Structural analysis of immunodominance in the antibody response to influenza virus haemagglutinin following natural infection.

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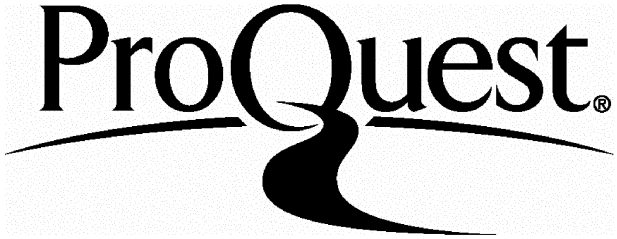

"I have tried too in my time to be a philosopher;

but, I don't know how,

cheerfulness was always breaking in."

Oliver Edwards 1711-1791

ABSTRACT

The protective immune response to influenza virus is directed against the surface glycoprotein haemagglutinin (HA) which undergoes continuous antigenic drift in order to evade immune recognition. The recognition sites for neutralising antibodies have been located to five surface accessible regions (sites A-E) on the membrane distal ectodomain of the HA1 subunit.

Although all five sites are recognised by neutralising antibodies, there is evidence for haplotype specific differences and immunodominance in the response to *natural infection.* BALB/c (H-2^d) Mab predominantly recognise HA1 198, and CBA/Ca (H-2^k) Mab, HA1 158.

Restriction of the immune response and the observed immunodominance raises several important questions concerning the mechanisms of repertoire selection, which this investigation aims to address using BALB/k (MHC congenic) mice, to evaluate possible effects of MHC background on selected repertoires.

The BALB/k antibody response to X31 HA was found to be a composite of both repertoires, recognising predominantly HA1 198(Ala→Glu) in four out of five donors and HA1 158(Gly→Glu) in the fifth, suggesting both non-MHC and MHC effects.

Previous studies on antibody gene usage have shown restriction for anti-haptenic responses and diversity for anti-protein responses. Generally, antibody specificity for proteins is diverse. Since I had found immunodominance in the response to influenza HA protein, it was possible that antibody gene usage would resemble a hapten-like response and thereby account for this highly focused response.

Extensive sequence analysis of antibody gene usage by influenza HA specific Mab revealed a *diverse* V_L usage but restricted V_H family usage by BALB/k HA1 198 specific Mab and *very restricted* V_L and donor dependent restricted V_H family usage by CBA/Ca HA1 158 specific Mab. V_L and V_H family usage was also mutually exclusive between the two strains and specificities. BALB/k HA1 158 specific Mab (a CBA/Ca-like response) were a composite of both repertoires, with *very restricted* V_H family usage, but with *diverse* V_L usage.

It was evident that the immunodominant response per individual was a consequence of clonal expansion of one to three progenitor cells from both BALB/k (recognition of HA1 198), and CBA/Ca (recognition of HA1 158), however antibody gene usage appears to be stochastic in the BALB/k HA1 158 specific response. Furthermore, the observation that the same V_L/V_H family combinatorial association by Mab of two different specificities from two donors and such diverse V_H/V_L gene family usage indicates that fine specificity and immunodominance *cannot* be attributed to antibody V gene usage *alone*.

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This thesis is dedicated to the ones I love.....

ABBREVIATIONS

Ab . . . . . antibody

Ag . . . . . antigen

AIDS . . . . . acquired immunodeficiency syndrome

APC . . . . . antigen presenting cell

Ars . . . . . arsonate

β_2m beta-2-microglobulin

BAM . . . . . BALB/k Mab selected escape mutant virus

BBS . . . . . borate buffered saline

BHA . . . . . bromelain digested haemagglutinin

BLASTN . . . . . basic local alignment search tool for nucleotide sequence

BSA . . . . . bovine serum albumin

C . . . . . constant

cDNA . . . . . complementary deoxyribose nucleic acid

CK . . . . . murine immunoglobulin constant region PCR primer

CDR . . . . . complementarity determining region

CDRnH . . . . . amino acid number in immunoglobulin heavy chain CDR region

CDRnL . . . . . amino acid number in immunoglobulin light chain CDR region

CEF . . . . . chick embryo fibroblasts

CL . . . . . cardiolipin

CTL . . . . . cytotoxic T-cell

D . . . . . diversity

DNP . . . . . dinitrophenyl (see NP)

dNTP . . . . . deoxy nucleotide triphosphate

ddNTP . . . . . dideoxy nucleotide triphosphate

d/dd ATP . . . . . deoxy/dideoxy adenosine triphosphate

d/dd CTP . . . . . deoxy/dideoxy cytosine triphosphate

d/dd GTP . . . . . deoxy/dideoxy guanosine triphosphate

d/dd TTP ..... deoxy/dideoxy thymidine triphosphate

ddH_2O ..... double distilled water

DEAE ..... diethylaminoethylcellulose

DMSO ..... dimethyl sulphoxide

DNA ..... deoxyribonucleic acid

dsDNA ..... double stranded DNA

DTT ..... dithiothreitol

EDTA ..... ethylene diamino tetra acetic acid

ELISA ..... enzyme linked immunosorbent assay

EM ..... electron microscopy

EMBL ..... european molecular biology laboratory

ER ..... endoplasmic reticulum

Fab ..... immunoglobulin antigen binding region fragment

Fc ..... immunoglobulin constant region fragment

FCS ..... foetal calf serum

FDC ..... follicular dendritic cells

FL 3-fucosyllactosamine

FR ..... immunoglobulin framework region

GCG ..... genetic computer group sequencing software

GDE ..... genetic data environment sequence handling software

H ..... heavy

HA ..... haemagglutinin

HAU ..... haemagglutination unit

HAT ..... hypoxanthin-aminopterin-thymidine

HEL ..... hen egg lysozyme

HI ..... haemagglutination inhibition

HIM ..... hyper IgM

HLA ..... human histocompatibility leukocyte antigen

Hn ..... amino acid number in immunoglobulin heavy chain

HnNn ..... influenza virus haemagglutinin/neuraminidase subtype

HSV ..... herpes simplex virus

H-2 . . . . . mouse major histocompatibility complex

Ia . . . . . I-region associated

Id . . . . . idiotype

Ii . . . . . MHC invariant chain

IFN . . . . . interferon

Ig . . . . . immunoglobulin (sIg surface, mIg membrane associated)

IL . . . . . interleukin

i.m . . . . . intramuscular

i.n. . . . . intranasal

i.p. . . . . intraperitoneal

Ir . . . . . immune response

i.v. . . . . intravenous

J . . . . . joining

JCB . . . . . CBA/Ca monoclonal antibodies and B-cell hybridoma cell lines

KCl . . . . . potassium chloride

KLH . . . . . keyhole limpet hemocyanin

L . . . . . light

LAH . . . . . lactalbumin hydrolase

LCMV . . . . . lymphocytic choriomeningitis virus

LD . . . . . CBA/Ca monoclonal antibodies and B-cell hybridoma cell lines from low dose infection

Ln . . . . . amino acid number in immunoglobulin light chain

Mab . . . . . monoclonal antibody

Mbq . . . . . megabequerel

MHC . . . . . major histocompatibility complex

MKV . . . . . murine immunoglobulin variable region PCR primer

μ m . . . . . membrane IgM

MRF . . . . . monoclonal rheumatoid factor immunoglobulin

mRNA . . . . . messenger ribonucleic acid

$MgCl_2$ . . . . . magnesium chloride

M1,M2 . . . . . influenza virus matrix protein

NA ..... neuraminidase

NaCl ..... sodium chloride

NaOAc ..... sodium acetate

NaOH ..... sodium hydroxide

NH_4Ac ..... ammonium acetate

NH_4Cl ..... ammonium chloride

NP ..... influenza virus nucleoprotein (in introduction)

NP ..... 4-hydroxy-3-(nitrophenyl) (in chapters 2,3 and discussion)

NS1,NS2 ..... influenza virus non-structural proteins

PA ..... acidic influenza virus polymerase protein

PB1,PB2 ..... basic influenza virus polymerase protein

PBS ..... phosphate buffered saline

PC ..... phosphorly choline

PCR ..... polymerase chain reaction

PEC ..... peritoneal exudate cell

PEG ..... polyethylene glycol

p.f.u. ..... plaque forming units

phOx ..... phenyl oxazolone

PKC ..... protein kinase c

PNK ..... polynucleotide kinase

PR8 ..... A/Puerto Rico/8/34 influenza strain

RAG ..... recombination activating genes

RIA ..... radioimmunosorbent assay

RNA ..... ribonucleic acid

RNAsin ..... placental RNAse inhibitor

RP ..... virus haemagglutination reactivity pattern of immunoglobulin

RT ..... reverse transcription

RTmix ..... reverse transcriptase mix

SDS ..... sodium dodecyl sulphate

SFA ..... BALB/k B-cell hybridoma cell lines and Mab

$S_{isotype}$ ..... class switching recognition sequence

TBE ..... tris-borate-EDTA buffer

Tc ..... cytotoxic T cell

TCR ..... T-cell receptor

TdT ..... terminal deoxynucleotidyl transferase

T_H ..... helper T cell

TMV ..... tobacco mosaic virus

TNF ..... tumour necrosis factor

TRBC ..... turkey red blood cells

TSK ..... tight skin mice

Tween 20 ..... polyoxyethylene-sorbitan monolaurate

u ..... unit

UV ..... ultraviolet

V ..... variable

V_H ..... immunoglobulin heavy chain variable region

V_L ..... immunoglobulin light chain variable region

V_κ ..... immunoglobulin kappa light chain variable region

V_λ ..... immunoglobulin lambda light chain variable region

vRNA ..... viral RNA

X31 ..... influenza virus with H3N2 external proteins and PR8 (H1N1) internal proteins

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GENERAL INTRODUCTION

A1. Immune recognition

The generation of antibodies reactive with antigen of an invading organism represents a major mechanism of defence against infection. The humoral immune response has developed to allow tremendous diversity in antibody specificities generated for protection against as wide a range of pathogens as possible. Nevertheless, production of antibody is tightly controlled to avoid generation of harmful autoreactive antibodies. B-cells generate a potentially infinite array of antibody specificities, whereas T-cells regulate secretion of antibodies and limit the probability of an autoimmune response.

Surface Ig (sIg) specificity is generated at random in the first instance, so it is quite possible for B-cells to express sIg reactive with self antigen. Similarly, T-cell receptors are also generated with random specificity, but these undergo extensive selection in the thymus to ensure self reactive T-cells are not released into circulation. The immune system thus avoids potentially damaging autoimmune antibody production because to respond to thymus dependent antigen, i.e. most protein antigens, a B-cell must interact not only with the antigen but also with T-cells specific for the same antigen (Noelle & Snow, 1990).

Interaction of B and T-cells requires the antigen to be processed and presented by B-cells in a form recognisable by T-cells, Thus lack of self reactive T-cells can directly limit autoreactive B-cells, and if B-cells encounter antigen in the absence of suitable T-cells, they may become anergic as a secondary mechanism for maintenance of B-cell tolerance. There is therefore an integral relationship between the immune repertoire of B and T-cells that is often overlooked by investigators concerned with the more defined aspects of cellular and molecular immunology.

Gell and Benacerraf were the first to show in 1959 differences in reactivity to antigen by cells of the immune system, that is, B-cells, recognising conformational determinants (humoral antibody) and T-cells, being able to recognise denatured proteins (cell mediated immunity), before anything was really known about the two classes of cells (Gell & Benacerraf, 1959). It has since been established that T_H cells only recognise

processed antigen as peptides on the surface of antigen presenting cells (APC), in association with MHC class II molecules (human HLA-DR or murine H-2 (I-A or I-E)), generally recognising continuous determinants presented as peptide fragments sitting in the MHC groove after processing (Schwartz, 1985; Mills *et al.*, 1986a, b; Burt *et al.*, 1989). It has therefore been assumed that the T-cell repertoire is far more restricted (by MHC), recognising a limited number of antigenic sites of a foreign protein, whereas antibody interacts with all surface accessible regions of a globular protein.

A2. B-cell development

Differentiation of stem cells to antigen specific B-cells involves modification of germline V-genes and subsequent selection of combinatorial variants. The selection pressure for this is not foreign antigen since this takes place before encounter with antigen (Jerne, 1971). Pluripotent stem cells differentiate into proB cells, then preB1 cells. Immunoglobulin D_H-J_H gene rearrangement occurs at this stage on both chromosomes resulting in one productive and one non-productive rearrangement (allelic exclusion) which ensures that a single type of functional antibody with two identical light and two identical heavy chains is expressed by each B-cell (Early & Hood, 1981). PreB1 cells become preB2 cells in which V_H genes rearrange to D_HJ_H , then preB3 (V_κ-J_κ rearrangement) and preB4 (V_λ-J_λ rearrangement) and finally, after immunoglobulin (Ig) gene assembly is complete, resting B-cells (antigen independent phase).

Resting B-cells circulating in the blood migrate across high endothelial venules to the secondary lymphoid organs (spleen, lymph nodes) where entrapped antigen is held (Clark & Ledbetter, 1994). B-cells with Ig specific for the entrapped antigen enter the T-cell rich paracortical region of lymphoid tissues and have the capacity to process and present antigenic fragments. Some T-cells may become activated here after recognising antigen presented by such APC in association with MHC. Following encounter with antigen and activated T-cells, B-cells migrate to B-cell follicles where they interact with follicular dendritic cells (FDC) which are thought to specialise in binding antibody-antigen complexes. B-cell proliferation and differentiation leads to dark zone formation in the germinal centre (there are no T-cells here).

Maturing B-cells move to the light zone (T-cells and FDC present here). In the basal light zone, V genes undergo somatic mutation to increase affinity for antigen within days of antigenic stimulation and only during germinal centre formation. FDC in the germinal centre have Fc receptors on their surface which bind antigen complexed with antibody and present antigen in an unprocessed form to antibody (B-cell receptor) (Berek & Ziegner, 1993). This is necessary for differentiation of B-cells into B memory cells in the apical light zone and important in long term maintenance of B memory. Interestingly antigen is present in an undegraded form on FDC for months post immunisation.

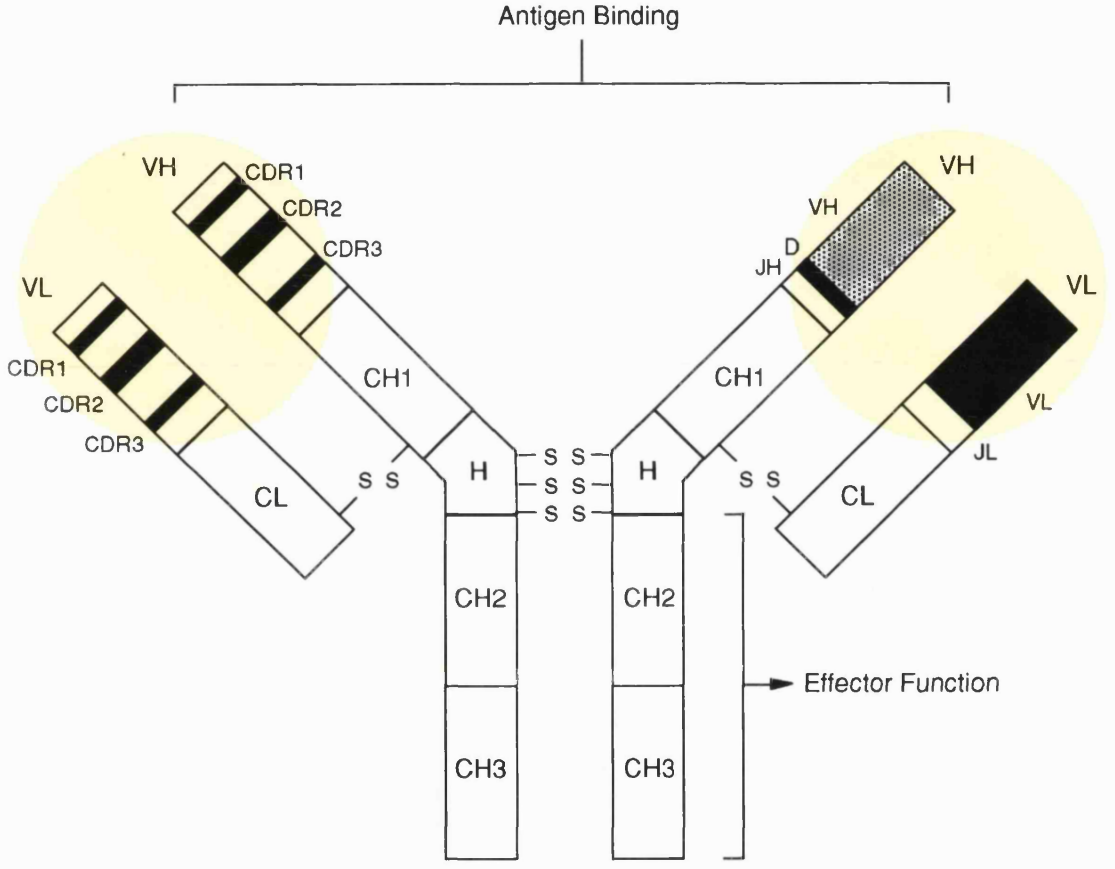
FDC induce somatic mutation of Ig V-region genes in B-cells and subsequent selection of high affinity antibody as well as their role as antigen depots, while germinal centre signals preventing B-cell apoptosis allow time for this to happen. B-cells expressing high affinity antibodies are selected to undergo class switching. Activated B-cells, $CD4^+$ T-cells and FDC are required for class switching. Development of humoral immunity involves quantitative and qualitative changes in pre-existing antibody by the two completely independent processes of class switching and somatic mutation during clonal expansion (Manser, 1989). These germinal centre B-cells eventually mature into memory B-cells and plasma B-cells (Ab secreting cells) in response to signals in the apical light zone.

A3. The antibody molecule

The B-cell surface immunoglobulin receptor consists of two identical heavy (H) chains and two identical light (L) chains held together by disulphide bonds to form a Y-shaped structure with two identical antigen binding sites at the variable end of the molecule (Figure 1). Genes encoding the Ig variable regions are assembled from multiple germline segments. There are two possible light chains, κ and λ. Kappa chains usually assemble in 95% of cases. Lambda assembly occurs only when κ fails. Kappa light chains are made by rearrangement of one of 300-1000 variable (V) genes, one of four joining (J) genes and a constant (C) gene all on chromosome 6 in mouse. Lambda genes are on chromosome 16. Heavy chains are formed by rearrangement of V, J, D (diversity) and C genes present on chromosome 12. There are over 300 V_H genes arranged into nine families based on sequence homology, approximately 16 D_H genes in three families, 5 J_H and 8 C_H genes.

Figure 1: Schematic representation of an IgG-type antibody molecule.
Adapted from Rathburn *et al.* (1989).

The antibody molecule consists of two identical light chains disulphide linked to two identical heavy chains. The antigen binding domain is shown as the yellow region at the tips of the molecule. This region (Fv) is the most variable part of the antibody, encoded by VJ_L and VDJ_H immunoglobulin genes. Diversity arises in this region by somatic hypermutation, particularly in the CDR regions "mutation hot-spots" (CDR1 and CDR2) and by junctional diversity at the V-(D)-J joining region, CDR3. The constant regon of the heavy chains is responsible for effector function and isotype (encoded by C_H).


Antigen Binding
VH
CDR1
CDR2
CDR3
VL
CDR1
CDR2
CDR3
CH1
CL
S S
H
S S
S S
S S
CH2
CH3
VH
VH
D
JH
VL
VL
JL
CH1
CL
S S
H
CH2
CH3
Effector Function

Heavy chain assembly occurs first with random rearrangement of one D to one J gene, then one V gene to the DJ segment, followed by VDJ to C. Once a functional H chain is expressed (all are $C\mu^+$ to begin with, prior to class switching), a light chain is assembled (Rathburn *et al.*, 1989). 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 the combination of any L to any H chain, a potential 6.4 million different possible antibodies (excluding junctional region diversity and somatic mutation) (Paige *et al.*, 1978; Perry & Kelley, 1979; Alt *et al.*, 1982; Parslow & Granner, 1982). So, there are five ways in all in which diversity of antibody specificity can be generated. These are, multiplicity of distinct germline V_H or V_L sequences encoding different amino acid sequences, combinatorial assortment of VDJ sequences, joining region junctional diversity, somatic mutation and finally, H-L pairing.

