2a predominated, and by day 14, in the secondary response, the order of antibody titre was γ2a > γ1> γ2b> γ3.

The γ2a antibodies predominate regardless of route of inoculation of influenza virus (i.n. verses i.m.) in serum and lungs of CBA/Ca mice. However, the response to inactivated virus consists of all four γ isotypes. BALB/c have higher titres of γ1/γ2a than C57BL/6 which has the highest titres of γ2b. Neutralisation activity of the different isotypes also varies with strain. For example, γ1 is twice as effective as γ2a, which is twice as effective as γ2b in BALB/c, but in C57BL/6, γ2a and γ2b are more effective than γ1 and γ3, and in CBA/Ca mice, γ2a has twice the neutralising activity of γ2b.

The present investigation, and previous studies in this laboratory have shown that secondary antibody responses in CBA/Ca, BALB/c or BALB/k mice to be predominantly of the γ2a, subclass (Smith, *et al.*, 1991),which is a characteristic trait of most viral infections in the mouse (Coutelier, *et al.*, 1988; Coutelier, *et al.*, 1987) and may illustrate the influence of antigen-specific CD4 T cells: Th1 cells secrete γ-IFN, which enhances γ2a, synthesis in vitro (Snapper, *et al.*, 1988), and has been shown to preferentially induce γ2a production (Mosmann and Coffman, 1989).

For neutralising antibodies to VSV or rabies virus, high affinity antibodies are essential at an early stage of infection since slow affinity maturation may allow chronic or severe systemic infection and prolonged

transmission of the virus. In contrast, influenza virus does not establish systemic infection, and is restricted to the respiratory tract due to a requirement for apical budding from epithelial cells into the bronchial lumen (Roth, 1983). Consequently, host IgM antibody responses may not be a significant determinant of immune protection and /or antigenic variation. Thus, protection from live virus challenge is associated with rapid clearance by both secretory IgA and serum IgG (by transudation) so that by day 6 post infection there is no significant lung titre for virus. This is also associated with rapid class-switching.

The most detail studies to determine the dynamics of class specific antibody responses to HA in serum and nasal secretions have been reported by Murphy and his colleagues, who used live attenuated vaccines. Serum antibody responses typical of primary viral infections were detected in naive children using the ELISA technique (Murphy, et al., 1982). IgM, IgA and IgG antibodies appeared in the serum within two weeks after inoculation of virus. The maximum serum IgG response was detected at approximately six weeks, while IgM and IgA antibody levels declined after two weeks. In nasal secretion, IgA was the predominant antibody and was present in the majority of individuals within two weeks of inoculation. IgG and IgM responses occurred less frequently and with low titres.

Changes in receptor binding site structure and function:

The selection of influenza virus variants with altered receptor binding properties has been reported previously by several groups. For the most part, such variants had either been selected for on the basis of their resistance to glycoprotein inhibitors of viral infectivity (Rogers, et al., 1983b), or because of their ability to grow in various host cells (Burnett and Bull, 1943; Cohen and Biddle, 1960; Crecelius, et al., 1984; Schild, et al., 1983). However receptor binding or adsorptive mutants have also been reported following selection with *sub-neutralising* levels of monoclonal antibodies (Daniels, et al., 1987; Temoltzin-Palacios and Thomas, 1994; Yewdell, et al., 1986).

Yewdell et al, (1986) reported the selection of mutants by sub-neutralising levels of a mixture of monoclonal anti-HA antibodies. The antibody mixture contained at least two antibodies specific for each of the four PR8 HA antigenic sites. Although, the mutants did not differ from wild type antigenically, variation was observed in the ability of individual antibodies to inhibit mutant and wild type viruses from agglutinating chicken erythrocytes. In contrast, very small differences were observed when human erythrocytes were used. That difference was probably due to the altered interaction of the virus with the cellular receptors and not due to the differences in antigenicity. Two of the residues (HA1 185 and 231) in the variants were found to be located near the receptor-binding site and could directly alter binding. The third residue (HA1 244) was located at the trimer interface. The variants were also able to agglutinate erythrocytes which were treated with twice the amount of neuraminidase needed to prevent agglutination by the wild type virus. Thus, it seemed that the antibody selected variants had higher affinity for cellular receptors.