Antibody gene assembly occurs in B-cell precursors (antigen independent phase), and results in a multitude of independent novel B lymphocyte clones each expressing one type of antibody molecule limiting B-cell clones to functional assembly and expression of a single L chain and a single H chain V region gene (allelic exclusion) (Early & Hood, 1981). Specificity of the antibody repertoire depends on antigen activation of a specific subset of the available clones, primarily by binding of antigen to the antibody molecule of "best fit", and then affinity maturation.

Antibody specificity is determined by hypervariable regions within the variable portion of the antibody molecule. The variable regions of H and L chains consist of framework regions interrupted by three hypervariable regions:- the complementarity determining regions, CDR 1-3 (Wu & Kabat, 1970). The hypervariable regions of the H and L chains interact in the three dimensional structure of antibody to form the antigen binding pocket. CDR 1 and 2 are encoded by V genes, and CDR 3 at the junctional region of combination of V-(D)-J (Poljak *et al.*, 1974).

A4. Mechanism of immunoglobulin V-region assembly.

(Poljak *et al.*, 1974; Tonegawa, 1983)

The rearrangement of V region genes is a controlled process. Variable region genes have specialised recombination recognition sequences. Each V gene is followed by a highly

conserved palindromic heptanucleotide sequence CACAGTG (or analogue) and a less conserved AT rich nonameric sequence ACAAAAACC (or analogue) separated by spacer sequences of 12 or 23 bases (equivalent to one or two turns of the helix). J and D genes are preceded by the nonamer GGTTTTTGT and heptamer CACTGTG, both of which are complementary to the V gene recognition sequences.

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 both 5' and 3' of D_H ensure D joins to J, then V to DJ. Direct V-J joining is not observed at a significant frequency due to the 12/23 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.

It is possible that these sequences are targets for 7-mer/9-mer specific proteins during recombination. Rearrangement is imprecise and addition or loss of nucleotides is common to IgH chains (and also T-cell receptors which assemble in a similar fashion). Nucleotide addition (N-region addition) is characteristic of terminal deoxynucleotidyl transferase (TdT) activity, so these recognition sequences may be targets for Ig recombinases. Nicks are made by TdT at the end of the signal directly adjacent to the 7-mer and two signal ends join. Nucleotides are added or excised between coding ends prior to joining.

Although TdT is integral in VDJ recombination, it is not an obligate participant since VDJ recombination can occur efficiently in its absence (Landau *et al.*, 1987). However, VDJ recombination only takes place in cells expressing the RAG-1 and -2 locus recombination activating genes. RAG locus expression only occurs in the lymphoid lineage, and the cerebellum and hippocampus of the brain. RAG-1 expression correlates precisely with recombination activity (Oettinger *et al.*, 1990), and RAG-1 mRNA is detectable only in early stages of T and B-cell development when VDJ recombination occurs (preB cells). Mice lacking RAG-1 or -2 (SCID) suffer from severe immunodeficiency disease due to their inability to initiate VDJ recombination in developing T and B-cells, providing evidence that RAG-1 (and RAG-2) expression is essential for recombination.

Once VDJ recombination has occurred, these join to the C region gene, initially to the most J_H proximal which is Cμ, so during early B-cell development, the cells always

express surface or membrane IgM which facilitates light chain gene assembly and expression. Tsubata *et al* (1992) suggested membrane μ (μ m) expressed by preB cells in association with surrogate L chains λ_5 and V_{preB} , and CD3-like proteins signal the onset of L chain rearrangement, but μ m expression may not be absolutely required. Cross-linking of μ m to λ_5 and V_{preB} on the preB cell surface may generate a signal that activates V_κ rearrangement. This complex in association with Igα and Igβ 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 suppresion of further V_H rearrangement and enhancement of Ig κ locus rearrangement. Similarly, μ^+ preB cells require interaction with stromal cells to undergo the transition to L chain assembly.

PreB cells synthesise μ H chains but not κ or λ L chains therefore complete IgM molecules are not expressed. μ chains are retained in the endoplasmic reticulum and degraded. 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, the B-cell allows IgM to move to the Golgi and undergo glycosylation processing and export to the cell surface.

Class switching follows VDJ-Cμ recombination. Five prime of each C_H gene there are S regions (switch) which are tandem repeats, for example, for Sμ $[(GAGCT)_nGGGGT]_m$, where n = 1-7 and m = up to 150. First Sμ and one other S region recombine and then a possible second switch occurs from, for example, Cγ to Cε or Cα downstream since the intervening DNA is deleted by looping out or splicing. This then gives the final isotype of the antibody.

A5. Antibody affinity maturation.

Following maturation of B lymphocytes and Ig gene rearrangement in the germinal centre, B-cells of the primary immune response to antigen are thought to randomly diversify their Ig genes by somatic hypermutation after which high affinity variants are selectively preserved as B memory cells for the response to secondary antigenic challenge (MacLennan & Gray, 1986; Nossal, 1992). B-cells carrying low affinity receptors are eliminated by apoptosis. Maturation requires that antigen binding sites in the matured repertoire are

structurally different from the primary response. There is often a shift in the repertoire of germline genes used in the primary and memory response (Kim *et al.*, 1985; Riley *et al.*, 1989; Hiernaux *et al.*, 1990; Nicoletti *et al.*, 1991; Berek & Ziegner, 1993; Yang *et al.*, 1994) as well as accummulation of mutations.

Changes in antibody gene usage have been observed in aged BALB/c mice in the T15 antibody response to phosphoryl choline (PC) (Yang *et al.*, 1994). T15 idiotype is shared by a series of BALB/c IgA/κ myeloma proteins derived from plasma cell tumours induced in BALB/c mice by *intraperitoneal* immunisation with mineral oil or pristane (Potter & Leiberman, 1970). These include MOPC 299, S63, S107, TEPC-15 and HOPC-8, all with binding affinity for PC. This idiotype is also found on all PC-specific antibodies generated by immunisation of BALB/c mice with S.pneumoniae R36a (Pn) strain or PC conjugated to a carrier protein (Eichmann, 1975). These antibodies resemble TEPC-15 in fine specificity (Mackenzie *et al.*, 1989) and are identical in H and L chain sequence to TEPC-15 (T15). This idiotype is controlled by at least two unlinked genes, V_H 1 (S107) and V_κ 22 (also D_HFL16.1, J_H1 and J_κ5).

In 2-4 month old mice greater than 85% of B-cells are $T15^+$, using V_H1 (S107 family) and V_κ22. At the age of 20-24 months, 35-85% are $T15^+$. $T15^+$ B-cells represent a minor population of less than 50%. The rest are C_μ^+ $T15^-$ and with loss of V_κ22 expression. $T15^+$ PC reactive B-cells in aged mice respond to antigen but do not dominate the response due to an intrinsic molecular change in aging B-cells (Riley *et al.*, 1989; Nicoletti *et al.*, 1991). Young mice used genes from the V_H family S107, old mice used genes from V_H families 7183, J558, X-24 or S107 and V_L genes other than V_κ22, and peripheral T-cells may play a role in shaping the antibody repertoire (Kim *et al.*, 1985).

In the C57BL/6 antibody response against nitrophenyl (NP), antibodies using heavy chain V_H 186.2 and light chain V_λ1 genes dominate the early primary response. Somatic mutation in clonotypes was observed as early as six days after primary NP immunisation (Yokochi *et al.*, 1982; Cumano & Rajewsky, 1986). The mutational change in hypervariable region CDR1 of tryptophan at position 33 to leucine increased antibody affinity for NP tenfold. This change was found in 75% of late primary and B memory clones. There was also 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 antibody 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; Jacob *et al.*, 1991).

A6. Antibody function as an effector molecule.

The X-ray crystal structure of antibody has resolved several questions concerning the structure and function of antibody-antigen complexes. Fine specificity of antibody is determined by the six hypervariable CDR loops (3 in H chains and 3 in L chains). Antigen may, or may not be in contact with all six (Davies *et al.*, 1990; Wilson & Stanfield, 1993), but any of the six are important. Padlan (Padlan & Kabat, 1991) showed that, in the 3D structure of an antigen-antibody complex there was no contact of antigen with residues 59-65 in CDR 2 of the heavy chain (CDR 2 consists of V_H reisues 50-65). Specific framework region residues can have a strong influence on antigen binding, by indirect or long range effects as shown by Brummel (1993), by changes in CDR 3H which affected affinity of binding of antibody to Salmonella serogroup B O-polysaccharide, and Chien (1989) by changes in J_H1 Asp→Ala101, 9Å from the PC binding pocket affecting binding.

Poljak (1991) found using HEL specific antibody and E.coli expressed V_HV_L , that neither antibody nor antigen undergo conformational change in their complex. Even though there are some very small changes, the tertiary structure is preserved. Small molecular movements occur which allow induced fit of antigen to antibody. The contact site of antigen and antibody is between fourteen amino acids from each covering an area of 60-70 nm, with 2-3 water molecules at the interface, held together by H-bonds and van der Waals forces. Single amino acid changes in either antibody or antigen can abrogate recognition by steric hindrance, preventing antigen entry into the antibody cavity, even by long range effects such as changes in framework regions, hence defining fine specificity of antibody.

Membrane Ig functions as a signal transducer to switch resting B-cells into cell cycle. Antigen is bound by antibody and this cross-linking in Ig results in increase in intracellular Ca^{2+}, hydrolysis of phosphatidyl inositol and the activation of protein kinase C. Tyrosine phosphorylation, also as a result of Ig cross-linking, is necessary for subsequent internalisation of membrane Ig-antigen complexes, processing, and presentation of peptides with MHC to T-cells (Pure & Tardelli, 1992).

Because Ig possess at least two identical antigen binding sites (10 for IgM), antibodies can bind two identical particles, made possible by flexibility about the hinge, and cause them to approach each other, an interaction usually counteracted by identical charge repulsion. The size of antibodies allow combining sites to be 10 nm (IgG) to 35 nm (IgM) apart, minimising such effects due to the exponential fall-off of charge with distance.

The antibody function of binding antigen is important in inactivation of toxins and prevention of penetration of bacteria, parasites and viruses into host cells, but also for cross-linking functions which activate the effector system *via* Fc and the activation of B and T lymphocytes by signal transduction.

Other functions of antibodies include activation of the complement cascade system. C1 can dissociate into its components, C1q and a tetramer of two C1r and two C1s. C1 is activated by antibody which binds the globular head of C1q. Upon activation, C1r undergoes limited cleavage and is transformed into a protease which cleaves C1s into a protease, and so the cascade begins. Antibody activates the C1 structure *via* the Fc portion. C1q can discriminate between aggregated and unaggregated Fc (IgG). Aggregated Fc activates C1 even after it has been proteolytically separated from the antigen combining sites of the antibody molecule. Increase in avidity of Fc region for the globular head by multivalent binding is a contributing factor as to why immune complexes in the presence of 1000 times greater quantities of uncomplexed antibody can activate the C1 structure, possibly by conformational change, on binding the globular head by antibody. It is possible that activation of C1 requires distortion of the stalks supporting the globular head and around which C1r and C1s are threaded (Metzger, 1986).

Mast cells and macrophages have Fc receptors on their surfaces which interact with immune complexes. Mast cells have FcR specific for IgE. Upon aggregation of cell bound Ig by multivalent antigen, the cells are stimulated to release preformed mediators and synthesise other potent products. Antibody confers specificity in this system. FcR for IgE is the initiating molecule for secretory events, and stimulation of phosphatidyl inositol turnover and influx of Ca^{2+} correlate perfectly with aggregation of these receptors.

Macrophages have different FcR types on their surface. Multivalent interactions with immune complexes are possible and contribute to the capacity of the cells to discriminate between immune complexes and the enormously higher concentrations of free antibody.

This enhanced binding can account for rapid internalisation of immune complexes because FcR spontaneously cycle between the cell surface and the interior (Suzuki *et al.*, 1982; Young, J. D. E. *et al.*, 1983). It has been suggested that FcR form ion channels in the cell membrane when combined with immune complexes, and have intrinsic phospholipase A_2 activity, although the relationship of these to activation of the cell remains to be clarified.

A7. T-cell recognition: the T-cell receptor.

B-cells and T-cells are morphologically similar and derived from common haematopoietic stem cell precursors. The B-cell molecule for specific recognition is immunoglobulin, either surface associated or secreted. The T-cell recognition molecule is membrane bound T-cell receptor (TCR), which recognises foreign antigen as peptides in association with self-MHC.

TCR is a disulphide linked glycoprotein heterodimer, non-covalently associated with at least five other polypeptides on the cell surface (Marrack & Kappler, 1980). Antigenic specificity is determined by clonally expressed heterodimers of α and β chains in most T-cells (or γ and δ chains) and is structurally similar (at the nucleotide level) to immunoglobulin H and L (Kronenberg *et al.*, 1986). Each polypeptide consists of seven regions, namely, an N-terminal hydrophobic leader region of 18-29 amino acids, a membrane distal V segment of 88-98 amino acids, a J region (14-21 amino acids), C region (87-133 amino acids), a connecting peptide of variable length, a transmembrane region (20-24 amino acids) and a small cytoplasmic tail of 5-12 amino acids. TCR $(VJD)_\beta$ and $(VJ)_\alpha$ are homologous to Ig V regions, and TCR C_α and C_β are homologous to Ig C regions. The remaining portion of TCR is in a group of three invariant dimers, CD3, involving γ, δ, ϵ, ζ and η chains physically linked at the cell surface to TCR $\alpha\beta$, and TCR-CD3 association is a prerequisite for cell surface expression (Moretta *et al.*, 1984).

The 3D structure of TCR has been modelled on immunoglobulin X-ray crystal structure (Kronenberg *et al.*, 1986) and by comparison of Ig and TCR primary sequences. The secondary and tertiary structure of TCR has been built up from primary sequence homology to Ig.

A large number of sequences of TCR have identical or similar residues at homologous sites to 40 amino acids crucial in the Ig molecule for conserving the structure of the V domain and association with the C domain. Sequence data suggests the TCR V region is folded into β-sheet structures resembling Ig V regions. The V domain of V_H and V_L consists of a framework of β-pleated sheets. Each domain has three loops (CDRs), linked to the β-sheets and adjascent to each other in space. TCR V region loops forming the antigen binding site are of a similar size, but not conformation to Ig antigen binding sites, and lack framework region residues in this region.

A8. Structure of MHC.

It is widely accepted that T-cells only recognise denatured protein in the context of self-MHC. MHC is a family of polymorphic cell surface glycoproteins which occur in two distict "forms", class I (H-2 in mice, HLA-A,-B and -C in humans) and class II (Ia in mice and HLA-D in humans), which present peptides to $CD8^+$ and $CD4^+$ T-cells respectively (Schwartz, 1985).

Recent resolution of the 3D structure of the human class I MHC molecule (HLA.A2) has identified the peptide binding site to be a cleft on the outer surface of the molecule between two α-helices.

The 3D structure of human class II, HLA-DR1 has recently been defined by Brown *et al* (1993) and found to be very similar to class I structure. A dimer of αβ can be seen, and Class II α_1 and α_2 superimpose closely on the corresponding class I α_1 and β_2m, and class II β_1 and β_2 on class I α_2 less closely. Both class I and II binding grooves consist of a floor of eight anti-parallel β sheets with two antiparallel α-helices at the sides. Location of bulges in the β structure and kinks in the α-helices are also similar.

Peptides bind to class I on the membrane distal ectodomain located between the α-helical regions of the polymorphic α_1 and α_2 domains, which are 18Å apart (as are CDR1 and CDR2 of Ig V_H and V_L), and at the base by β sheets from both domains (Bjorkman *et al.*, 1987a, b). In class II, peptide is bound in an extended conformation that projects from both ends of an "open-ended" antigen binding groove, and a prominant non-polar pocket into which "anchoring" peptide side chain fits, near one end of the molecule. When peptide

is present in the binding cleft, it is probable that the top surface is fairly flat as suggested by cocrystallisation of HLA-A2 with endogenous peptide, and changes in the residues around the peptide binding cleft could influence the conformation adopted by bound peptide or the orientation of peptide in the cleft (Mosley & Wyckoff, 1946; Bjorkman *et al.*, 1987a, b; Brown, J. H. *et al.*, 1988, 1993; Davis & Bjorkman, 1988).

A9. Antigen processing and presentation.

Processing of exogenous antigen involves endocytosis, partial proteolytic degradation and peptide binding to MHC class II. Endogenous antigen undergoes proteolysis, and then presentation and recognition bound to class I (Bjorkman *et al.*, 1987a, b). T-cell recognition of processed antigen expressed by macrophages and B-cells occurs after antigen catabolism which appears to be a prerequisite for antigen recognition by T-cells. For MHC dependent presentation of antigen, catabolism must be partial and antigen fragments recycled to the surface of APCs before complete degradation (Townsend *et al.*, 1986).

Class I α-heavy chain and β_2m associate in the endoplasmic reticulum (ER). This complex is unstable in the absence of peptide and is inefficiently transported out of the ER. Empty α-heavy chains may also be degraded in the ER. This complex is stabilised when peptide is present in the binding cleft and efficiently transported to the cell surface. Proteins synthesised within the APC and degraded in the cytoplasm are the primary source of class I charging but peptide in the cytoplasm may also get access to ER *via* specific transporters (Braciale & Braciale, 1991).

Class II α and β chains normally assemble in the ER with invariant chain (Ii) to form stable trimolecular complexes. Ii possesses a short internal segment that occludes or closes the binding cleft thereby inhibiting unproductive interactions with segments of intact protein and binding of peptide to α-β-Ii in the ER. These complexes are efficiently transported out of the ER and targeted to the post Golgi compartment. They then traffic to an endocytic compartment where Ii is proteolytically cleaved. Loss of a portion of Ii may allow peptide to bind and may then signal further cleavage of Ii, resulting in egress of peptide charged α-β complex to the surface. $\alpha\beta$ can assemble in the ER in the absence of

Ii and be transported to the surface, but it is not clear whether it can associate with peptide in the ER since $\alpha\beta$ lacking Ii may have an altered conformational state. Peptides charging class II are derived from exogenous protein proteolytically cleaved in endosomes, although some endogenous proteins also appear to be processed in the intracellular compartment (Germain, 1994).

The evolution of two separate pathways for antigen processing and presentation confers cellular advantage to APCs by allowing CTL to recognise and lyse infected cells in which protein is produced endogenously, such as viral gene products during infection, and prevents lysis of class II APC presenting foreign antigen to helper T-cells (Townsend *et al.*, 1985; Germain, 1986).

A10. Cognate T-B interaction.

Recent advances in recombinant DNA technology and crystallographic data for MHC (class I and class II) and the cell biology of antigen processing have provided us with a clear understanding of the elements of cellular co-operation between antigen, B and T-cells, resulting in lymphocyte activation, cytokine release and differentiation of B-cells into antibody secreting plasma cells.

There are two ways in which T-cells and B-cells interact: by direct physical contact *via* surface receptors and ligands, and by secreted factors (lymphokines), both of which are essential for an immune response.

(a) Cell co-operation *via* cytokines.

Distinct populations of $CD4^+$ T-cells exist in the mouse as characterised by production of different cytokines. Cytokines affect many immune cells, (and cells of other lineages) and each cytokine mediates several effects on a single target cell. T_H cells can be divided into two groups based on their pattern of cytokine secretion. T_h1 cells produce IL-2, IFN-γ and lymphotoxin (or TNF β), whereas T_h2 cells produce IL-4, IL-5 and IL-6, the functions of which are summarised in Table 1.

As an example of cell co-operation *via* cytokines , IL-4 produced by T-cells induces B-cell proliferation and differentiation (Rabin *et al.*, 1986; Mosmann & Coffman, 1989) and

an increase in surface MHC class II expression (Reynolds *et al.*, 1987; Noelle & McCann, 1989), an example of T-cell activation of B-cells. Resting B-cells are however only responsive to the effects of lymphokines when entering cell cycle, induced after physical T-B interaction (Bartlett *et al.*, 1989), hence close proximity of T_H to B-cells is required for T_H dependent B-cell activation by lymphokines (IL-2 dependent and independent phases) and secretion of interleukin is directed towards the site of contact with the B-cell (Poo *et al.*, 1988). After class II restricted antigen specific interaction with B-cells, T_H become activated and reciprocally bind to and activate B-cells *via* non-restricted mechanisms. Activated T-cells express more CD3 and trigger lymphokine release which activates B-cells (Bartlett *et al.*, 1990; Noelle *et al.*, 1991).

It has been suggested that T_h0 cells are the precursors for T_h1 and T_h2 mature T-cells, but this has yet to be demonstrated. Different T-cell types predominate in different responses, for example, in mice immunised with Nippostrongylus brasiliensis, increased IL-4 dependent polyclonal IgE levels and IL-5 dependent eosinophilia are observed and there is an increased tendency to isolate T_h2 clones, whereas with Brucella abortus, T_h1 clones are exclusively isolated from infected mice (O'Garra, 1993).

T_h1 cells predominate in viral infections, but CTL also produce cytokine patterns similar to T_h1 cells. Viral infections result in increased CTL activation which may favour T_h1 activation, leading to IFN-γ and lymphotoxin production, both of which have antiviral activity. Studies on infectious murine cytomegalovirus suggest IFN-γ itself does not directly control replication of the virus but mediates its protective effects by upregulation of class I and II expression, which are essential for T-cell recognition.

Coutelier *et al* (1987), studying antibody isotype profiles in three different strains of mice, CBA/Rj, 129/Sv and C57BL/6, in response to a panel of eleven RNA and DNA viruses, including mouse adenovirus, polyoma, sindbis and sendai viruses, found a predominance of IFN-γ induced complement-fixing cytotoxic IgG2a antibodies (65-92% of total antibody) in all three strains and to all eleven viruses, elicited by both *intranasal* infection and *intraperitoneal* immunisation, in contrast to the IgG1 responses to soluble proteins and IgG3 to carbohydrates in parallel control studies. This distinct isotypic profile is thought to be related to the viral infection and selective recruitment of T_h1 cells.

Table 1: Cytokine effector functions.

Th1 PRODUCED CYTOKINES:	EFFECTOR FUNCTION
IL-2	T-cell growth factor, induction of T-lymphocyte cytotoxicity and stimulation of NK activity.
IFN-γ	Anti-viral effects, enhanced phagocytosis by macrophages, selective induction of complement-fixing cytotoxic IgG2a and inhibition of Th2 cell type proliferation.
Lymphotoxin (TNF β)	Anti-viral and anti-parasitic effects, cell cytotoxicity, granulocyte differentiation, enhanced chemotaxis, phagocytic and cytotoxic activities of macrophages and induction of IL-1, IL-6 and GM-CSF production.
Th2 PRODUCED CYTOKINES:	EFFECTOR FUNCTION
IL-4	B-cell activation, upregulation of MHC class II and FcεR on resting B-cells, costimulation for B-cell proliferation and induction of Ig class switching to IgG1 and IgE.
IL-5	Eosinophil and B-cell growth and differentiation factor and induction of maturation of B-cells to IgA secretion.
IL-6	B-cell stimulatory factor, controls growth and proliferation of haemopoietic progenitor cells and costimulation of thymocytes and mature T-cells.
IL-10	Inhibits cytokine production by Th1 cells, up-regulation of MHC class II on resting B-cells, costimulation of mast cells, thymocytes and mature T-cell growth.

(b) Cell co-operation *via* direct physical contact: receptors and ligands.

Specific recognition of antigen by surface Ig induces B-cells to proliferate and differentiate into antibody secreting plasma cells, or memory cells. This process requires help from activated T-cells and is dependent on the interactions between T-cells and specialised APCs (which can themselves be B-cells). Activated T-cells promote B-cell activation by T-cell derived cytokines, as discussed previously, or direct intercellular contact . The molecular details of the way in which these two cell types interact have only recently become apparent.

Bone marrow derived dendritic cells are very effective at presenting MHC class II bound peptides to $CD4^+$ T-cells as they express high levels of class II and CD40, and also the key accessory molecules CD54 (ICAM 1), CD58 and CD80, formerly B7. Although affinity of TCR for MHC class II bound peptide is low (Matsui *et al.*, 1991), the CD4 and CD2 coreceptors on T-cells help signalling through the receptor, after binding to class II and CD58 (LFA-3), their respective receptor ligands on dendritic cells.

Cross-linking of TCR and associated coreceptors leads to rapid activation of the CD11a/18 (LFA-1) complex (Dustin & Springer, 1989), which can then bind to ligands such as CD54. Resting B-cells lack these receptors, but all three are expressed by bone marrow derived dendritic cells, and can therefore activate naive T-cells whereas resting B-cells cannot. Once B-cells are activated by T-cells and FDC, they express an array of surface receptors and can present antigen to T-cells effectively.

Cell surface adhesion molecules such as integrins (CD11a/18), selectins and CD58/59 are broadly distributed and likely to affect migration and adhesion of lymphocytes in general, and modulate lineage specific signals. Other molecules are restricted to APC, T or B-cells and must therefore function in processes unique to lymphocyte maturation, for example CD40 and CD80, which are neither integrins or selectins. These bind coreceptors whose levels are affected by the state of the T-cell expressing them, so receptor-ligand pairs allow T and B-cells to "sense the state of their partners activation".

An example of a receptor-ligand pair involved in T-B cellular co-operation is CD40 and its ligand CD40L, through which T-cells activate resting B-cells and to class switch from IgM to IgG, A or E antibody secreting cells. CD40 is expressed on late preB cells in bone marrow, mature B-cells and accessory cells (FDC and bone marrow derived dendritic

cells). Cross-linking CD40 promotes B-cell proliferation (Liu *et al.*, 1992), prevents apoptosis of germinal centre B-cells (Clark & Ledbetter, 1986) and promotes Ig class switching (Jabara *et al.*, 1990). Its ligand, CD40L, alternatively known as gp39, sc8 or TRAP, is expressed only on activated T-cells and enables B-cells to respond to activated T-cells (Armitage *et al.*, 1992; Hollenbaugh *et al.*, 1992) but it is not clear where these interactions occur. They can theoretically take place either in the T-cell zone or basal light zone.

X-chromosome linked genetic defects in the gene encoding CD40 ligand on T-cells have been reported which result in a condition known as hyper-IgM syndrome (HIM). Because of the defective CD40L, T-B contact *via* CD40/CD40L fails to produce the full complement of antibodies needed to fight off pathogens and only IgM is produced because of failure to initiate class switching (Marx, 1993). Interestingly, HIM patients suffer from infections such as Pneumocystis carinii, prevalent in T-cell deficiencies e.g. A.I.D.S. (Notarangelo *et al.*, 1990).

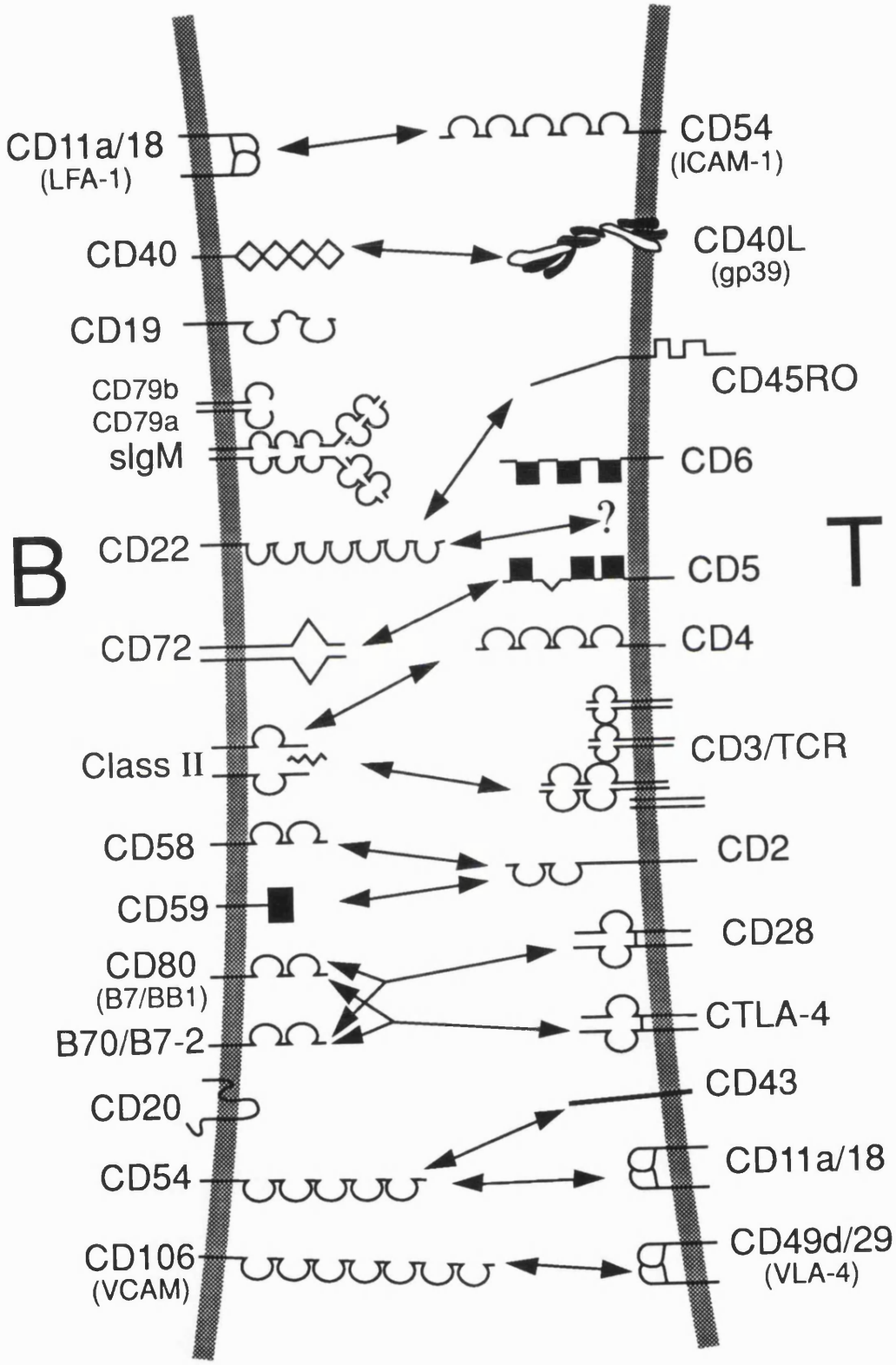
A reciprocal example is CD80-CD28/CTLA-4 interaction. This allows peripheral T-cells to respond to activated B-cells by dividing and producing cytokines required for T-cell differentiation (Yokochi *et al.*, 1982; Freeman *et al.*, 1989). CD28 is present on both resting and activated T-cells (Linsley *et al.*, 1990) but CTLA-4 is present only on activated T-cells (Linsley *et al.*, 1991). Interference with CD80-CD28 interaction can prevent T-cell proliferation (Linsley *et al.*, 1990) and B-cell maturation induced by T-cell derived cytokines.

CD80 expression is induced after cross-linking MHC class II, allogeneic T-cell interactions with bone marrow derived dendritic cells, and during autoreactive T-cell interaction with B-cells (Nabavi *et al.*, 1992; Young, J. W. *et al.*, 1992), therefore interactions with T-cells which cross-link MHC may thus induce CD80 expression on B-cells so that CD80 can in turn signal to T-cells *via* CD28 (Nabavi *et al.*, 1992). CD80 expression may coincide with CD40L expression on T-cells responding to MHC class II bound peptides (Nabavi *et al.*, 1992).

Signals delivered by both CD28 and CD40 promote cell proliferation *via* antigen receptor cross-linking. These are more efficient after stimulation through the antigen receptor, thereby ensuring that the interaction is specific, and activation of self-reactive or

Figure 2: Receptor-ligand pairs on T and B lymphocytes.

Cognate interaction between T and B-cells via these receptors and ligands initiates both T and B-cell activation. **Adapted from Clark and Ledbetter, 1994.**


CD11a/18
(LFA-1)
CD54
(ICAM-1)
CD40
CD40L
(gp39)
CD19
CD45RO
CD79b
CD79a
sIgM
CD6
?
B
CD22
CD5
T
CD72
CD4
Class II
CD3/TCR
CD58
CD2
CD59
CD28
CD80
(B7/BB1)
CTLA-4
B70/B7-2
CD43
CD20
CD54
CD11a/18
CD106
(VCAM)
CD49d/29
(VLA-4)

bystander cells is prevented (June *et al.*, 1990). CD28 cross-linking prevents specific unresponsiveness or apoptosis that would otherwise occur on stimulation of TCR (Groux *et al.*, 1992; Harding *et al.*, 1992). Similarly, signals delivered by CD40 on contact with T-cells can prevent apoptosis of immature B-cells normally induced by cross-linking their surface Ig receptors (Tsubata *et al.*, 1993).

CD40-CD40L and CD80-CD28/CTLA-4 receptor-ligand pairs are not the only means by which T-cells and B-cells interact. There are many known receptor/ligand pairs involved in T-B interaction. These include TCR/MHC, LFA-1/ICAM, CD2/LFA-3, CD28/B7, Ly1/lyb2 (Springer, 1990) and B7.2/CD28 and CTLA-4 (Freeman *et al.*, 1993) as shown in Figure 2, and each pair of interacting cell surface molecules play an important role in collaboration between T and B-cells, affecting different stages in activation and differentiation.

A11. Protein antigenicity and immunogenicity.

(a) Factors intrinsic to the protein affecting immunogenicity.

Immunogenicity is defined as the potential to stimulate an immune response, humoral and/or cell mediated, which is influenced by the history and genetic attributes of the immunised animal. Antigenicity is defined as the ability to be recognised by a product of an immune response, i.e. antibody or T-cells. While most immunogens are also antigenic, a substance does not have to be immunogenic to be antigenic. Consider for example, haptens; these are small molecules which are not immunogenic alone, but when attached to an immunogenic carrier protein, will elicit antibody that can bind to the free hapten, therefore free hapten is antigenic without being immunogenic. What makes any protein immunogenic or antigenic? Can a region of a protein be called an immunogenic or antigenic site in its own right, or only with respect to the particular antibodies which bind to that site, or a host that can make such specific antibodies?

There are two currently held opinions on this: first, that certain parts of a protein are inherently antigenic sites and that this property is intrinsic to the nature of the protein molecule and independent of the host to be immunised (Twining *et al.*, 1980, 1981). Intrinsic factors such as surface accessibility for antibodies to bind (Colman *et al.*, 1983),

hydrophilicity (Hopp & Woods, 1981, 1983), and segmental mobility of the polypeptide backbone (Westhof *et al.*, 1984) all contribute to antigenicity of a protein. Antigenic flexibility may also contribute to the ability of the immune system to provide defence against an enormous diversity of antigens. Specificity of primary antibody is genetically predetermined before antigen ever enters the system. Antigen can then activate and expand specific antibody. The second view is that virtually any accessible part of a protein is a potential antigenic site and the choice of site eliciting the immune response in a particular case depends largely on bias of the immune system of that particular host, determined by factors extrinsic to the protein (Benjamin *et al.*, 1984).

Antigenic sites fall into two structural categories, the segmental continuous type which is a linear sequence of amino acids that binds with a higher affinity in a preferred conformation (Berzofsky & Schechter, 1981), or the assembled topographic or discontinuous conformational type which consists of groups of amino acids, not necessarily contiguous in the primary sequence, but brought into close proximity by secondary structure (Sachs *et al.*, 1972).

The method used to study epitopes will determine whether linear continuous or topographic sites are observed. Crumpton *et al* (1965) studied the antigenicity of sperm whale myoglobin using synthetic peptides or proteolytic fragments and only detected segmental sites with polyclonal sera. This lead them to conclude that there were five antigenic sites on myoglobin of six or seven amino acids each, irrespective of the immunised species. With the advent of Mab technology and the use of native myoglobins of known sequence differences, the sites were found to be topographic and not in the regions delineated by synthetic peptides (Hurrell *et al.*, 1977; East *et al.*, 1980; Lando & Reichlin, 1982). Differences in affinity of binding of Mab were found for myoglobins from different species e.g. sheep or dog immunised with bovine myoglobin, depending on how similar the immunogen and homologous self protein were. Similarly for lysozyme C (Cecka *et al.*, 1976), cytochrome C (Nisonoff *et al.*, 1970), serum albumin (Kamiyama, 1977) and influenza HA and NA (Colman *et al.*, 1983), all of which have multiple (sometimes overlapping) determinants.

In influenza HA (and NA), the antigenic sites cluster at the membrane distal ectodomain of the molecules (Wiley *et al.*, 1981; Underwood, 1982; Colman *et al.*, 1983;

Staudt & Gerhard, 1983) and are considered to be four overlapping antigenic regions on HA. This lead to the multi determinant-regulatory hypothesis, which suggests that the surface of a protein is a continuum of potential antigenic sites, and selection of sites that elicit antibody production in an animal depend on a number of regulatory influences in the host, i.e. protein extrinsic factors affecting immunogenicity/antigenicity.

(b) Factors extrinsic to the protein affecting immunogenicity.

It is apparent from numerous studies that although a great deal of the surface of a protein is potentially antigenic, only a small subset of potential sites elicit an immune response (Ab) and this is due to host (extrinsic) factors. When differrent animals are immunised with the same antigen, e.g. sperm whale myoglobin, they elicit an antibody response able to bind a particular peptide which cannot be bound by antibodies elicited by the same antigen in a different animal, strain or possibly individual (East *et al.*, 1980). So what are the host factors governing immunogenicity?

The immune system has the potential to make auto-antibodies but does not due to regulatory mechanisms, therefore if an immunogen is homologous to a self protein, it might be assumed that antibodies are directed to regions of the protein which are different to self. A rabbit immunised with horse or mouse cytochrome C generates antibodies against the side chain which differs between species since the backbones are essentially the same (Nisonoff *et al.*, 1970), thus tolerance to self limits the potential antibody response to a few sites.

MHC restriction might be a factor contributing to protein immunogenicity, and peptide antigenicity for the T-cell repertoire. The T-cell response to a protein will be restricted to processed peptides that can be presented by self MHC. This will be limited directly by peptide binding to the MHC groove (Fathman & Frelinger, 1983; Delisi & Berzofsky, 1985; Schwartz, 1985), and single amino acid substitutions e.g. 109Asp→Glu substitution in horse myoglobin compared to sperm whale myoglobin can abrogate binding and recognition by T-cells (Berkower *et al.*, 1982). This has also been observed in the T-cell response to influenza HA, where for example, a BALB/c T-cell clone which recognises peptide HA1 56-76 is not able to recognise an analogous peptide with a single substitution at amino acid 63 (D→N or Y) and any natural variant viruses with this substitution (A/ENG/69, PC/73, VIC/75, TEX/77, BK/79 or CN/84) (Thomas *et al*, 1989), similarly a

CBA/Ca clone, specific for peptide 68-83 is sensitive to a substitution at HA1 78 (Burt *et al.*, 1989; Barnett *et al.*, 1990).

In the case of antibodies, Ir genes act indirectly by influencing levels of specific T_H cells, which are essential for activation of B-cells and antibody production, therefore influencing site specificity of antibodies raised to protein antigens. B-cells bind specific antigen *via* surface immunoglobulin (sIg). If B-cell uptake of antigen is *via* receptor mediated endocytosis, an antigen-antibody complex may be processed rather than free antigen. Each B-cell with different sIg might process a different part of the protein complex and thereby sterically "protect" from proteolysis different antigenic domains. Thus each different B-cell might present a different peptide and co-operate with T_H cells of a different specificity (Cecka *et al.*, 1976; Adorini *et al.*, 1979).

In addition to Ir gene influence, antibody specificity may also be restricted by structure of the antigen binding site of the antibody molecule which is generated by Ig gene rearrangement and heavy and light chain pairing. Heavy chains are made by V-D-J recombination and light chains by V-J recombination, and both undergo somatic hypermutation (Bekoff *et al.*, 1982; Kawamura *et al.*, 1984), so that sufficient genetic diversity can be generated between strains to influence the specificity of antibody response to the same minimal antigenic determinant, or hapten, e.g. immunodominant antibody responses to 4-hydroxy-3-nitrophenyl acetyl group in BALB/c mice use V genes from one V_H family while antiboties from C57BL/6 use a different V_H family (Loh *et al.*, 1983) (but may focus the response of antibodies from different strains of mice to the same region of a protein).

A12. Influenza: A paradigm for infection-immunity studies.