Daniels et al, 1987, reported an IgG1 mAb (HC63) that was broadly cross-reactive for a majority of natural H3N2 isolates. A number of variants of X31 and of a receptor-binding mutant of X31 (X31-HS: HA1 226 L→Q) were selected by that antibody in eggs and in MDCK cells. Those variants principally involved substitutions in positions HA1 218 G→R, HA1 193 S→N, 226 L→P, or deletion of HA1 224-230 (RGLSSRI). In most instances, the variant was recognised by the selecting antibody in ELISA although there was resistance to Ab neutralisation, or haemagglutination inhibition. The variants also showed altered receptor binding properties compared to X31: they either agglutinated erythrocytes containing the SA α 2,3 Gal linkage (HA1 218 G→E), or showed resistance to inhibition by α_2-macroglobulin (del HA1 224-230; HA1 193 S→N, 226 L→P) or exhibited transient haemgglutination of all derivatised cells.

Temoltzin-Palacios and Thomas (1994) employed a somewhat different approach to select receptor-binding variant viruses: certain HA1 antigenic residues (63, 144, 158, 193) were found to be immunodominant in the neutralising Ab response of CBA/Ca mice to X31 infection. Following sequential mAb selection, in ovo a variant of X31 was obtained (IMUT-4) with substitutions at each of these positions, and was used to infect naive CBA/Ca recipients, and to obtain neutralising mAbs. Each of the mAbs failed to select antigenic variants of IMUT-4 but, following treatment with *sub-neutralising* levels of mAb, a receptor-binding mutant was obtained with two further changes (HA1 190 E→D, 226 L→Q). The variant was resistant to horse serum inhibition and was still recognised by the selecting mAb in ELISA. It is of some interest that these same two residue changes (HA1 190, 226) have been reported in recent H3 isolates (e.g. BEIGING/92 - World Influenza Centre; NIMR).

The selecting mAb used in this study still recognised X31, and was used to select an X31 variant virus. This differed from wild type by a single residue change, HA1 155 Thr→Ile, which also conferred altered receptor-binding specificity (for N-glycolylsialic acid of horse RBC).

The structure of the receptor binding pocket was discussed in the introductory section (1.8.7 and fig 5) and I have indicated, in a space filling model (Fig 15), the topographic relationship of residue changes in the laboratory variants m 7.1 (HA1 145, 226) or m-21.2 (HA1 135, 225) or m-1.1 (HA1 135, 158) or m-4.1 (HA1 135) (fig 15). Residues 134 -138 form the right side of the site and residues 224-228 form the left side and the bottom of the binding site is formed by Tyr 98 and Trp 153. The residues Glu 190, Leu 194 project down from a short α-helix which, together with His 183 and Thr 155 define the rear of the depression. What residue changes therefore, within this region of the molecule, affect receptor specificity?

It should be emphasised that, despite a consistent finding that antigenic residue changes are located proximal to the binding pocket, some antigenic