Influenza virus is a useful model for studying the immune system. The complete sequence (Verhoeyen *et al.*, 1980) and 3D-structure (Wilson *et al*, 1981) of its surface immunogenic protein, haemagglutinin (HA) is known and a large panel of natural variants and laboratory mutants with changes corresponding to those featuring in antigenic drift (for

B-cell studies), as well as synthetic peptides corresponding to the entire HA sequence (for T-cell studies) are available.

A relevant animal model system also exists, namely the mouse, which can be infected with influenza giving pathological symptoms similar to those in the human population (Andrewes *et al.*, 1934; Francis, 1934; Loosli, 1949; Roth *et al.*, 1979). This provides us with a model system that allows dissection of the immune response to a natural infection and with which parallels can be drawn to the human response to the same infection, also providing us with the opportunity for haplotype studies and the effect of major histocompatibility complex (MHC) polymorphism on the selected immune repertoire, which is an important consideration for future vaccine design. This model has been used in the past to define both antibody and T-cell epitopes.

A13. Influenza virus

Influenza virus is an enveloped animal virus belonging to the Orthomyxoviridae family. It was one of the first viruses to be studied with the electron microscope (Taylor, A. R. *et al.*, 1943) and provided the first demonstration of viral assembly and budding from the cell membrane (Murphy & Bang, 1952).

Circulating influenza viruses are continually evolving in the population, altering predominantly their surface glycoproteins, but changes also occur in each of the eight RNA segments of the viral genome. Influenza viruses have a range of hosts and were first isolated from chickens in the early twentieth century, pigs in the 1920's, humans in the early 1930's, horses and ducks in the 1950's, terns in 1961 and a range of water fowl and other animals from 1974 to date (Easterday, 1975; WHO memoranda, 1980; Guo *et al.*, 1983). Influenza is divided into three types A, B and C, based on differences in internal nucleoprotein (NP) and matrix protein (Pereira, 1969; Schild, 1972) of which type A has a range of hosts as described above, type B occurs in man alone and type C in man and pigs. Type A influenza can be further subdivided into subtypes based on antigenic differences in their surface glycoproteins.

Influenza virions are pleiomorphic particles, later becoming roughly spherical with a diameter of 80-120 nm (Mosley & Wyckoff, 1946), although filamentous particles have

been reported (Chu *et al.*, 1949; Choppin *et al.*, 1960), predominating in newly formed and newly isolated strains, and in human infections for which the spherical form is preferentially selected following several passages through eggs. The virion consists of a helical nucleocapsid and an inner shell of matrix protein enclosed within a host derived lipid bilayer envelope in which virus encoded glycoprotein spikes 10-12 nm long are embedded.

A14. The Viral Genome

(Figure 3)

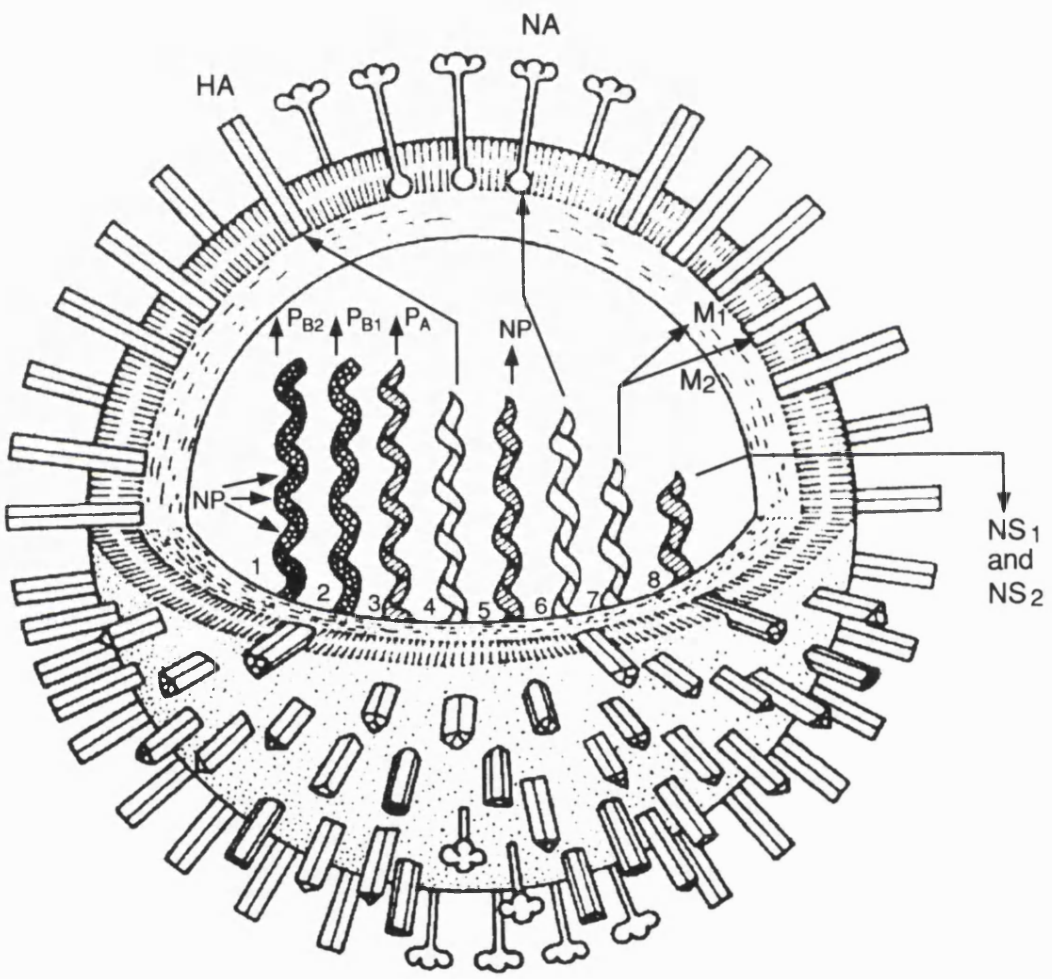
The influenza virus genome consists of eight unique segments of negative polarity single stranded RNA (Lamb, R. A., 1989) all of which are needed for infectious virus. These are loosely encapsidated by multiple NP molecules in a helix and complexes containing viral polymerase proteins are situated at the ends of the nucleocapsids. These eight RNA segments encode ten products: the RNA polymerases, structural and non-structural proteins, and the surface antigenic glycoproteins which will be discussed in detail later.

Segment 1 encodes PB2, one of the viral RNA dependent RNA polymerases. PB2 functions during initiation of viral mRNA transcription by recognising and binding to 5' cap I structures of host cell mRNAs for use as viral mRNA transcription primers. PB2 is also involved in endonucleolytic cleavage of caps. PB1 polymerase is encoded by segment 2 and is responsible for elongation of primed nascent viral mRNA and as an elongation protein for template RNA and viral RNA synthesis. The polymerases all function together as a protein complex with the third viral polymerase PA, encoded by segment 3. PB2 and PA polymerase are localised in the nucleus of infected cells, to where newly synthesized PB1 also migrates. The role of PA polymerase in viral RNA synthesis is unclear, but it may have possible protein kinase or helix-unwinding functions (Lamb, R. A., 1983).

Segment 4 encodes the viral surface glycoprotein haemagglutinin (HA). HA is responsible for virus binding to host cell receptors and fusion. It is also the influenza virus major antigenic protein. HA is a 75-80 kDa protein accounting for 24% of total virus protein with 600-930 molecules per virion (varying with different stains and subtypes).

Figure 3: Diagram of the influenza virus.
Adapted from (Oxford & 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 (see text). Haemagglutinin is present on the surface as a trimer and neuraminidase as a tetramer. Both undergo extensive mutation resulting in antigenic drift and shift. M2 is a memebrane protein which forms ion channels. The membrane and matrix shell of M1 protein enclose the viral RNA, nucleoprotein and polymerases.


NA
HA
P_{B2}
P_{B1}
P_A
NP
M_1
M_2
NP
NS_1
and
NS_2
1
2
3
4
5
6
7
8

It is synthesised as a single primary gene product, HA0, which undergoes three types of post-translational modification. First, it is cleaved to remove the 14-18 amino acid amino terminal hydrophobic signal sequence for transport to the cell membrane. Secondly, it is glycosylated, and finally palmitic acid is added to cysteines near the carboxy terminus. HA0 is then proteolytically cleaved by host produced trypsin-like proteases into two polypeptides, HA1 (50 kDa) and HA2 (28 kDa) (Lazarowitz *et al.*, 1971; Brand & Skehel, 1972) by removal of arginine at position 329. These two polypeptides are held together by disulphide linkage between amino acids HA1 14 and HA2 137 (Ward, 1981; Lamb, J. R. *et al.*, 1987). The cleavage of HA0 to HA1 and HA2 is important for infectivity since virus-cell fusion is mediated by the free amino terminus of HA2 (Klenk *et al.*, 1975; Lazarowitz & Choppin, 1975).

Segment 5 encodes nucleoprotein, NP, the second most abundant protein in the virion representing 17-26% of the total protein (Compans *et al.*, 1970; Skehel & Schild, 1971). Influenza type classification is partly based on NP. Newly synthesized NP is transported to the infected cell nucleus where it binds and encapsidates viral RNA (Pons *et al.*, 1969; Compans *et al.*, 1972). Other functions include switching viral RNA polymerase activity from mRNA synthesis to cell RNA and viral RNA synthesis, and NP is also the target for cytotoxic T-cell action. This protein contains no carbohydrate but is phosphorylated (Kamata & Watanabe, 1977) in a host cell dependent manner, which may have a role in host range restriction.

Segment 6 encodes the other antigenic surface protein neuraminidase (NA) which has esterase activity to cleave terminal sialic acid from glycoproteins and glycolipids (Gottschalk, 1957) to free virus particles from host cell receptors and facilitate virus progeny escape, hence spread of the virus (Gottschalk, 1957). NA is also glycosylated and has an amino terminal hydrophobic sequence which functions both as a signal sequence for transport to the cell membrane and as a transmembrane domain. NA accounts for only 3.7% of the total viral protein and is present on the virus surface as a mushroom shaped homotetramer of 50-60 kDa with a 5.0 x 8.5 nm oblong head and 8 nm fibrous stem with a 4 nm diameter knob at the base. These appear as 4 x 4 nm diameter spheres in coplanar square array and are present on the viral surface at a fifth the density of HA with only 120 molecules per virion.

The matrix proteins M1 and M2 are both encoded by bicistronic segment 7. M1 is produced by colinear transcription and is the most abundant viral protein (33-46% total) and is type specific. It is located in the nucleus and cytoplasm of infected cells and is speculated to play an important role in the initiation of progeny virus assembly as well as its structural role forming a shell under the virion lipid bilayer. M2 is an integral membrane protein derived from segment 7 colinear transcript by splicing. M2 is present as a tetramer on the virus surface and functions as a proton channel, controlling the pH of the trans-Golgi network during HA synthesis and the acidification of the interior of the virion during uncoating.

Finally, segment 8, also bicistronic, encodes the two non-structural proteins NS1 and NS2. NS1 is synthesised in large amounts only in infected cells (Lazarowitz *et al.*, 1971; Skehel, 1972) and has been found associated with polysomes. NS1 may be involved in the shut off of host cell protein and viral RNA synthesis (Lazarowitz *et al.*, 1971; Compans, 1973). The function of NS2 is unknown. It occurs in the cytoplasm of infected cells as the result of splicing of the segment 8 transcript, but is not incorporated into progeny virions.

A15. Virus Replication Cycle (Krug *et al.*, 1989)

The infectious cycle begins when a virus particle binds to the host cell sialic acid receptor. The virus is endocytosed into a low pH (5) endocytic vesicle which results in a conformational change in the HA to facilitate insertion of the hydrophobic free amino terminus of HA2 into the vesicle membrane. Following fusion of the viral and vesicle membranes, the virion contents are released into the cytoplasm of the cell.

The nucleocapsid migrates to the host cell nucleus where primary transcription of mRNA begins, and the transcripts used for translation of NP and NS1 early in infection. Host mRNA translation is blocked and newly synthesized NP and NS1 migrate to the nucleus. The resulting increase in NP concentration triggers a shift to cell RNA and viral RNA synthesis. Newly synthesized viral RNAs are encapsidated in NP and function as templates for secondary transcription of viral mRNAs.

M1, HA and NA are translated later in infection. HA and NA are post-translationally modified, travel to the cell surface and integrate into the cell membrane. Nucleocapsids

migrate out of the nucleus into the cytoplasm for progeny assembly as M1 protein builds up, then encases the nucleocapsids. These then bud out through areas of the host cell membrane with embedded HA and NA spikes, enveloping the nucleocapsids. HA0 is then cleaved to HA1 and HA2 and the infection cycle begins again.

Two roles for M2 protein in viral replication have been proposed. These are, (a) uncoating in an endocytic pH5 compartment and (b), maintaining the appropriate ionic environment for maturation, and transport of *de novo* synthesised HA to the plasma membrane, at least for avian (H7) viruses that are cleaved in the Golgi. M2 protein forms an ion channel which preserves the conformation of native acid-sensitive HA during transport from the *trans* Golgi compartment to the plasma membrane of the infected cell by regulating pH within the *trans* Golgi network. HA of the human H1, H2 and H3 are cleaved in a post-Golgi compartment and are not dependent on M2 function for trafficking to the plasma membrane (Mackenzie *et al.*, 1989).

A16. Structure of influenza HA

The 3D structure, at 3Å resolution was obtained by Wilson, Wiley and Skehel in 1981 and has provided a paradigm for infectious disease studies since antigenically significant regions of the molecule, recognised by neutralising antibody can be located at the molecular level, with respect to receptor binding site and more recently, crystallographic data available for HA complexed with Fab fragments of such antibodies.

Haemagglutinin consists of two disulphide linked subunits, HA1 and HA2 (Figure 4). It is present on the surface as a homotrimer (Wiley *et al.*, 1977). Each homotrimer spike is 13.5 nm long and consists of a globular head region of HA1 residues in eight anti-parallel β-sheet structures and a stalk 8 nm long of both HA1 and HA2 residues in two α-helices. The carboxy terminus of HA2 has a hydrophobic transmembrane sequence and a terminal cytoplasmic anchor sequence where palmitate is attached. The globular head region (HA1) is antigenically important and also has the receptor binding cavity.

The receptor binding site is a shallow concave pocket at the distal end of the HA molecule and contains several highly conserved residues within it (98Tyr, 153Trp, 183His, 190Gln, 194Leu and 195Tyr). Residue 226 is important for receptor binding and host range

restriction. A leucine residue is necessary at position 226 for binding of human influenza virus isolates to host cell sialic acid receptors in an α(2-6) galactose linkage but a glutamine at this position is necessary for avian and equine isolates to bind their receptors to α(2-3) linkages; a virus possessing a glutamine at 226 will not be able to infect humans thus restricting host range. Conversely, horses and guinea pigs have a receptor analogue, α_2 macroglobulin which competitively inhibits human H3 HA binding (Rogers & Paulson, 1983; Rogers *et al.*, 1983; Ryan-Poirier & Kawaoka, 1991). Host range is also restricted by host specific phosphorylation of NP affecting replication of influenza (Kistner *et al.*, 1985, 1989; Scholtissek *et al.*, 1985), and changes in NA can affect virulence (Mahy & Barry, 1978; Suguira & Ueda, 1980).

There is also a conserved second shell behind the pocket (97Cys, 99Pro, 139Cys, 147Phe, 195Tyr and 229Arg) to stabilize the architecture of the binding pocket. The conserved receptor binding pocket is surrounded by highly antigenic regions against which neutralising antibodies are directed. While changes have occurred all around the receptor binding site, the pocket itself has not changed since 1968, although receptor binding mutant viruses have been selected in the laboratory (Rogers & Paulson, 1982; Temoltzin-Palacios & Thomas, 1994).

A second binding site has recently been located (Sauter *et al.*, 1992). This concave pocket at the interface of HA1 and HA2 was structurally identified by X-ray crystallography, using a range of sialic acid compounds, some of which bound only to site 1 and others which bound to a second site also, but with a quarter of the affinity.

Both HA1 and HA2 are glycosylated but the number and positions of glycosylation sites vary between strains/subtypes generally clustering around the antigenic sites on the globular head. N-glycosylation occurs where an Asn-X-Ser/Thr motif exists. In H3 viruses, for example, this is at Asn-8, 22, 38, 81, 165 and 285 on HA1, and Asn154 on HA2 (Ward & Dopheied, 1980). There are several consequences of N-glycosylation:- (i) masking antigenic sites (Wiley *et al.*, 1981; Wilson *et al.*, 1983) and (ii) to mask potential proteolytic cleavege sites for HA0 to HA1 and HA2, therefore regulating virulence (Caton *et al.*, 1982; Verhoeyen *et al.*, 1983; Skehel *et al.*, 1984).

Resistance to infection correlates with serum anti-HA antibody levels (and secretory IgA) which are subtype specific (Hobson *et al.*, 1972; Dowdle *et al.*, 1973; Couch & Kasel, 1983) and B-cell memory can persist for decades, so that immunity is retained for over

Figure 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 (Wiley *et al.*, 1981). **Carbohydrate is shown in green.**

The antigenic sites are as follows:

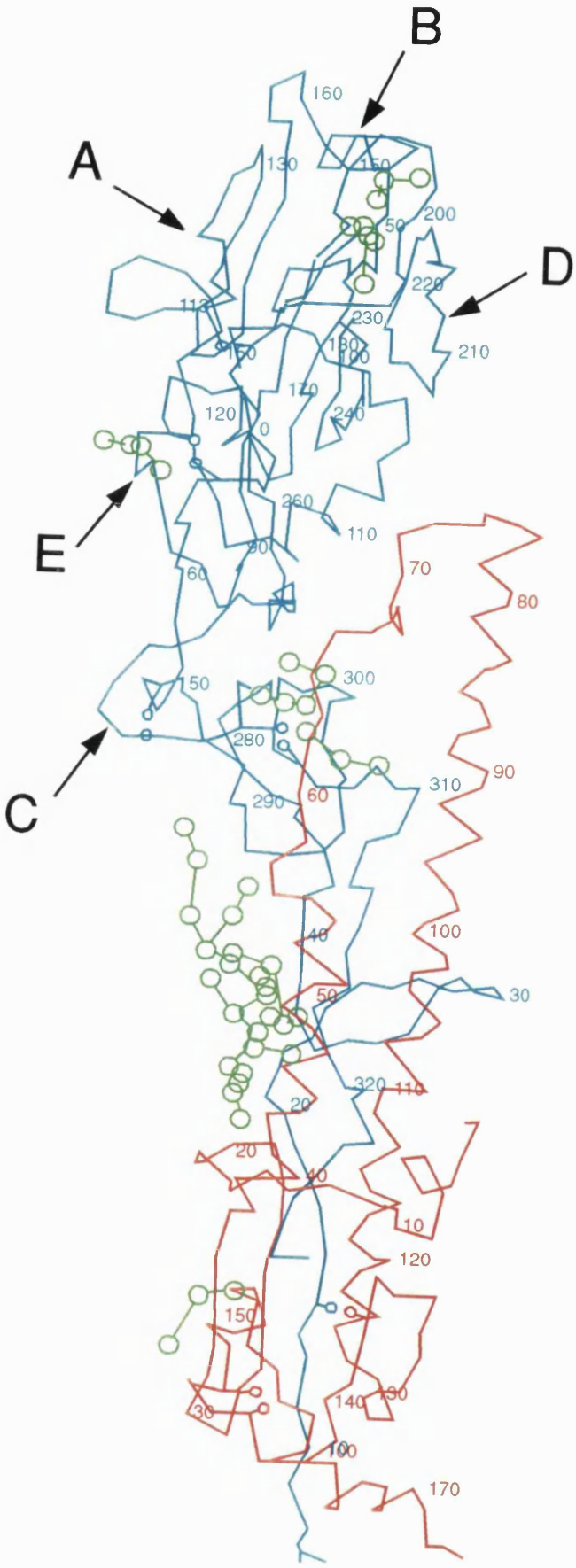
Site A: this is a prominant loop protruding 8Å from the local molecule surface covered by HA 1 aa140-146 and adjacent surface.

Site B: at the distal tip of HA 1 adjacent to the binding pocket, this site comprises a loop formed by HA-1 aa 155-160 and an α-helical region at HA 1 aa187-199.

Site C: a surface bulge 60Å from the distal tip in a region of highly conserved disulphide bonding.

Site D: a region of β-pleated sheets near the trimer interface.