Fig 15:   Space filling model indicating molecular location of the amino acid substitutions in the laboratory variants mentioned in this thesis.
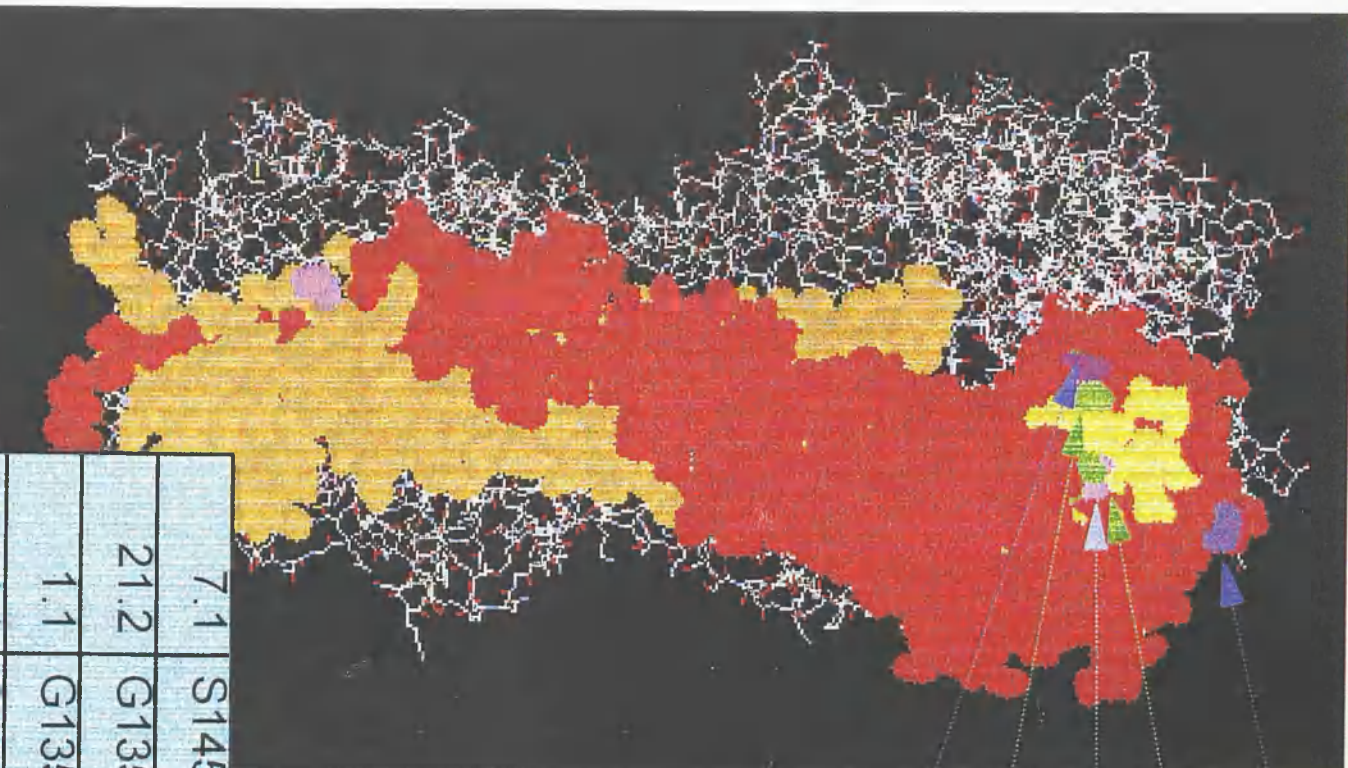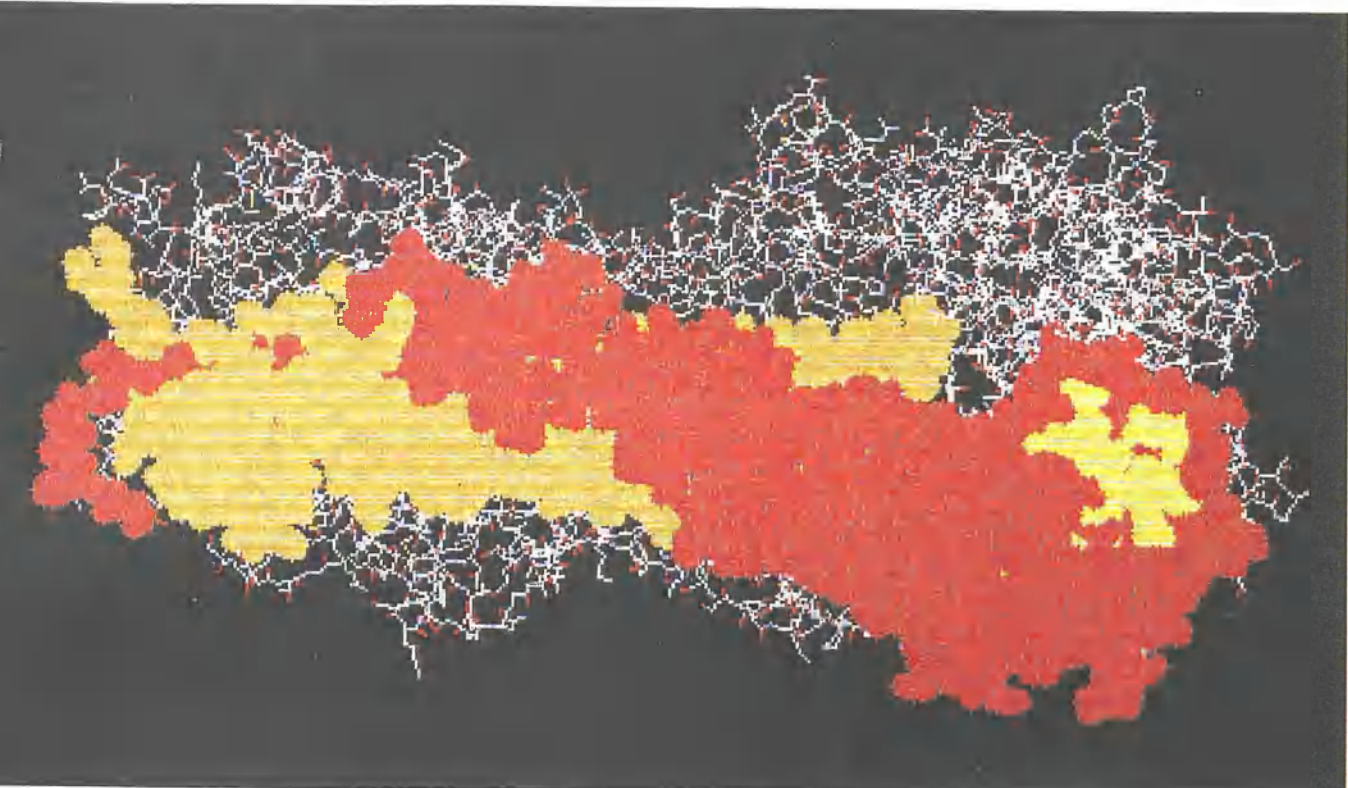
7.1	S145N	L226P
21.2	G135R	
1.1	G135R	G225D
	G158E	

158
145
135
226
225

residue changes <u>do</u> affect receptor-binding specificity. For instance, the drift substitution HA1 155 Thr→Tyr in natural isolates confers increased affinity for N-glycolyl sialic acid residues, present on horse RBC. Furthermore, Temoltzin-Palacios and Thomas (1994) have reported a laboratory variant of X31, HA1 155 Thr→Ile that is able to agglutinate horse RBC; and the same residue change has occurred in natural H3 isolates (CAEN/83 or SOF/84; data from the World Influenza centre, NIMR). Similarly, HA1 158 G→E is a defined antigenic site for both laboratory variants and natural isolates (e.g. TEX/77) and thus residue change differentially affects susceptibility (in agglutination assays) to periodate treatment of RBC.

Exposure to mild periodate treatment (in the absence of light) selectively cleaves cis-hydroxyl groups, present in the N-glycan side chain; and attacks terminal sialic acid residues, at the C7-C8 cishydroxyl tail, thereby effectively reducing the quantity of available sialic acid residues that can participate in agglutination reactions. (An alternative procedure would have been mild NANAse treatment of RBC).

Rather than antibody selection, inhibition by equine α_2-macroglobulin was used to select for a receptor-binding variant and which was found to differ from wild type virus by a single residue change HA1 226 L→Q (large non-polar to large polar residue). Changes in receptor-binding specificity were confirmed by altered specificity for α, 2-3 and α,2-6 sialyl derivatised RBC in agglutination assays. The crystal structures of both wild type and variant HAs complexed to sialyllactose have been deduced by Weis et al (1988) and the authors conclude that minimal changes have occurred in the receptor pocket so as to accommodate the introduction of a large polar residue and further potential for establishing hydrogen-bonded contacts. I am not qualified to express an opinion on these findings but can only assume that there is no

"straightforward" structural relationship between HA1 226 L→Q change and specificity for the α, 2-3 and α,2-6 linkage.