Site E: as defined by natural variants and laboratory mutants at amino acids 61, 63, 78, 81 and 83. Mutation at HA1 aa63 abrogates antibody recognition by the introduction of an N-glycosylation site.


B
A
D
E
C
160
130
150
200
50
220
230
210
120
0
240
260
110
70
80
300
150
280
90
310
290
60
100
40
30
50
320
110
20
20
40
10
120
150
140
130
30
100
170

thirty years providing protection against subsequent infection by previously encountered virus strains. Mechanisms of virus neutralisation are discussed further in "Protective immunity".

A17. The antigenic nature of HA.

The surface distal globular domain of the HA1 subunit contains five antigenic sites recognised by neutralising antibodies, designated sites A-E (Wiley *et al.*, 1981) (see Figure 4). The molecular location of these antigenic regions has been defined by structural analysis of Mab-selected laboratory mutants differing from the immunising virus by one, or two amino acid substitutions in HA1, which have also featured in natural variant viruses. These five sites correspond to the most surface exposed regions of HA1 and cluster around the receptor binging pocket.

Site A consists of a prominant loop and adjacent surfaces of amino acids HA1 140-146, protruding 8Å from the local molecule surface.This site has been defined by laboratory mutants with substitutions at HA1 128, 135, 143(Pro→His/Ser/Thr/Leu), 144(Gly→Asp), 145(Asn→Lys) and 146. Strains of epidemic significance with substitutions in this region include A/Memphis/102/72 (144Gly→Asp and 122Thr→Asn), A/Victoria/1/75 (145Ser→Asn) and A/Victoria/3/75 (137Asn→Ser).

Site B is around the distal tip of HA1 adjacent to the binding pocket.This comprises a loop formed by amino acids 155-160 and an α-helical region at amino acids 188-199. This site has been characterised by substitutions in epidemic strains A/Memphis/102/72 (188Asn→Asp and 155Thr→Tyr) and A/Victoria/3/75 (189Gln→Lys and 193Ser→Asn). Laboratory mutants with single substitutions at HA1 156, 157, 158, 188, 189, 193, 198, and 199, confirm the designation of this site.

Site C is a surface bulge in the 3D structure 60Å from the distal tip of HA1 at the disulphide bond between Cys52 and Cys277, in a region of highly conserved disulphide bonding. Epidemic viruses with substitutions in this region include A/Memphis/102/72 (275Asp→Gly), A/Victoria/1/75 (53Asn→Asp) and A/Victoria/3/75 (278Ile→Ser). In the 1968 strain HA structure, Asp275 is hydrogen-bonded to Asn53 and Ile278 and packed against Asn54, and Cys52 is disulphide bonded to Cys277, and changes in the types of

contacts between these residues may be the structural alterations to which antibody binding is sensitive. The antigenicity of this region has been confirmed by laboratory mutants at HA1 54(Asn→Lys), (186Sre→Ile and 220Arg→Ile) which is also sensitive to HA1 54 specific antibody, and more recently by this laboratory, 273(Pro→Leu) (Smith *et al.*, 1991).

Site D consists of a region of β-pleated sheets near the trimer interface, defined by laboratory mutants 201(Arg→Gly), 205(Ser→Tyr), 220(Arg→Leu) and natural variants A/Memphis/102/72 (207Arg→Lys) and A/Victoria/3/75 (201Arg→Lys and 217Ile→Val).

Site E, also a region of β-pleated sheets, has been defined on the basis of natural drift substitutions at 61, 63, 78, 81 and 83. Substitution at HA1 63 (Asn→Asp) abrogates antibody recognition by the introduction of an N-glycosylation site ($Asn_{63}Cys_{64}Thr_{65}$), and 83Thr→Lys abrogates N-glycosylation at HA1 81.

A18. Antigenic variation in influenza viruses.

Both of the surface glycoproteins HA and NA are protective antigens. Consequently, it is these two proteins that undergo proportionally more changes/amino acid subsitutions to evade immune recognition. The neutralising antibody response is focused predominantly to the ectodomain of both HA and NA molecules and is directed against sites on HA which cluster around the receptor binding pocket, thus preventing attachment of the virus to the host cell and infection (Colman *et al.*, 1983).

Although antibodies can be, and are, made against any structural protein of influenza viruses (HA,NA,M1,NP), virus neutralisation only occurs if antibodies are directed against the surface HA, and the main focus of the host immune response is indeed against the surface antigenic glycoproteins HA and NA. For the virus to survive and maintain its presence in the population, it must evade immune recognition. The way type A influenza viruses have accomplished this is by antigenic variation.

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 occurs in two ways: by drift or shift.

Antigenic shifts are sudden dramatic changes, occurring every 10-15 years, resulting in new pandemic or epidemic infections since HA or NA or both are completely different to previously circulating strains. These usually arise by recombination between isolates of different species e.g. human with avian/equine/porcine (Webster *et al.*, 1971) in either one or both of the glycoproteins with up to 80% sequence change (Webster *et al.*, 1982). High RNA recombination rates have been shown (Simpson & Hirst, 1961; Hirst, 1962) and a recombination frequency of 97% demonstrated by Burnett and Lind (1951) and others (Laver, W. D. & Kilbourne, 1966; Laver, W. G. & Webster, 1966).

There are, to date, fourteen known subtypes of HA and nine NA in avian viruses, although in the human there are only three subtypes, (H1N1, H2N2, H3N2) and not all of these will be circulating in the population at a given time. For example, the present circulating human strain is of the H3N2 type which did not emerge until 1968 and has been circulating in the population since. The H3N2 virus was preceded by H2N2, prevalent between 1957 and 1968, and the H1N1 before that, first isolated from humans in 1933 (Laver, W. D. & Kilbourne, 1966; Laver, W. G. & Webster, 1966), all of which have been responsible for major pandemics and millions of deaths worldwide. Although there can be clear cut dates for the emergence and disappearance of the new strains, they do occassionally re-emerge to cause epidemic infections among the immunologically naive susceptible population, such as in the resurgence of the H1N1 (Asian) subtype recently (overlapping with the currently circulating predominant H3N2 subtype).

Drift substitutions are less drastic changes in sequence, usually as single point mutations, giving rise to different strains within a subtype. These minor changes provide the variant virus with a selective growth advantage over the parent virus, evading antibody neutralisation during interpandemic years. Gradual accummulation of these subtle changes in antigenicity, however, sporadically yield pandemic and epidemic strains.

Drift mutations occur primarily in the HA1 subunit of HA at positions corresponding to five antigenic sites A-E (Gerhard *et al.*, 1981; Wiley *et al.*, 1981; Caton *et al.*, 1982; Skehel *et al.*, 1984; Daniels *et al.*, 1985; Raymond *et al.*, 1986), at a very high frequency of 0.3% amino acid change per year (Daniels *et al.*, 1985; Kawaoka *et al.*, 1989), suggesting immune selective pressure in the generation of new variants.

The molecular location of antigenic variation has been investigated using HA-specific monoclonal antibody (Mab) selected mutants, and single amino acid substitution differences to wild type viruses have identified key residues in the 3D structure (Knossow *et al.*, 1984), confirmed by electron microscopy of antibody-HA complexes (Gerhard *et al.*, 1981; Wrigley *et al.*, 1983). These Mab-selected variants have single amino acid changes corresponding to residues that frequently change in natural variants, confirming the importance of antibody selection in antigenic variation (Lubeck & Gerhard, 1981; Underwood, 1982). Each new virus isolate of epidemiological importance has generally four or more amino acid substitutions in at least two of the five antigenic sites, with one antibody epitope completely changed between epidemic years (or 20% of each of the five sites), such as A/Chile/83 (H1N1) differing from the previous strain A/England/80 by six amino acids in sites B, D and E, and A/Sichuan/87 by seven amino acids at sites A, B and E compared to A/Mississippi/85 before it. Antigenic variants of H3N2 between 1968 and 1986 vary by 0.8% in sequence homology (interepidemic) whereas H2 and H3 (epidemic) are only 36% homologous in HA1 and 50% in HA2 (Gething *et al.*, 1980; Verhoeyen *et al.*, 1980).

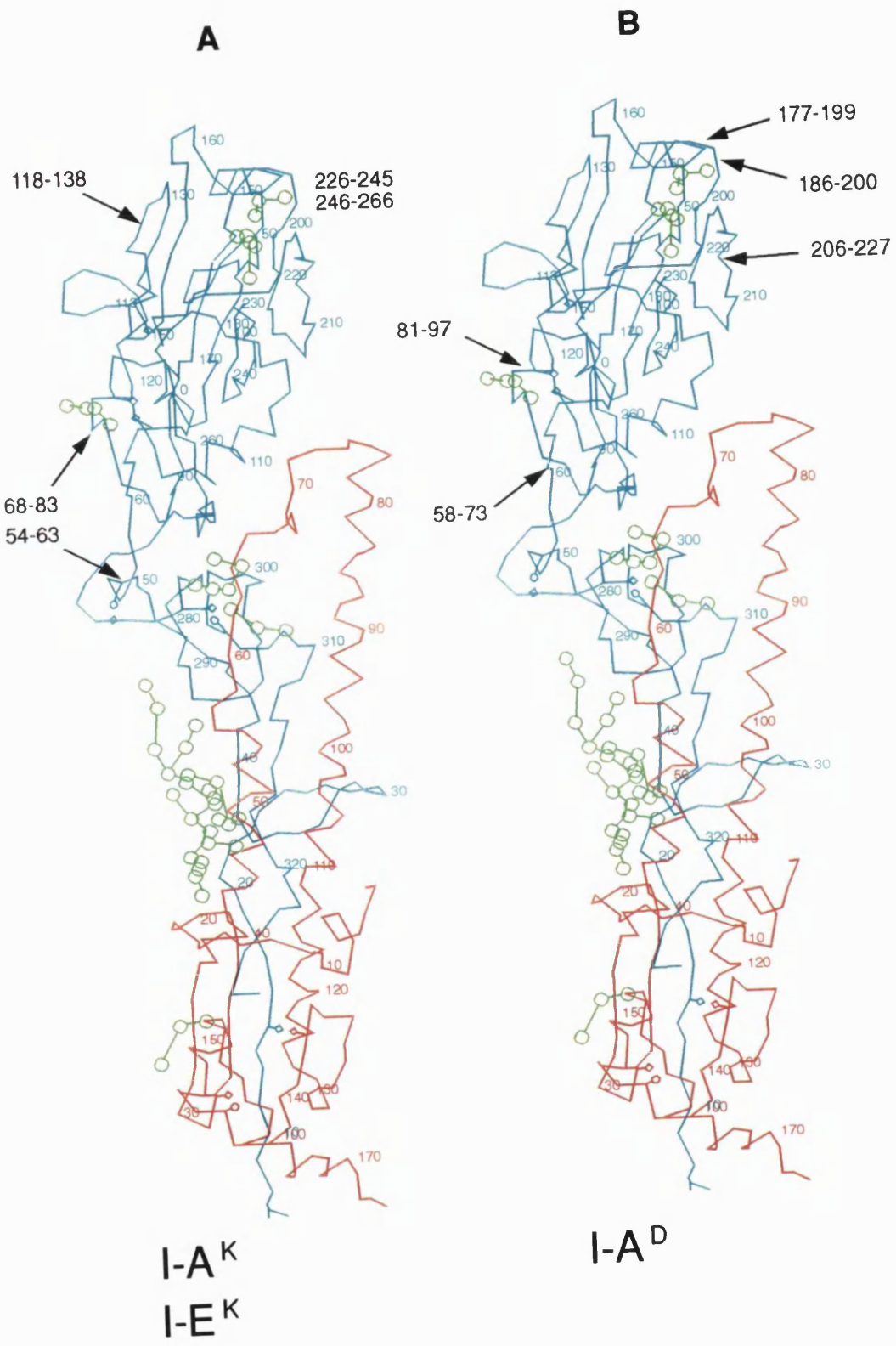
The class II restricted T-cell recognition sites of HA have also been defined (Hurwitz *et al.*, 1984; Barnett *et al.*, 1989a, b; Burt *et al.*, 1989) and frequently found to be located within, or proximal to hypervariable regions of the HA1 polypeptide recognised by neutralising antibodies. The majority of these have featured in antigenic drift.

A19. T-cell recognition sites of influenza HA.

The class II restricted T-cell recognition sites of influenza HA for both Ia^d and Ia^k restricted T-cell clones have been defined in this laboratory, using overlapping synthetic peptides spanning the entire HA sequence. Multiple non-overlapping T-cell epitopes recognised in the context of a single class II restriction element have been identified as illustrated in Figure 5 (Thomas, D. B. *et al.*, 1989), and a considerable degree of overlap found with regions of antibody recognition, contrary to the observations of Hurwitz *et al*, who found that the T-cell response of BALB/c mice immunised with PR8 virus focused on different determinants to the antibody response, and that the T-cell repertoire was more restricted than the B-cell repertoire (Hurwitz *et al.*, 1984). No evident structural similarities

Figure 5: Schematic representation of the 3D structure of an H3 HA monomer indicating the surface location of $CD4^+$ T-cell epitopes. (Thomas, D. B. *et al.*, 1989)

The α carbon backbone of HA1 (blue) and HA2 (red) is shown. I-A^k restricted epitopes by clones from CBA/Ca mice are shown on the left monomer (A), and I-A^d restricted epitopes by clone from BALB/c mice on the right monomer (B). The I-E^k restricted epitopes are within conserved buried regions at HA 1 226-245 and 246-265.


A
B
118-138
226-245
246-266
68-83
54-63
177-199
186-200
206-227
81-97
58-73
I-A K
I-E K
I-A D

have been identified in these peptides, nor do they compete for antigen presentation to individual $CD4^+$ clones, therefore the peptide binding site of class II may accommodate a variety of HA peptides. The majority of these T-cell epitopes on influenza HA have featured in antigenic drift and T-cell recognition is abrogated by mutations within these regions either in natural variants or antibody selected laboratory mutants (Thomas, D. B. *et al.*, 1989).

An interesting finding in this laboratory has been repertoire diversity for a given haplotype but restriction to one or a few peptides for individuals from the same inbred strain. Differences in peptide immunogenicity for different MHC genetic backgrounds have also been shown by the different arrays of peptides recognised by mice of the $H\text{-}2^d$ and $H\text{-}2^k$ haplotypes (Kawamura *et al.*, 1984).

A20. Antibody recognition sites of influenza HA.

Antigenic drift mutations occur in specific regions of the HA molecule as deduced by structural analysis of laboratory mutant viruses selected with neutralising monoclonal antibodies (Mab), and mirroring those seen in natural variants (Laver, W. G. *et al.*, 1979) thereby confirming the importance of antibody selection pressure in antigenic variation.

Five major antigenic regions in HA1 have thus been defined and designated site A-E (Gerhard *et al.*, 1981; Lubeck & Gerhard, 1981; Wiley *et al.*, 1981; Caton *et al.*, 1982; Underwood, 1982) as discussed previously, and shown in Figure 4. Each of these sites fulfill the structural requirements specified by Jameson and Wolf (1988) as being typical of antibody recognition sites. Virus neutralisation results from antibody binding to one or more of these regions surrounding the receptor binding site of HA1, thereby preventing interaction with host membrane receptors.

Initial studies on antibody recognition of influenza HA were pursued by immunisation of BALB/c mice with PR8 (H1N1) or X31 (H3N2) viruses, and assigned to the 3D structure by haemagglutination inhibition reactivity patterns with natural variants. Using this methodology, extreme diversity in the secondary antibody response to influenza HA has been found.

Previous studies by Cancro *et al* (1978), defining paratopes of HA-specific antibodies by antibody fine specificity to a panel of antigenically related influenza A viruses, demonstrated extensive paratypic diversity in the BALB/c HA-specific response using a panel of eight epidemic influenza A viruses in a fine specificity analysis.

Staudt and Gerhard (1983), redefined paratypic diversity using a panel of 39 distinct PR8 (H1N1) single amino acid mutants, along with 12 epidemic strains. They characterised fine specificities of 125 BALB/c HA-specific antibodies. The antibodies were divided into four broad spectrum groups of related fine specificities corresponding to four sites on H1 (Gerhard *et al.*, 1981) consistent with the four antigenic sites defined from the 3D structure of HA previously (Wiley *et al.*, 1981; Wilson *et al.*, 1981).

Hybridomas were made by fusion of individual spleens or mediastinal lymph nodes from 14 adult BALB/c donors (8-16 weeks old) following secondary immunisation i.p. with A/PR8/8/34 virus. From these, 125 hybridoma antibodies were shown to be specific for HA of PR8 based on reactivity with recombinant influenza viruses with known reassortments of genes of PR8 and distantly related A/HK/68 (H3N2). These were tested by viral radioimmunoassay, that is, the antibodies were incubated with virus and radioiodinated rabbit anti-mouse $F(ab)_2$ antibody and radioactivity measured. Fine specificity was detected by differences in radioactivity counts with variant viruses relative to PR8.

Overall, 104 reactivity patterns (RP) to 51 viruses were found in the 125 antibodies generated from 14 donors. For example, seven HA-specific antibodies were generated from donor 8, all with different reactivity patterns, 5 from donor 17 with 4 distinct RPs, 11 antibodies from donor 18 with 8 RPs, 13 antibodies from donor 36 with 11 RPs and 54 from donor 37 with 43 RPs. Using the statistical method of Wybrow and Berryman (1973), they predicted from these results that the adult BALB/c repertoire consisted of 1500 different paratopes, a very diverse response.

It is interesting, however, to note that while Staudt and Gerhard report "unequivocal evidence of large diversity of the adult BALB/c anti-HA antibody response" they also state that "there are clear paratypic similarities among antibodies from mouse 37, suggesting that groups of these antibodies have structurally related V domains" and the secondary antibody response of mouse 36 has ten out of thirteen antibodies binding to site Sb (i.e. the response is limited), of which 22% use the same V_κ gene (V_κ 21C) and "this antibody panel is rather restricted in specificity".

A single amino acid substitution within an antibody recognition site may affect binding of the antibody to an amino acid paratope. In another study using antibodies generated from BALB/c mice (immunised *intraperitoneally*) 125 H1-specific Mabs were divided into ten groups, based on reactivity patterns to sixteen natural variants, all mapping to antigenic sites on HA (Gerhard *et al.*, 1981; Smith *et al.*, 1991). Each group contained a core region including residues which affected binding of a number of antibodies, and a periphery containing residues which influenced binding of particular antibodies within this number (also seen for other proteins e.g. myoglobin (East *et al.*, 1980)). It was found that binding of antibody to HA1 205 was affected by mutations at 218 and 226 (Brown, L. E. *et al.*, 1990).

Clarke (1990) also reported highly diverse specificities in the secondary antibody response to influeza HA site Sb (H1 equivalent of H3 site B) in contrast to the primary antibody responses that were structurally and functionally similar. The secondary repertoire reflects the expansion of clonotypes present in the primary repertoire (Cancro *et al.*, 1978), and it has been suggested that the increased diversity is due to the use of both a larger repertoire of antibody V-region genes and somatic mutation (McKean *et al.*, 1984; Caton *et al.*, 1986).

Underwood (1984), studying the secondary response of BALB/c mice immunised i.p. with H3N2 virus by cross-reactivity patterns, 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 may be restricted to one or two target areas.

Recent studies from this laboratory employing an alternative protocol, that is, Mab generated from individual CBA/Ca mice following natural infection, and definition of antibody recognition sites by sequencing of Mab-selected virus escape mutants *in ovo* (Smith *et al.*, 1991), have shown the secondary antibody response to be unexpectedly limited. The majority of antibodies generated not only recognised the same region of the HA molecule, but also selected for the same amino acid residue change HA1 158 in laboratory mutants. This is more an issue of route of inoculation and method of epitope mapping rather than the antigenicity of HA, since minor specificities for other regions of

the molecule, within the five designated antigenic regions A-E were also observed, and presumably in a heterogeneous population, all antigenic sites would be recognised by protective antibodies.

Antibodies against the HA2 subunit have been detected in rabbit sera and human convalescent sera (Vareckova *et al.*, 1993) but these do not inhibit haemagglutination or neutralise virus.

Anti-neuraminidase antibodies can protect against lethal influenza viruses (Webster *et al.*, 1988) but these also do not neutralise virus. Some antibodies inhibit neuraminidase activity thereby preventing virion release from the host cell and thus terminating infection (Air *et al.*, 1989). These "neutralising" antigenic determinants are also located at the top of the tetrameric head around the active site crater (as determined by escape mutants selected *in ovo* as for HA mutants with Mab).