Various assays for differentiating variant viruses on the basis of their receptor binding characteristics involve either chemical or enzymatic modification of the receptors on erythrocytes (RBC). Daniels et al, 1987 performed such haemagglutination tests by adsorbing viruses to derivatised (α 2,3 or α 2,6) erythrocytes that contained amounts of sialic acid optimal for detecting differences in receptor binding properties. X31 was shown to be specific for the SA α-2,6 Gal linkage and bound weakly to SA α-2,3-derivatised cells even at the highest level of incorporation examined. In contrast, X31/HS (226 L→Q) bound to both SA α-2,6 and SA α-2,3 derivatised erythrocytes (Daniels, *et al.*, 1987).

In the present investigation, receptor binding specificity was analysed by ELISA using either α-2,6 or α-2,3 N-acetyl-lactosaminyl-BSA (SLB) as substrates. All variants exhibited reduced binding for α-2,6 SLB, particularly m-7.1 (145, 226), and this was consistent with a critical role for HA1 226 in receptor specificity. Interestingly, m-21.2 (135, 225) showed an intermediate binding for both α-2,6 and α-2,3 SLB. Another notable finding was that X31-HS failed to bind α-2,6 SLB in contrast to X31. This may be because of the different assay system used by myself and Daniels et al (RBC agglutination). Variant m-1.1 (135, 158) and m-1.1N (145, 158) also exhibited altered receptor binding characteristics. HA1 158 is present on the opposite side of the receptor binding pocket and has been implicated in receptor binding specificity.

Thus, Hu transgenic IgM antibodies would appear to preferentially select variant viruses with changes in conserved residues of the receptor binding pocket. How do I account for such preferential selection?

A likely mechanism may relate to the fact that most IgM antibodies are of low affinity, in contrast to neutralising IgG mAbs that have been used to characterise HA antigenically. However, to establish that antibody *affinity* is

indeed the determinant for selection of such variants, the next step should be measurement of antibody affinity and /or on-rate kinetics. Antibody affinity is defined as the strength of interactions between an antigenic determinant and the homologous antibody combining site and represents the summation of attractive and repulsive forces involved in interaction. Antibody avidity, on the other hand, depends in part on affinity but also involves other factors such as antibody valency. This means that a higher affinity antibody would bind larger amounts of antigen in a shorter period of time (faster on-rates) compared to a lower affinity antibody.

Affinity can be measured directly by ELISA, and on-rate kinetics can be determined by measuring the velocity of virus neutralisation. A range of measured values of affinity constants for the antibody binding site is enormous from below 10^5 M^{-1} to above 10^{12} M^{-1}. The measurement requires determination of free and antibody-bound antigen at equilibrium. Previously, sera containing a heterogeneous population of antibodies of various classes and of different affinities, were used and therefore interpretation of results were based on an overall affinity estimation which were less accurate. Subsequently monoclonal antibodies provided the opportunity to study individual homogenous antibodies of known affinity, class and concentration.

Because of the simplicity of the ELISA technique it has been used to estimate equilibrium constants directly from solid phase binding of antibody (Beatty, et al., 1987; Li, 1985). These measurements estimate a 'functional affinity' (Li, 1985)or avidity, the magnitude of which is directly dependent upon surface effects. The lack of surface diffusion of antigen and slow diffusion rate of 'bulky antibody molecules' slows down the association rate of antibody and antigen (Beatty, et al., 1987; Li, 1985). For IgG antibody, multivalent binding has been shown to produce a 1000-fold increase in avidity, while for IgM the enhancement factor was 10^6 (Hornick and Karush, 1972). Thus, if both the reactants, antigen and antibody are multivalent, accuracy will not be achieved. This necessitates the preparation of Fab fragments of

antibody. However, this is where it is problematic to measure "on-rate" kinetics for IgM antibodies. IgMs are decavalent and it is difficult to prepare Fab fragments of such antibodies. My intention was to compare affinities of IgM mAbs that I had generated (and selected double mutants) with IgM mAbs from CBA/Ca or BALB/c donors (that had selected X31 variants with single changes in known antigenic sites) to see if there was any difference in the "on-rate" kinetics. However, *very* preliminary attempts to employ a plasmon resonance technique (BIACOR) have been unsuccessful, due to non-specific high backgrounds.

I now wish to consider the findings in the second part of my thesis which may be of some relevance to the phenomenon of *Original antigenic sin* - a skewed response during secondary infection such that the antibody specificity is directed primarily towards the antigen encountered in the original infection (Francis, et al., 1953). Epidemiological studies in humans have shown that infection by one subtype confers little or no protective immunity to other subtypes, and sera from humans or experimentally infected animals do not cross-react with viruses of different subtypes (Knight and Kosel, 1973). After infection, individual sera contain antibodies to determinants on the HA of the infecting strain that are strain specific as well as cross-reactive antibodies to determinants shared by variants of that subtype. In human immune sera that have been virus absorbed the proportions of strain-specific and cross-reactive antibodies depend on the individual's previous experience of influenza virus infection. In sera from unprimed and naturally infected children, the predominant antibodies were strain-specific with only a small amount of cross-reactive antibody present (Oxford, *et al.*, 1979a).

In adults who have previously been exposed to an earlier variant, the predominant antibodies after infection are cross-reactive but strain-specific (Oxford, *et al.*, 1979b). These studies suggest that antibodies induced by recurrent infection with influenza virus were primarily directed toward those determinants.

This brings me to the second part of my thesis where I have tried to "mimic" the human situation in mice: individual donors were infected with X31 and, following hemisplenectomy the recognition specificity for X31 HA was examined by the production of mAbs, and the selection and sequencing of their corresponding variant viruses. Thereafter mice were re-challenged with an X31 variant virus (HA1 145 S→I, 158 G→E, del 224-230). My initial aim was to see whether the immunodominance observed after initial X31 infection had any influence on the repertoire to subsequent challenge with a variant virus.

The potential antibody repertoire of an individual, i.e. the total number of possible V-domains that can be generated from the germline is dynamic, and of the order of 10^9 different V-domains (Berek, et al., 1985; Tonegawa, 1983). The emergent repertoire produced daily in the bone marrow is approximately $3-5 \times 10^7$ different clones (Opstelten and Osmond, 1983). The available repertoire, i.e. the number of domains currently expressed, is limited to a maximum of 10^8 specificities, and the actual repertoire which is the V-domains used by the effector Ig-secreting cells are about 10^6 cells (Benner, et al., 1982). However, the normal immune system is dynamic, with high rates of cell production and high turnover rates allowing rapid substitution of the major part of the immunocompetent cell pool. The size of different antibody repertoires, and the dynamic properties of lymphocytes suggest that the immune system only uses a minor fraction of its potential diversity. Repertoires can undergo continuous qualitative changes throughout life, and such changes can occur at many stages of development. The response to antigenic challenge depends on the *expressed* repertoire, and is therefore of some interest to establish whether these are dictated by stochastic events, or germ-line bias, or post "antigenic experience".

Thus, in a sequential infection there might be two possible scenarios: (a) either T-memory cells, with a broader specificity than the B-cells, are triggered by the cross-reactive antigen and are able to help B-virgin lymphocytes to secrete antibodies, or (b) B-memory cells with specificity for

the first antigen are directly triggered by the cross reactive antigen. In the present study, donors were able to generate a neutralising antibody response to the variant virus that was also cross-reactive for the wild type virus. In earlier studies of original antigenic sin, after a sequential infection with two different but antigenically related strains of influenza virus, the antibody stimulated by the second infection reacted more strongly with the primary virus than with the one actually eliciting the response (Fazekas, and Webster, 1966 a; Fazekas, and Webster, 1966 b) - which is what I find!

In the present study, I have examined *directly* the recognition specificity of "recall" mAbs for X31 and its variant virus, by the selection of further laboratory variants. X31 variants selected with such antibodies (particularly mAb B-3G1), had multiple amino acid substitutions (HA1 193 S→N, 198 A→E, 226 L→R) or (HA1 198 A→E, 223 V→I). A43 variants had a further two substitutions (HA1 145 I→S, 189 Q→K), or (145 I→S, 196 V→A). How can I explain the finding that X31 variants had multiple changes both in conserved residues and in antigenic regions? I would *speculate* again that Ab affinity may have played some role in the selection process, as also seen in the earlier findings with HuIg mAbs.