A21. Protective immunity

The immune response to influenza infection involves both B and T lymphocytes, and immunogenicity requires cognate recognition by B-cells and T helper $CD4^+$ (T_H) lymphocytes (Mitchison, 1971). Although the anti-HA antibody response is dependent on T lymphocyte help (Virelizier *et al.*, 1974a, b; Lamb, R. A., 1989), induction of neutralising antibody might not always require cognate B-T cell recognition of HA itself; "intermolecular help" from T-cells specific for internal proteins could be sufficient to elicit antibody response to HA.

Protection against influenza is dependent on the presence of serum IgG against HA (Virelizier, 1975; Virelizier *et al.*, 1976) although IgM and IgA also contribute (Breschkin *et al.*, 1981). Neutralisation of virus by HA specific antibody occurs by inhibition of attachment of viruses to the terminal sialic acid residues of host cell glycoproteins thereby preventing internalisation and replication (Taylor, H. P. & Dimmock, 1985a, b).

IgA has been described as the sole mediator of mucosal immunity to influenza reinfection. Influenza specific IgA given *intravenously* is transported to nasal secretions and protects against reinfection, but administering anti-IgA antibody *intranasally* abrogates

protection and renders previously immune mice susceptible. When IgM or IgG specific antibodies are given in the same way, mice remain immune to reinfection (Renegar & Small, 1991).

Cytotoxic T-cells (T_c or CTL), the other arm of effector immunity, are specific for the cross-reactive type specific antigens of influenza, M1 and NP (Lamb, J. R. *et al.*, 1987). They are not protective (Virelizier *et al.*, 1979) although they might be important for recovery from viral infection by limiting the spread of infection and clearing virus.

Askonas and colleagues (Taylor, P. M. & Askonas, 1986), and others (Ada *et al.*, 1981; Yewdell *et al.*, 1985; Townsend *et al.*, 1986; Doherty *et al.*, 1989; Ulmer *et al.*, 1993) found that adoptive transfer of murine primary or restimulated spleen cells caused significant reduction of infectious virus levels in lungs of mice and prevented death from *intranasal* challenge with a lethal dose of influenza A virus. $CD8^+$ CTLs were identified as the effector cells (Doherty *et al.*, 1989), and subsequently, Townsend and Skehel identified NP as the dominant viral determinant recognised by these cells (Townsend *et al.*, 1986). A positive correlation of CTL cytotoxic activity *in vitro*, with clearance rate of virus in the lungs was observed (Mackenzie *et al.*, 1989). CTL induced by *in vivo* immunisation resulted in poor protective immunity, therefore CTL naturally induced by infection *in vivo* were less effective in anti-viral function, *in vivo*, than those from adoptive transfer experiments. This was thought to be due to insufficient activation and poor recruitment of effector cells to the site of viral infection (Andrew *et al.*, 1987; Stitz *et al.*, 1990; Epstein *et al.*, 1993).

T_H cells play a major but indirect role in the antibody response to influenza by delivering a signal for differentiation able to transform primed HA-specific populations of B lymphocytes into antibody secreting cells. Transfer of immune serum confers protection against influenza infection, providing confirmation that the humoral immune response is more important than cell mediated immunity against influenza infection (especially anti-HA) (Loosli *et al.*, 1953; Portnoy *et al.*, 1973; Virelizier, 1975), and protective immunity is exclusively dependent on the presence of neutralising antibodies against HA. T_H-cells are also a major source of IFN-γ which may have a local protective effect during infection.

A22. Immunodominance within diversity of recognition.

Five antigenic sites (Wiley *et al.*, 1981; Wilson *et al.*, 1981; Wiley & Skehel, 1987) for antibody responses to influenza HA have previously been characterised by immunisation of mice but immunodominance has also been observed in T and B responses to natural infection.

The phenomenon that T-cells recognise a limited number of dominant epitopes has been observed in all haplotypes and for different proteins: immunodominance in the T-cell repertoire has been reported for lysozyme (Katz, M. E. *et al.*, 1982), pigeon cytochrome C (Solinger *et al.*, 1979), β-galactosidase (Krzych *et al.*, 1982), staphylococcal endonuclease (Finnegan *et al.*, 1986), myoglobin (Berkower *et al.*, 1984) and bacteriophage lambda cI repressor protein (Perkins *et al.*, 1991) each having a single immunodominant T-cell epitope, e.g. with greater than 95% of T-cells recognising peptide 12-26 of cI in BALB/c mice.

Immunodominance may be influenced by peptide conformation, which affects the configuration of peptide binding to MHC. Events prior to T-cell recognition involving protein unfolding, proteolytic cleavage sites, antigen processing or competition for binding to MHC may have significant effects on immunogenicity. Immunodominance is not simply a function of the primary amino acid sequence, but is a function of the context of the epitopes within the protein molecule. This will affect the available T-cell epitopes, hence limit the T-cell repertoire.

Immunodominance is also observed in the BALB/c and CBA/Ca T-cell response to influenza HA after natural infection. Despite the potential diversity of the responses, the BALB/c (H-2^d) T_H cells recognise predominantly HA1 177-199 (Barnett *et al.*, 1989a) corresponding to site B (Caton *et al.*, 1982; Staudt & Gerhard, 1983), or 56-76 (Graham *et al.*, 1989), and the CBA/Ca (H-2^k) T_H cells recognise HA1 118-138, or 226-245 or 54-63 (Burt *et al.*, 1989). In total however, five different peptides are seen by T-cells from these mice in the context of a single MHC molecule. It is no longer accepted therefore that one MHC haplotype can present only one peptide. For these T-cells, one haplotype can present five different peptides, and the peptides recognised by different strains or haplotypes differ (as does the immunodominant epitope), but overlap with antibody recognition sites nevertheless.

Even though much of the accessible surface of the globular head of the HA molecule may consist of overlapping epitopes capable of binding antibodies, *in vitro* only certain of these epitopes may be immunodominant, or even immunogenic in a given individual. Studies have shown that in the human, children exposed to a primary infection mount a limited response to influenza virus, which for HA is restricted to only one or two antigenic sites (Haaheim, 1980; Natali *et al.*, 1981; Wang *et al.*, 1986), and a different spectrum of antibodies is produced per individual, thus viruses may evolve in a stepwise fashion through sequential infection of a population to new epidemic strains. New viruses usually need at least one mutation in each of the five antigenic sites to be epidemic strains (Wiley *et al.*, 1981).

In natural infection studies, the CBA/Ca antibody response is focused on HA1 158 (for seven out of eight antibodies from a single donor) (Smith *et al.*, 1991), a specificity not reported for Mab obtained by immunisation, suggesting that the route of administration of antigen as well as genetic background (MHC or immunoglobulin V region genes) are significant factors in selection of the immune response. The natural route of infection may elicit a different and more restricted cellular response from B and T-cells.

A23. T-B commonality of epitopes.

Because the T-cell repertoire is limited due to MHC restriction and the B-cell repertoire assumed to be very diverse, T-cell and B-cell epitopes were originally thought to be dissimilar antigenic sites (Anders *et al.*, 1981; Lamb, J. R. *et al.*, 1982; Lamb, J. R. & Green, 1983; Atassi & Kurisaki, 1984; Katz, J. M. *et al.*, 1985) and that T-cells recognised conserved regions of HA1, not recognised by antibodies. Differences in T-cell and B-cell specificities had been observed in systems (Berzofsky, 1978; Corradin & Chillen, 1979; Maizels *et al.*, 1980; Thomas, J. W. *et al.*, 1981) using proteins with some homology to self, a factor which would itself restrict the possible T and B repertoires, but commonality of epitopes has been observed in the immune response to influenza virus HA following natural infection.

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

$CD4^+$ T_H cell recognition patterns have also been studied and shown to coincide with antibody binding sites (Hurwitz *et al.*, 1984; Mills *et al.*, 1986a; Thomas, D. B. *et al.*, 1987; Brown, L. E. *et al.*, 1988; Barnett *et al.*, 1989b; Burt *et al.*, 1989; Graham *et al.*, 1989), thus amino acid changes within these antigenic sites abrogate both T-cell and B-cell recognition.

A24. T-B reciprocity: why do T-cells focus on B-cell sites?

There has been some debate on the possible focusing of B and T-cells to common antigenic sites. Berzofsky (1983) speculated that B-cell MHC (Ia) limits T-cell specificity, which in turn limits antibody specificity, and that B-cells can present to T-cells in an effective form only certain epitopes on the anitgen (determinant selection) for which there are two lines of evidence. Firstly, T-cell epitopes are MHC restricted and B-cell blasts (lyb 5^+) do not require genetically restricted help but resting B-cells (lyb 5^-) do, that is, a T-cell must recognise antigen in association with the appropriate Ia on a B-cell to provide help.

Secondly, when T-cell specificity is limited by inducing T-cell tolerance in rabbits to cross-reactive lysozyme, these rabbits were found to make antibody of limited specificity. Moreover, B-cells can bind antigen *via* surface Ig which is internalised, processed and re-expressed on the B-cell surface in association with class II. Presented peptides i.e. T-cell epitopes might therefore be influenced by Ig specificity. If Ig bound regions of a protein were protected from proteolytic enzymes and preferentially re-expressed on the surface, this would be instrumental in the selection of T-cell epitopes in close proximity to antibody recognition sites.

Lanzavecchia and others (Lanzavecchia, 1985; Watts & Davidson, 1988) have suggested a possible selective role for surface Ig receptors of B-cells in antigen presentation. Antigen specific B-cells recognise conformational features of native protien which is internalised by receptor mediated endocytosis and then intracellularly processed in an endocytic compartment, followed by subsequent expression and presentation of these or closely related regions as linear peptides at the surface to T-cells in association with MHC.

Does class II restriction influence the B-cell repertoire such that antibody is focused on a limited number of antigenic sites per individual? Clonal populations of antigen specific B-cells from different individuals may generate different fragments intracellularly from the

same protein, and therefore present different fragments as shown for processing of protein by EBV-transformed B-cells from different individuals. Since mice of different haplotypes recognise different regions of influenza haemagglutinin, MHC polymorphism and restriction of the T-cell repertoire may, in turn, influence the neutralising antibody response of an individual, directing the antibody to a limited number of the available antigenic sites. If this is the case, then which response directs which?

A25. The objective in this investigation.

This study addresses the possibility of a causal link between MHC polymorphism and the selected antibody repertoire, by characterisation of the antibody recognition specificities to natural infection of MHC congenic BALB/k mice (of H-2^k haplotype on a BALB/c (H-2^d) background), since the antibody sites for CBA/Ca (H-2^k haplotype), and BALB/c are already known under these conditions of infection.

For our purposes, haplotype studies are important for the elucidation of the variety of immune responses generated within a population, to a primary infection of a naive population. Antibodies see five major sites. Each epidemic strain of influenza has changes in all five sites for the human population. The virus escapes immune recognition by mutation, therefore the immune response is applying selective pressure on influenza, but does each individual select one site or more, and do individuals of the same haplotype select the same sites or different ones? In which case what is the restriction element responsible?

The BALB/k response might follow one of three directions. (a) It could either mimic the CBA/Ca (H-2^k) response because of a common MHC haplotype, or (b) the BALB/c response because of non-MHC factors common to both, or (c) an intermediate response or combination of the two because both elements are active in BALB/k mice.

In this investigation, definition of antibody recognition sites for individual BALB/k mice following natural infection might resolve some of these questions and further our understanding of the underlying mechanisms behind selection of the immune response following natural infection.

MATERIALS AND METHODS

B1. Mice

BALB/k mice were bred under specific pathogen free conditions at the National Institute for Medical Research (NIMR) and used at three months of age.

B2. Viruses

All influenza viruses were grown in the allantoic cavity of 10 day embryonated hen eggs at 33°C, the allantoic fluid harvested after 2 days and stored at -70°C (Hirst, 1942; Beveridge & Burnet, 1946). Virus titres were determined in haemagglutination assays (Salk, 1944) using turkey erythrocytes (TRBC) and expressed as HA units/ml. Briefly, two-fold serial dilutions of a 50µl virus sample in PBS were made in a round bottom 96 well microtitre plate (Nunc) and 50µl of 1% TRBC in PBS added to each well. The highest concentration showing complete haemagglutination after 30 minutes at room temperature was equivalent to 1 HAU.

X31 is a recombinant virus between A/AICHI/2/68 and A/PR/8/34 with A/Aichi glycoproteins (H3N2) and PR8 internal proteins (Kilbourne, 1969).

Natural variants were all of the H3N2 subtype isolated from major influenza outbreaks between 1968 and 1984, provided by the World Influenza Centre at NIMR.

Mutants were cloned by the plaque purification method (as described in section 2.2) and individual plaques isolated, grown up in eggs and stored at -70°C.

B3. Viral fragments.

Haemagglutinin was released from purified X31 by bromelain digestion and purified on a sucrose density gradient (BHA: Brand & Skehel, 1972). The HA1 portion of the HA (28-328) designated "tops" was prepared by pH5 treatment of X31 and a trypsin digestion (Skehel *et al.*, 1982), followed by separation of the soluble fraction, tops, from the remainder of the virion, insoluble aggregate, by sucrose density gradient centrifugation.

BHA and tops were kindly provided by members of the Virology division at NIMR (Dr. Steve Wharton, Rose Gonzalves and Dave Stevens).

B4. Chick embryo fibroblast plaque purification (Simpson & Hirst, 1961)

B4.1. Complete Geys medium

Chick embryo fibroblasts were grown in complete Geys AB Tris medium with 5% calf serum (Gibco), 0.8 g/l protease peptone (Difco), 4% lactalbumin hydrolysate (LAH), 112.5 mg/l Geys C (sodium bicarbonate), 54.6 mg/l penicillin and 91.0 mg/l streptomycin.

B4.2. Chick embryo fibroblasts (CEF)

CEF monolayers were prepared using the Simpson and Hirst method. Briefly, fifteen (for 300 ml) 10 day old chick embryos were minced after removal of eyes and intestines, trypsinized, and the cell suspension seeded onto plastic petri dishes (Nunc) at 5 ml/5 cm plate and incubated overnight at 35°C for a confluent monolayer to establish.

B4.3. Plaque purification and cloning of virus.

The confluent CEF monolayers were washed with BSA/saline (20 ml/500 ml) and inoculated with 1 ml per plate of virus diluted from 10^{-5} to 10^{-8} of seed stock in saline/BSA. After an adsorption period of 30 minutes at room temperature, the excess inoculum was aspirated and the monolayer covered with 5 ml/plate of trypsin overlay containing 2x199 medium pH 7.0 (Gibco medium 199 powder with EBSS), 0.5% BSA (Sigma), 0.01% dextran DEAE (Sigma), 2.5 µl/ml trypsin (Sigma), 27.3 mg/l penicillin, 45.5 mg/l streptomycin and 1.5% Noble's agar (Difco). The overlay was left for 5-10 minutes at room temperature and the plates then incubated at 35°C 6% CO_2 for 72 hours until visible plaques formed. If by the end of the incubation period the plaques were not visible to the eye, the plates were stained with 0.01% neutral red in saline for 3-4 hours in the dark, and plaques then picked up into 1 ml/plaque of a PBS A, 3% protease peptone and penicillin and streptomycin mixture, and the virus grown up in eggs again at 0.1 ml of mixture per egg.

Figure 6: Strategy for definition of B-cell epitopes by antibody neutralisation escape mutant viruses.

Intranasal infection of mice with X31 influenza virus

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Intravenous boost 6-8 weeks post infection

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Generation of B-cell hybridomas by fusion of splenocytes with myeloma cells 3 days post boost

↓

Cloning of HI positive antibody secreting hybridomas

↓

Generation of haemagglutinin-specific monoclonal antibodies

↓

In ovo Mab selection of X31 escape mutant viruses

↓

Harvesting from eggs and purification of escape mutant virus

↓

Extraction of viral RNA

↓

Sequencing of HA region

↓

Single amino acid change defines B-cell epitope

B5. Generation of B-cell hybridomas and definition of antibody binding specificities.

B5.1 Culture media.

(a). Standard medium.

B cell hybridomas and myelomas were grown in RPMI 1640 medium (Flow), supplemented with heat inactivated, FCS (foetal calf serum; Flow), 2 mM Glutamine (Flow), $5x10^{-5}$ M β-mercaptoethanol (Sigma), 100 U/ml Penicillin, 100 μg/ml Streptomycin (Flow).

(b). Selective medium.

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

B5.2. B-cell hybridomas and monoclonal antibody production.

BALB/k mice were infected *intranasally* with 4 HAU of X31 and boosted *intravenously* with 1000 HAU 6-8 weeks later. Three days after boosting, splenic lymphocytes from 10 individual donors were fused with the BALB/c derived myeloma line P3-X63-Ag8653 (Kohler & Milstein, 1975, 1976; Kearney & Valik, 1986) , following the Fazekas de St. Groth and Scheidegger (1980) protocol.

Equal number of washed spleen cells and myeloma cells were fused in a solution of 500μl PEG (polyethylene glycol; Sigma) in RPMI 1640 and 50μl 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. 50μl aliquots of fused cells were added

to 150µl of PECs (peritoneal exudated cells) in selective medium, in flat-bottom-96-well-microtitre plates, and cultured at 37°C in 6% CO_2: $2x10^7$ spleen cells and PECs from one mouse, were dispensed onto each plate. Cultures were fed on day 5 with 100µl of selective HAT medium 20% FCS, on day 7 with HT medium 20% FCS, and on day 14, and when required (a good indication of this is medium colour change from orange to yellow) with standard medium 10% FCS.

Culture supernatant from wells were screened for HA-specific neutralising antibody in a haemagglutination inhibition (HI) assay (World Health Organization 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 (as determined by HA) in 25µl volumes was added to each well and the plate incubated at room temperature for 30 minutes. 25µl of 2% TRBC was added to each well and inhibition of haemagglutination scored 30 minutes later. 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.

B5.2 (a). 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 microtiter plates were seeded with 100 µl of a PECs suspension in standard medium 20% FCS. The hybridoma cells were diluted to a final concentration of 5 cell/ml (equivalent to 0.5 cells/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 Mab were generated by growing hybridomas as ascitic tumours in mice. Briefly, (CBA/Ca x BALB/c) F1 mice were given a single 0.5 ml *intraperitoneal* injection of Pristane (2,6,10,14 tetra methyl pentadecane; Koch Light) one week before the intraperitoneal injection of at least $1x10^6$ hybridomas cells per mouse in 1.5 ml of PBS (phosphate-buffered saline, pH 7.0). Each Mab was used to select a neutralisation escape mutant for which the HA gene was sequenced to determine the epitope recognised.

B5.2 (b). Mab isotype determination.

The Mab isotype determination was performed using standard indirect ELISA techniques (Ishiguro *et al.*, 1983; Kendall *et al.*, 1983). Briefly, plastic 96 well microelisa plates (Nunc) were coated with 1 μg/well BHA in borate buffered saline pH 7 (BBS), 0.17 M borate (BDH), 0.12 M NaCl, for 2 hr at 37°C and washed three times with PBS-Tween 20 (0.05%; Sigma) allowing a 1 minute soak between washes. 50μl hybridoma culture medium supernatant 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 (3x). Mabs bound to BHA were detected by adding 50μl/well 1:1000 biotinylated goat anti-mouse Ig isotype specific antibodies (Southern Biotech) for 2 hr at room temperature. The plates were washed as before and then 50μl/well streptavidin-alkaline phosphatase conjugate (1:2000) (Southern Biotech) was added for 1 hr at room temperature. Plates were then developed with 50μl/well 0.1M paranitrophenyl phosphate (Sigma) in 1M diethanolamine buffer pH 9.8 (Sigma), the reaction stopped with 5 M NaOH (BDH) and read at 405 nm.

B5.3. Definition of Mab fine specificity by selection of Mab neutralisation escape mutant viruses.

Monoclonal antibody selected mutants were produced by growing X31 in the presence of neutralizing antibody (HI positive) in eggs. Equal volumes (usually 1ml) of ascitic fluid and X31 were incubated together at room temperature for 30 minutes, and 500μl aliquots injected into ten day old embryonated hens eggs. Allantoic fluid was harvested from individual eggs on day two and quantified by HA, then tested separately for neutralisation escape-mutant virus by HI assay. Escape mutant viruses were cloned by limiting dilution as described previously.