The relationships between the amino acid sequences of immunoglobulins and the structures of their antigen-binding sites are important for understanding the molecular mechanisms for the generation and maturation of the immune response. The specificity and affinity of the binding sites are governed by the structures of six hypervariable regions (Wu and Kabat, 1970). Because hypervariable regions have different sequences in different antibodies, each region adopts a different conformation in different antibodies. The conformations are determined by the interactions of a few residues at specific sites in the hypervariable regions (Chothia, *et al.*, 1989).

Thus, affinity of an antibody for its antigen influences its biological activity. High affinity antibody forms more stable association with antigen than lower affinity antibody. Not only do antibodies from different subclasses differ

in their affinities, but affinity differences are found within a given subclass (Sarvas, *et al.,* 1983). Thus, it is possible that the IgG antibodies generated after challenge with the variant virus had lower affinity compared to the ones generated earlier. This is speculation on my part and requires further investigation and data measurements of affinity for Fab fragments.

To summarise, I have shown that (probably) low affinity antibodies against X31 may preferentially select for substitutions within or proximal to the receptor binding pocket. In contrast, moderate to high affinity antibodies for the most part select for a single amino acid change within antigenic regions of the HA molecule.

Finally, I investigated the recognition specificity of a mAb B1-9.1 that was generated in a BALB/c donor following natural infection. This mAb selected a variant with changes at HA1 135 G→V, 226 L→Q, and failed to recognise its mutant by ELISA thereby qualifying it as an antigenic variant. In previous chapters, I have outlined the importance of residue 226 and how it affects receptor binding specificity of the virus. I therefore did further selection of X31 variants with mAb B1-9.1 at different times, and with varying mAb concentrations. I found that 6 out of 6 variants had the *same* amino acid changes, HA1 135 G→R, 158 G→E. Interestingly, the selecting mAb could still recognise the laboratory variants HA1 135 G→R or HA1 158 G→E in HI assays which suggested that the variant had to have *both changes* in order to abrogate recognition by that particular antibody.

I therefore decided to use mAb B1-9.1 to select a variant of HA1 135 G→R, and found that the further substitution was indeed HA1 158 G→E. However, in addition to HA1 135, 158, the variant also had an amino acid substitution at HA1 251. Change at HA1 158 is known to alter receptor binding specificity of the virus.

In summary I have found both IgM (from transgenic donors) and IgG mAbs (following recurrent infection) preferentially select laboratory variants with multiple substitutions in the HA1 subunit and altered receptor-binding

specificity. Affinity measurements of these antibodies might help to establish whether low affinity antibodies do indeed select variants with changes in the conserved residues that constitute part of the receptor-binding pocket (fig 5: structure of receptor binding pocket) .

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APPENDIX I

(Buffers)

Borate Buffered Saline (BBS), pH 8.6

H_3BO_3	Boric acid	100mM
$Na_2B_4O_7$	Sodium tetraborate	40mM
NaCl	Sodium chloride	70mM

Diethanolamine buffer

$(HOCH_2CH_2)_2NH$	Diethanolamine	9.7% v/v
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate	0.49mM
NaN_3	Sodium azide	0.02% w/v

Phosphate buffered saline (PBS)

Na_2HPO_4	Di-sodium hydrogen phosphate	100mM
KH_2PO_4	Potassium di-hydrogen phosphate	1.8mM
NaCl	Sodium chloride	171mM
Kcl	Potassium chloride	3.4mM

Tris/Borate buffer (TBE)

Tris-base	89mM
Boric acid	89mM

EDTA	2mM

Tris/Glycine buffer (pH 8.3)

Tris-HCl	25mM
Glycine	384mM
SDS	0.1%

Tris/saline buffer

Tris-HCl	10mM
EDTA	1mM
SDS	1% w/v

Loading buffer (non-reducing)

Urea	8.3M
SDS	0.6% w/v
Coomassie blue	0.01% w/v

Loading buffer (reducing)

Urea	8.3 M
SDS	0.6% w/v
Coomassie blue	0.01% w/v
2-Me	0.46M

(All chemicals from BDH laboratoriy supplies)