B5.3 (a). Purification of viruses (Skehel & Schild, 1971).

X31 and Mab selected variants were purified from allantoic fluid using the method described by Skehel and Schild, 1971. Briefly, polyethylene glycol 6000 (PEG;BDH) to

give a final concentration 5%, was added to the harvested allantoic fluid which had been clarified by centrifugation at 1500rpm for 20 min. The solution was pelleted at 1600rpm overnight at 4°C, or 4000rpm for 3 hours at 4°C. The pellet from 1 litre of allantoic fluid was then resuspended in 15 ml of 10 mM Tris-saline (BDH) homogenised and sonicated for 5 min., the solution was layered onto a 20 ml discontinuous gradient of 30% and 60% sucrose (BDH) and centrifuged at 25,000rpm for 90 min. at 4°C. The band containing virus was collected and then diluted with 30 ml of 10 mM Tris-saline and centrifuged at 25,000rpm for 90 min at 4°C. The pellet was resuspended in 4 ml of 10 mM saline, homogenized, sonicated and layered onto a 30 ml continuous gradient of 15%-40% sucrose gradient. The virus was centrifugated at 25,000rpm for 90 min. at 4°C. The viral pellet was resuspended in 0.5 ml of 10 mM Tris-saline and stored at 4°C.

B5.3 (b). Viral RNA extraction

All solutions used are sterile.

Viral RNA was exracted according to Maniatis *et al* (1982). 2-10 µg/ml purified virus was solubilized in 60 mM sodium acetate pH 5 (BDH), 6 mM ethylene diamine tetracetic acid pH 7 (EDTA:BDH) and 1% sodium dodecyl sulphate (SDS:Sigma). The RNA was extracted 2-3 times with an equal volume of phenol/chloroform (Radburn Chemicals) the aqueous phase recovered and the RNA precipitated from it with 70mM NaCl (BDH) and twice the volume of -20°C absolute ethanol on dry ice for 1 hour. The pellet was collected by centrifuging at 13000g for 20 muinutes, 4°C, vacuum dried for approximately 5 minutes, redissolved in sterile double distilled water (15µl) and the concentration (mg/ml) determined by O.D. at 260nm and purity determined by the $O.D._{.260nm}$:$O.D._{.280nm}$ ratio and stored at -20°C.

B5.3 (c). Nucleotide sequence analysis of the HA gene.

The HA gene was sequenced using the dideoxy nucleotide chain terminating method of Sanger *et al* (1977) as used by Daniels *et al* (1983) for this purpose.

Figure 7: Nucleotide sequence of X31 HA gene encoding HA1 and HA2 subunits.

Positions of the six primers used for sequencing of influenza virus HA1 gene of laboratory monoclonal antibody selected escape mutant viruses, spanning the region from nucleotide base number 1 to 950 are shown. The oligonucleotides were designed and numbered according to the sequence of X31 haemagglutinin cDNA (Jameson & Wolf, 1988). Primer sequences and binding sites are as follows:

PRIMER	PRIMER BINDING SITES
P21	^{5}AAAGCAGGGGA^{15}
DP	^{191}TGCTACTGAGCT^{202}
P98	^{367}GTTACCCTTATG^{378}
P40	^{518}TTCAGTAGACT^{529}
P99	^{627}ACCCGAGCACG^{638}
P6	^{778}GGACAATAGT^{786}

```
1       P21                          30                                  60
AGCAAAAGCA GGGGATAATT CTATTAATCA TGAAGACCAT CATTGCTTTG AGCTACATTT
                                    90                                 120
TCTGTCTGGC TCTCGGCCAA GACCTTCCAG GAAATGACAA CAGCACAGCA ACGCTGTGCC
                                   150                                 180
TGGGACATCA TGCGGTGCCA AACGGAACAC TAGTGAAAAC AATCACAGAT GATCAGATTG
                  DP               210                                 240
AAGTGACTAA TGCTACTGAG CTAGTTCAGA GCTCCTCAAC GGGGAAAATA TGCAACAATC
                                   270                                 300
CTCATCGAAT CCTTGATGGA ATAGACTGCA CACTGATAGA TGCTCTATTG GGGGACCCTC
                                   330                                 360
ATTGTGATGT TTTTCAAAAT GAGACATGGG ACCTTTTCGT TGAACGCAGC AAAGCTTTCA
                P98                390                                 420
GCAACTGTTA CCCTTATGAT GTGCCAGATT ATGCCTCCCT TAGGTCACTA GTTGCCTCGT
                                   450                                 480
CAGGCACTCT GGAGTTTATC ACTGAGGGTT TCACTTGGAC TGGGGTCACT CAGAATGGGG
                                   510                 P40             540
GAAGCAATGC TTGCAAAAGG GGACCTGGTA GCGGTTTTTT CAGTAGACTG AACTGGTTGA
                                   570                                 600
CCAAATCAGG AAGCACATAT CCAGTGCTGA ACGTGACTAT GCCAAACAAT GACAATTTTG
                                        P99                            660
ACAAACTATA CATTTGGGGG ATTCACCACC CGAGCACGAA CCAAGAACAA ACCAGCCTGT
                                   690                                 720
ATGTTCAAGC ATCAGGGAGA GTCACAGTCT CTACCAGGAG AAGCCAGCAA ACTATAATCC
                                   750                     770
CGAATATCGG GTCCAGACCC TGGGTAAGGG GTCTGTCTAG TAGAATAAGC ATCTATTGGA
 P6                                810                                 840
CAATAGTTAA GCCGGGAGAC GTACTGGTAA TTAATAGTAA TGGGAACCTA ATCGCTCCTC
                                   870                                 900
GGGGTTATTT CAAAATGCGC ACTGGGAAAA GCTCAATAAT GAGGTCAGAT GCACCTATTG
                                   930                                 960
ATACCTGTAT TTCTGAATGC ATCACTCCAA ATGGAAGCAT TCCCAATGAC AAGCCCTTTC
                                   990                                1020
AAAACGTAAA CAAGATCACA TATGGAGCAT GCCCCAAGTA TGTTAAGCAA AACACCCTGA
AGTTGGCAAC AGGGATGCGG AATGTACCAG AGAAACAAAC TAGAGGCCTA TTCGGCGCAA
TAGCAGGTTT CATAGAAAAT GGTTGGGAGG GAATGATAGA CGGTTGGTAC GGTTTCAGGC
ATCAAAATTC TGAGGGCACA GGACAAGCAG CAGATCTTAA AAGCACTCAA GCAGCCATCG
ACCAAATCAA TGGGAAATTG AACAGGGTAA TCGAGAAGAC GAACGAGAAA TTCCATCAAA
TCGAAAAGGA ATTCTCAGAA GTAGAAGGGA GAATTCAGGA CCTCGAGAAA TACGTTGAAG
ACACTAAAAT AGATCTCTGG TCTTACAATG CGGAGCTTCT TGTCGCTCTG GAGAATCAAC
ATACAATTGA CCTGACTGAC TCGGAAATGA ACAAGCTGTT TGAAAAAACA AGGAGGCAAC
TGAGGGAAAA TGCTGAAGAG ATGGGCAATG GTTGCTTCAA AATATACCAC AAATGTGACA
ACGCTTGCAT AGAGTCAATC AGAAATGGTA CTTATGACCA TGATGTATAC AGAGACGAAG
CATTAAACAA CCGGTTTCAG ATCAAAGGTG TTGAACTGAA GTCTGGATAC AAAGACTGGA
TCCTGTGGAT TTCCTTTGCC ATATCATGCT TTTTGCTTTG TGTTGTTTTG CTGGGGTTCA
                                  1710                                1738
TCATGTGGGC CTGCCAGAGA GGCAACATTA GGTGCAACAT TTGCATTTGA GTGTATTA
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B5.3 c (i) Primers.

Five oligonucleotide primers were made by D. Chase using an applied Biosystems 380 DNA synthesiser. The oligonucleotides were used to prime dideoxynucleotide chain termination sequencing reactions and were designed to correspond to regions within the HA1 gene sequence (primers DP1, P98, P99, P6 and P41) according to the sequence of X31 HA1 cDNA (Verhoeyen *et al.*, 1980). The sequence of the primers, length and binding sites on HA1 are shown in Figure 7.

(ii) Primer labelling and vRNA hybridization.

The 5' terminus of the primers at 1µl O.D.1/virus were phosphorylated at 37°C for 1 hour with 1µl/virus 10µCi (0.37 MBq) gamma ^{32}P-ATP (Amersham) by 10 units T4 polynucleotide kinase (Pharmacia) in 0.05M tris(hydroxymethyl)methylamine pH 8.0 (BDH), 0.01M $MgCl_2$ (BDH) and 0.02M fresh 2-mercaptoethanol (Sigma). 20µg glycogen (Boehringer Mannheim) was added and the labelled primer precipitated with 1M pH 5.0 ammonium acetate (BDH) in -20°C absolute ethanol on dry ice for 15-30 minutes. The primer was pelleted at 13000g for 20 minutes, 4°C, vacuum dried for 5 minutes and resuspended in 10µl/virus double distilled water.

The labelled primer and 1µg vRNA were then coprecipitated with 20µg glycogen, 10mM NaCl and cold ethanol, pelleted and vacuum dried as before and resuspended in 9µl of reverse transcription (RT) mix of 75mM tris pH 8.3, 15mM $MgCl_2$, 80mM KCl, 30mM fresh dithiothreiotol (DTT:BDH), 25u/µl RNase inhibitor (Amersham) and 20u/µl reverse transcriptase (Anglican Biotech).

(iii) Sequencing reaction.(Russel & Moffat, 1969)

2µl of primer/vRNA/R.T. mix solution were added to each of four tubes (per virus) containing 1µl of 1.2mM deoxynucleotide/0.3mM dideoxynucleotide mixes (A,C,G and T mix:Pharmacia) and incubated at 42°C for 1 hour for primer extension, the reaction stopped with 6µl formamide dye solution (0.02 bromophenol blue (BDH) and xylene cyanol (BDH)), heated for 5 minutes at 105°C in a heating block for strand separation and then 3µl of each sample loaded onto an 8% polyacrylamide (Boerhinger Mannheim) and 8.3M urea (BDH) sequencing gel.

Figure 8: Strategy for immunoglobulin V gene assignment for Mab for investigation of a structural basis for immunodominance by V gene usage.

X31 *intranasal* infection of five BALB/k mice

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Secondary boost *intravenously/intraperitoneally*

↓

B-cell hybridoma production (splenocyte/myeloma fusion)

↓

Monoclonal antibody production

↓

Definition of Mab specificity by *in ovo* selection of X31 escape mutant viruses and sequencing of HA gene

↓

Identification of immunodominant epitopes

↓

Selection of Mab of immunodominant recognition specifity for sequencing
(i) One donor, different isotypes and (ii) one isotype, different donors

from BALB/k	from CBA/Ca
↓	↓
26 Mab	11 Mab

↓

Extraction of hybridoma total cellular RNA for antibody gene sequencing

↙ ↘

Heavy chain	Light chain
↓	↓
Direct sequencing of mRNA using C_H region primers	Poly(dT) cDNA synthesis (in triplicate) Three independent PCR amplifications with 11 MKV and 1 CK primers. Gel purification of all PCR products. Column extraction of PCR products from gel. Direct sequencing of PCR products (both strands) with primers used for amplification.

↓

Manual multiple sequence alignments.
GCG DISTANCES sequence homology calculation.
GCG BLASTN family assignment.

B6. Analysis of antibody gene usage

B6.1. Extraction of total cellular RNA from B-cell hybridomas.

Total cellular RNA was extracted from $2x10^7$ cells at a time from culture after washing twice with PBS in a universal tube. The cells were centrifuged for five minutes at 1000rpm and the pellet resuspended in 3ml 6M urea / 3M LiCl and sonicate for 1 minute to break up the cells, then left for RNA to settle out overnight at 4°C. The homogenate was then centrifuged at 3000rpm for 20 minutes at 4°C to pellet the RNA. Supernatant was removed and the pellet resuspended in 1ml LiCl/Urea, transferred into an eppendorf tube, and centrifuged for 3 minutes, 4°C. This pellet was then dissolved in 300µl TES (10mM Tris/HCl pH 7.5, 1mM EDTA, 0.5% SDS: BDH). 3M NaOAc pH5 was then added to a final concentration of 0.3M (approximately 33µl). Following phenol/chloroform (TE buffered) extraction, the RNA was precipitated from the aqueous phase with $2^1/_2$x volume of cold absolute ethanol at -20°C for 30 minutes. The RNA was pelleted by centrifuging for 5 minutes, washed by careful addition and immediate removal of 70% ethanol without disturbing the pellet, which was then resuspended in 50µl double distilled water. Yield and purity of RNA were determined by $O.D._{260/280nm}$ ratios. RNA integrity was routinely checked for every sample by running a 1 µl aliquot on a 1% agarose (Sigma type 1 low EEO) gel containing 5M urea in TBE (100mM Tris, 100mM boric acid, 5mM EDTA pH8 :BDH) at 30mA. The gel was stained with 3µl/ml ethidium bromide in TBE for 10 min. The RNA was stored at -20°C.

B6.2. Oligonucleotide primers.

B6.2 (a). Design.

Oligonucleotide primers used for PCR and DNA sequencing were kindly made by Ms. D. Chase using an Applied Biosystems 380 DNA synthesiser. Oligonucleotides for Ig RNA sequencing were designed to be complementary to previously published sequences of the 5' ends of the constant region genes, for each isotype (Cγ1,2a and 2b, Cγ3, Cµ or Cα) (Kabat *et al.*, 1991).

Primers used for PCR and DNA sequencing were designed to correspond to the 3' ends of different light-chain signal sequences as a set of 11 degenerate primers (MKV 1-11), or be complementary to the 5' end of the light-chain constant region (CK) (primers were adapted from Jones & Bendig, 1991). See tables 2 and 3.

B6.2 (b). Purification of primers from ammonia solution.

Oligonucleotide primers (1 μM) were provided in 2ml 35% ammonia with the 5' trityl group removed. These were cooled at -20°C for 30 minutes. 337.5μl of each primer was transferred to a fresh eppendorf tube and 37.5μl 3M NaOAc added to this and mixed. 1.125ml absolute ethanol was added to the tube and the contents mixed by inversion, then left to precipitate at -20°C for 1 hour (or -70°C for 10 minutes). The oligonucleotide was then centrifuged for 10 minutes, supernatant decanted and the pellet carefully washed in 1ml 80% ethanol, centrifuged for 5 minutes, vacuum dried and finally, resuspended in 50μl ddH_2O. Concentration was determined by $O.D._{260:280nm}$ and the primers stored at -20°C. An aliquot of each PCR primer was diluted to a working concentration of 5μM and stored in several smaller aliquots.

B6.3. Sequencing of antibody Heavy chains.

Isotypes of antibodies were predetermined by ELISA as described. Antibody H-chains were sequenced directly from 20μg of total cellular RNA using the same protocol as for viral HA-RNA sequencing. The primers used were designed to bind in the constant (C) region and were isotype specific (Table 2). Primers Cμ, Cα, $C\gamma_3$ or $C\gamma_1$ (for γ1, γ2a and γ2b) were all used at a concentration giving an $O.D._{260nm}$ of 1.

Special "XL mixes" of deoxynucleotides/dideoxynucleotides (2mM d/0.069mM dd) were made for long extension products of greater than 350 bases from the sequencing reaction and the XL reaction products run on 5% hydrolink Long Ranger (J.T. Baker) gels containing 8M urea and TBE.

Table 2: Nucleotide sequence of murine immunoglobulin heavy chain constant domain RNA sequencing primers.

PRIMER	5'	3'	-mer
Cμ	TTG GAA GGA CTG ACT CTC		18
Cα	GGG ATT TCT CGC AGA CTC		18
Cγ1	GGG GCC AGT GGA TAG AC		17
Cγ3	ATG GGG CTG TTG TTG TA		17

These primers were designed to bind in the immunoglobulin constant region from amino acid number 124 onward.

The primer used to sequence RNA was determined by ELISA isotyping of Mab.

B6.4. Sequencing of antibody Light chains.

Antibody L-chains were sequenced following cDNA synthesis from total RNA, PCR amplification of specific Ig L-chain DNA, and DNA product purification as described below.

B6.4 (a). Precautions to minimise cross-contamination.

Precautions were exercised for cDNA synthesis and PCR amplification of Ig light-chains based on those described by Kwok and Higuchi (1989). Since specific DNA amplification by PCR is sensitive to contamination with exogenous template DNA, all PCR reactions were set up in a biosafety lamina flow hood equipped with a UV light source. Separate pipettes were dedicated for setting up PCR. Pipettes, autoclaved pipette tips, tubes and racks were UV irradiated in the hood for 5 minutes before use to destroy contaminating exogenous DNA. All reagents other than primers, RNA, cDNA, dNTPs and enzymes were autoclaved before use.

All reagents were aliquoted into small volumes to minimise contamination by repeated sampling. Disposable gloves were worn. When setting up a reaction, each primer was dispensed and tubes closed and put away before the next primer tube was opened. All reagents were dispensed before template was introduced into the hood. Each aliquot of template was thawed, briefly centrifuged and dispensed and all tubes overlayed with oil and closed before the next antibody cDNA template was opened. Finally a "no DNA" negative control was used in each PCR reaction containing all reaction components except DNA.

Each antibody was subject to three independent PCR amplifications as products were to be sequenced. Four antibodies were amplified at a time therefore combinations were varied so the same four antibodies were never amplified together more than once, and a single antibody (11 primers) never amplified more than once at a time. Different batches of cDNA per antibody were also used for each amplification.

B6.4 (b). Synthesis of cDNA from total cellular RNA.

Adapted from Compans, 1973 and Gubler & Hoffman, 1983.

cDNA was synthesised from 10µg of total cellular RNA in a 50µl volume of buffer containing 100mM Tris pH8.3, 10mM $MgCl_2$, 200mM KCl, per antibody, with 40u Reverse Transcriptase (Boehringer Mannheim), 25u RNAsin (Amersham), 0.1µg poly dT (Boehringer Mannheim) and 2.52µl/50µl reaction of dNTP mix (final concentration of 10mM each) at 42°C for 1 hour. Samples were then boiled at 100°C for 1 minute, then quenched immediately on dry ice.

Samples were thawed and cell debris sedimented by centrifugation for 10 minutes in a microfuge. The cDNA-containing supernatant was cleaned by phenol/chloroform extraction (equal volume) followed by ethanol precipitation on dry ice with $2^1/_2$ x volume cold absolute ethanol and 100mM NaOAc for 30 minutes. After pelleting by centrifugation for 10 minutes, the cDNA was resuspended in 330µl ddH_2O for use in PCR. There are 11 L-chain primers, so cDNA was resuspended in a volume sufficient for 3 PCR reactions with all 11 primers for each antibody.

B6.4 (c). Antibody L-chain specific cDNA amplification by PCR.

cDNA corresponding to the κ light-chains was amplified using a modification of the method of Mullis and Faloona (1986, 1987). Two primers, one specific for κ light-chain leader sequence gene segments (11 different degenerate MKV primers) and the other for the Cκ gene segment were used (see table 3).

Eleven PCR reactions were set up for each antibody using one of the 11 different MKV primers in each reaction with the CK primer. Each reaction was set up in a 500µl capped tube in 100µl volumes. 5µl of each MKV primer was dispensed into each tube. A "master-mix" of 110µl of 10x reaction buffer (500mM KCl, 100mM Tris-HCl pH 8.3 and 15mM $MgCl_2$), 55µl glycerol (Sigma), 110µl dNTP mix (2.5mM each dNTP final concentration in the reaction mix), 55µl of 5µM CK primer, 7.2µl Taq polymerase (5u/µl : Perkin Elmer), 598.4µl ddH_2O and 110µl cDNA template was made up for each antibody, vortex mixed, briefly centrifuged and 95µl aliquots added to each of the 11 tubes containing

Table 3: Nucleotide sequence of murine immunoglobulin light chain variable and constant domain PCR and DNA sequencing primers.

The design of these primers is based on leader sequence associated with V regions belonging to one or more Kabat groups (Kabat *et al.*, 1991).

MKV primer 1 is based on leader sequence from Kabat group II
MKV primer 2 is based on leader sequence from Kabat group III
MKV primer 3 is based on leader sequence from Kabat group V
MKV primer 4 is based on leader sequence from Kabat group V
MKV primer 5 is based on leader sequence from Kabat group IV,V and VI
MKV primer 6 is based on leader sequence from Kabat group II
MKV primer 7 is based on leader sequence from Kabat group V
MKV primer 8 is based on leader sequence from Kabat group V
MKV primer 9 is based on leader sequence from Kabat group V
MKV primer 10 is based on leader sequence from Kabat group I
MKV primer 11 is based on leader sequence from Kabat group IV and V

MKV	5'																				3'	-mer
1	ATG	AAG	TTG	CCT	GTT	AGG	CTG	TTG	GTG	CTG												30
2	ATG	GAG	TCA	GAC	ACA	CTC	CTG	TTA	TGG	GT												29
		A						C														
3	ATG	AGT	GTG	CTC	ACT	CAG	GTC	CTG	GCG	TTG												30
									G													
4	ATG	AGG	GCC	CCT	GCT	CAG	TTT	TTT	GGC	TTC	TTG											33
		A					A	C		A	A											
5	ATG	GAT	TTT	CAG	GTG	CAG	ATT	TTC	AGC	TTC												30
			A						A													
6	ATG	AGG	TTC	TTT	GTT	CAG	TTT	CTG	GGG													27
			G	CC		C	G		C C		A											
7	ATG	GGC	TTC	AAG	ATG	GAG	TCA	CAT	TTT	CTG	G											31
		A							G	ACC		A										
8	ATG	TGG	GGA	TCT	TTT	TTC	CCT	TTT	TCA	ATT	G											31
				C		G		C	AA													
9	ATG	GTA	TCC	TCA	CCT	CAG	TTC	CTT	G													25
		G		A		G																
10	ATG	TAT	ATA	TGT	TTG	TTG	TCT	ATT	TCT													27
11	ATG	GAA	GCC	CCA	GCT	CAG	CTT	CTC	TTC	C												28
CK	ACT	GCT	CAC	TGG	ATG	GTG	GGA	AGA	TG													26

MKV primer 1 is a single oligonucleotide
2 is a mixture of 4 oligonucleotides
3 is a mixture of 2 oligonucleotides
4 is a mixture of 32 oligonucleotides
5 is a mixture of 4 oligonucleotides
6 is a mixture of 256 oligonucleotides
7 is a mixture of 64 oligonucleotides
8 is a mixture of 32 oligonucleotides
9 is a mixture of 8 oligonucleotides
10 is a single oligonucleotide
11 is a single oligonucleotide

Total number of oligonucleotides = 405

the MKV primers, then each tube overlayed with 2 drops of mineral oil (Sigma) to prevent evaporation. A negative control tube was also made up at the samt time, containing everything but template DNA in a 100µl volume. Four antibodies were PCR amplified at a time in a Perkin Elmer Cetus DNA thermal cycler heating block through 25 cycles using the following template profile (Files 30-32) :-

Cycle 0	94°C	$1^1/_2$ min. for cDNA strand separation and primer annealing
Cycles 1-25	94°C	1 min. for strand separation,
	50°C	1 min. for primer annealing,
	72°C	1 min. for elongation
Cycle 26	72°C	10 min. for final elongation

Once amplified, products were stored at 4°C. PCR products were visualised by running a 5µl sample together with 1µl sucrose loading dye (45% sucrose, 0.25% bromophenol blue) on a 1% agarose gel in 1x TBE (100mM Tris, 100mM boric acid, 5mM EDTA pH8, 0.5mg/ml ethidium bromide) at 100V until the dye front had migrated $^2/_3$ down the gel. Molecular weight markers used were 1µl each of Boehringer Mannheim molecular weight markers V and VI .

B6.4 c (i). Purification of PCR products for sequencing.

Purification of PCR products was undertaken to remove unincorporated dNTPs, primers and degenerate products of the incorrect size range. Following amplification, a 20µl sample of the aqueous phase was transferred to a well of a mini 60 well microtitre plate (Nunc) with 6µl sucrose dye mix and loaded equally into two adjacent wells of a 1% low melting point agarose (FMC SeaPlaque : Sigma) gel in TBE (containing ethidium bromide at 1.5µg/ml). Molecular weight markers V and VI were also loaded onto the gel which was run at 100V as before. The gel was then viewed on a low intensity UV light box and bands corresponding to PCR products of the correct size (350-450 bases for Igκ) exised and placed in 1.5 ml eppendorf tubes.

DNA was recovered from the gel slices by melting the agarose at 75°C for 30 minutes followed by column purification using the Promega "Magic™ PCR Preps DNA

purification system for Rapid Purification of DNA Fragments" in accordance with the manufacturers' instructions.

Briefly, 1ml of Magic PCR Preps resin (guanidinium thiocyanate:GuSCN), prewarmed for 30 minutes at 37°C to dissolve any crystals, was added to the melted agarose slice and vortex-mixed. This was pipetted into a 2ml syringe barrel and gently pushed into the Magic PRC Prep Mini-column (one column per product) with the plunger. The syringe was detatched from the column, plunger removed and the syringe replaced on the column. To wash the column, 2ml of 80% isopropanol (BDH) was pipetted into the syringe and gently pushed through. The mini-column was rewashed and then centrifuged for 1 minute to dry the resin, and transferred onto a fresh 1.5 ml eppendorf tube. 50µl of ddH_2O was added directly onto the column which was centrifuged 10 minutes later for 1 minute to elute the DNA from the column. This was repeated and the elutants pooled. A 2µl 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 enable estimation of concentration of DNA in the sample for sequencing. Purified DNA samples were stored at -20°C.

B6.4 c (ii). Direct sequencing of PCR products.

The sequences of PCR amplified Ig L-chains were determined using a modified chain termination method (Sanger *et al.*, 1977). Sequencing was carried out from both the 5' end of the Ig V-region, using the same MKV primers used for amplification, and the 3' end using the CK primer, for each product.

2µl of purified PCR product and up to 1µl of primer were mixed to a template:primer ratio of between 1:35 and 1:70, and 3µl annealing mix (125mM Tris-HCl pH7.5, 50mM $MgCl_2$, 100mMNaCl, 20% DMSO) added. After incubation at 100°C for 3 minutes to denature the template, the samples were immediately quenched on dry ice to minimise renaturation. The samples were thawed, briefly centrifuged and 4µl labelling mix added to each (25mM DTT, 2.6u Sequenase 2.0™ T7 DNA polymerase and 10µCi ^{35}S-dATP (1µl) per template). This was then divided equally between 4 wells of a mini 60 well disposable conical bottom microtitre plate (Nunc) per sample, each containing 2µl of 160µM dCTP, dGTP, dTTP, 100mM NaCl, 20% DMSO, and 160µM dATP (well A), 8µM

ddCTP (well C), 8μM ddGTP (well G) or 8μM ddTTP (well T). The plate was then incubated at 37°C for 5 minutes. 2μl of chase mix (0.25mM of each dNTP, 50mM NaCl, 10% DMSO) was added to each well and the plate incubated for a further five minutes at 37°C before the reaction was stopped by addition of 6μl per well of formamide gel loading buffer "stop mix" as for RNA sequencing and either stored at -20°C (for no longer than two days) or run immediately on a denaturing 8% polyacrylamide gel containing 8M urea in TBE, or a 5% hydrolink gel, as for RNA sequencing, after boiling for 3 minutes. Gels were dried for $1^1/_2$ 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 manually.

B6.5. Sequence analysis

Sequences were aligned using a Genetic Data Environment 2.2 (GDE) software package on the Sun Unix system, and multiple sequence homologies calculated using an in-house adapted "readseq" programme to generate pir format, "Clustalv" for multiple sequence format (both by courtesy of Nigel Douglas, N.I.M.R.), and "Distances" in GCG (Genetics Computer Group sequence analysis software package version 7.3-Unix, Madison,Wisconsin) to calculate homology between sequences, without gaps and to the length of the shorter sequence, with a value of 1.0 representing complete sequence identity. Sequence comparison to other EMBL (database release 38) Ig sequences and assignment of V-region families were also possible using BLASTN (basic local alignment search tool for nucleotide sequences : Altschul *et al.*, 1990) in GCG, and Kabat and Wu (1991).

Chapter 1

Structural analysis of secondary antibody response of BALB/k mice to influenza virus haemagglutinin following natural infection.

C1. INTRODUCTION

Five major antigenic regions have previously been defined on the HA1 polypeptide. Earlier studies, defining Mab specificities obtained by immunisation of BALB/c mice, documented extensive diversity in the secondary antibody response based on haemagglutination inhibition reactivity patterns for variant viruses (Staudt & Gerhard, 1983; Underwood, 1984), with the response comprised of an estimated 1500 paratypically distinct antibodies. However, a recent study in this laboratory (Smith *et al.*, 1991) indicated striking immunodominance in the secondary antibody response of *CBA/Ca* mice following *natural infection*, as deduced by sequencing the HA genes of *in ovo* Mab-selected escape mutant viruses.

This study established first, that the majority of antibodies generated from five individual donors selected mutant HA1 158(Gly→Glu), and second, that minor specificities recognised different regions of HA1 than BALB/c mice. Previous work has suggested that BALB/c antibodies are directed mainly against site B. Recent investigations in this laboratory have found that BALB/c neutralising antibody response to HA, following natural infection predominantly recognise site HA1 198 (C.A.Smith, personal communcation).

Since $CD4^+$ T-cell recognition specificities have also been defined for these two strains, Ia^k focusing on sites A and C, and Ia^d on sites B and E, the question of a causal association between MHC and the selected antibody repertoire arises.

Since we have complete and extensive knowledge of CBA/Ca Ia^k and BALB/c Ia^d antibody and T-cell responses to influenza HA, and these two strains differ in the recognition sites for both antibodies and T-cells, I wished to investigate possible effects of MHC on the selected neutralising antibody repertoire.

For this purpose, I undertook a structural analysis of the neutralising antibody recognition sites for BALB/k MHC congenic mice by sequencing of the HA genes of laboratory mutant viruses selected with Mab from individual donors following X31 infection.

C2. RESULTS

C2.1. Generation of influenza HA specific B-cell hybridomas from BALB/k mice.

Protective immunity to influenza infection is mediated by neutralising antibodies against its surface glycoprotein HA, focusing on the HA1 polypeptide and clustering around the receptor binding site. Previous studies had identified the neutralising antibody recognition sites of two inbred strains of mice, CBA/Ca (H-2^k) and BALB/c (H-2^d) and found immunodominance, and focusing of antibodies from the different strains to different regions on HA1, CBA/Ca to HA1 158, and BALB/c to HA1 198.

Investigation of the neutralising antibody response to HA under identical conditions of infection, of an MHC congenic strain, BALB/k (H-2^k) was undertaken to assess possible MHC effects on the selected antibody repertoire, as described in this chapter.

The following strategy was employed: BALB/k mice were infected *intranasally* with live X31 influenza virus (H3N2), boosted i.p. six to eight weeks later with inactivated virus and spleens taken 3 days later for B-cell hybridoma and monoclonal antibody production. Splenocytes from five individual donors were fused with murine myeloma cells to generate B-cell hybridomas secreting HA-specific neutralising Mab, as determined by inhibition of haemagglutination (HI) of turkey erythrocytes.

Ninety-two HA1 specific Mabs were derived from hybridomas of the SFA series from five individual donors. Hybridomas were cloned by limiting dilution for Mab production. A total of forty-seven HA-specific Mab producing hybridomas (7-13 per donor) were used to select neutralisation escape mutant viruses *in ovo* for assignment of antibody fine specificities.

C2.1 (a). Optimisation of plaque purification cloning and *in vitro* Mab selection of escape mutants of X31 virus.

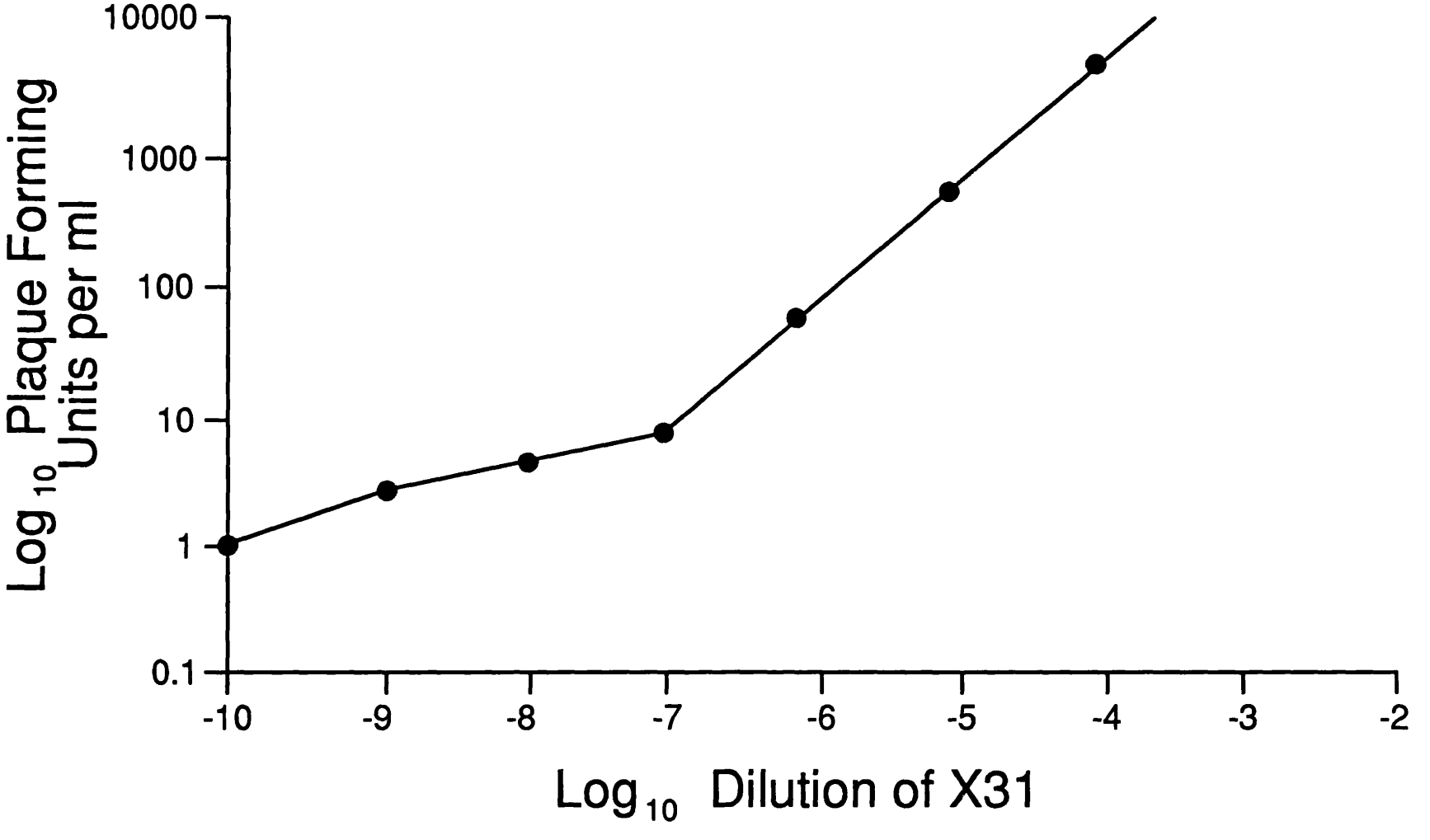
The plaque purificaton method was used for cloning of *in ovo* Mab selected escape mutants since the clonality of virus from eggs at various dilutions is questionable, whereas

Table 4: Plaque purification standard titration of X31 virus on CEF monolayers (p.f.u./ml per X31 dilution).

Dilution of X31 virus	Plaque forming units per ml
10^{-2}	confluent
10^{-3}	confluent
10^{-4}	confluent
10^{-5}	376
10^{-6}	51
10^{-7}	6
10^{-8}	3
10^{-9}	2
10^{-10}	1

Figure 9: Plaque purification standard titration curve of X31 virus grown on chick embryo fibroblast (CEF) monolayers.

Dilutions of X31 from 10^{-2} to 10^{-10} versus plaque forming units per ml per $5cm^3$ plate are shown on a logarithmic scale. Dilutions between 10^{-6} and 10^{-8} give single discrete plaques.


10000
1000
100
10
1
0.1
Log 10 Plaque Forming Units per ml
-10
-9
-8
-7
-6
-5
-4
-3
-2
Log 10 Dilution of X31

by plating at the correct dilutions a single plaque should result from a single virus particle infection which is clearly detectable on the monolayer.

Before the plaque purification method could be used routinely for cloning virus (by limiting dilution) the assay had to be maximised for efficiency and the optimum conditions established for our purposes. This involved the titration of X31 virus to define the dilution, relative to HAU necessary for obtaining single plaques, hence clonal virus populations on CEF monolayers.

Dilutions of X31 in saline/BSA 10^{-2} to 10^{-10}, were plated and plaques counted three days later. The concentration of virus in HAU/50µl was measured by haemagglutination of turkey erythrocytes (HA), and a titration curve of virus dilution (hence HAU/50µl) versus $\log_{10}$ pfu/ml plotted (Table 4, Figure 9). From this graph, the optimum dilution for yielding single plaques was calculated to fall in the 10^{-6} to 10^{-8} range where single discrete plaques were observed for X31.

A correlation between pfu and HAU was found from this titration in that, at the optimal dilution for single plaques (6 at 10^{-7} dilution) for a virus that has 1280 HAU/50µl and $6x10^7$ pfu/ml, there are approximately 2000 pfu/HAU. By this method, it was also possible to deduce the dilution necessary for single plaques, i.e. X31 has 1280 HAU/50µl which is approximately $2x10^5$ HAU/ml and hence $4x10^8$ pfu/ml and at 1 ml per plate of virus, a dilution of 10^{-8} would give 4 plaques, and 10^{-7} would give 40 plaques, also separate single plaques at this plating level, which is in agreement with the results from the X31 titration of dilution factor 10^{-6} to 10^{-8} range for single plaques.

C2.1 (b). Antibody isotype distribution of BALB/k derived HA-specific Mabs.

Forty-seven Mabs were used to select mutants *in ovo*. The isotypes of these were determined by ELISA (Table 5, Figures 10 and 11) and were found, overall, to be predominantly IgG2a (36%), followed by the other IgG isotypes (IgG1=21%, IgG2b and IgG3=13% each) and 8.5% each of IgA and IgM, typical of a secondary response to a viral infection (Coutelier *et al.*, 1987, 1991)

At the individual level, antibodies from donors 2B, 3B, 6B and 9B were predominantly of the IgG2a isotype, whereas antibodies from donor 7B were predominantly IgG3.

Table 5: Isotypes of BALB/k HA1 specific Mab from five individual donors in the secondary response to X31 influenza natural infection.

BALB/k Donor									
SFA 2B		SFA 3B		SFA 6B		SFA 7B		SFA 9B	
B-cell hybridoma clone	monoclonal antibody isotype	B-cell hybridoma clone	monoclonal antibody isotype	B-cell hybridoma clone	monoclonal antibody isotype	B-cell hybridoma clone	monoclonal antibody isotype	B-cell hybridoma clone	monoclonal antibody isotype
1.1	γ1	1.1	γ1	1.1	γ1	6.1	γ2a	8.1	γ1
7.2	γ1	3.1	γ1	7.1	γ1	9.1	γ2a	3.1	γ2a
8.1	γ1	12.1	γ1	6.2	γ2a	19.1	γ2a	2.1	γ2a
9.1	γ1	2.1	γ2a	8.1	γ2a	11.1	γ2b	4.1	γ2b
3.1	γ2a	10.1	γ2a	3.1	γ3	3.1	γ3	6.1	γ2b
2.1	γ2a	4.1	γ2a	5.1	α	5.1	γ3	1.1	γ3
4.2	γ2a	6.1	γ2a	4.2	μ	8.1	γ3	5.1	μ
5.2	γ2a	9.1	γ2b			13.1	γ3		
6.1	γ2a	7.1	γ2b			10.2	α		
10.2	γ2a	14.1	γ2b			1.2	α		
						7.1	α		
						2.1	μ		
						15.1	μ		

Figure 10: Dot plot of monoclonal antibody isotype profiles.

Distribution of monoclonal isotypes in the secondary response of five individual BALB/k mice to X31 influenza infection. The predominant isotype per donor is indicated. Each dot represents one Mab.

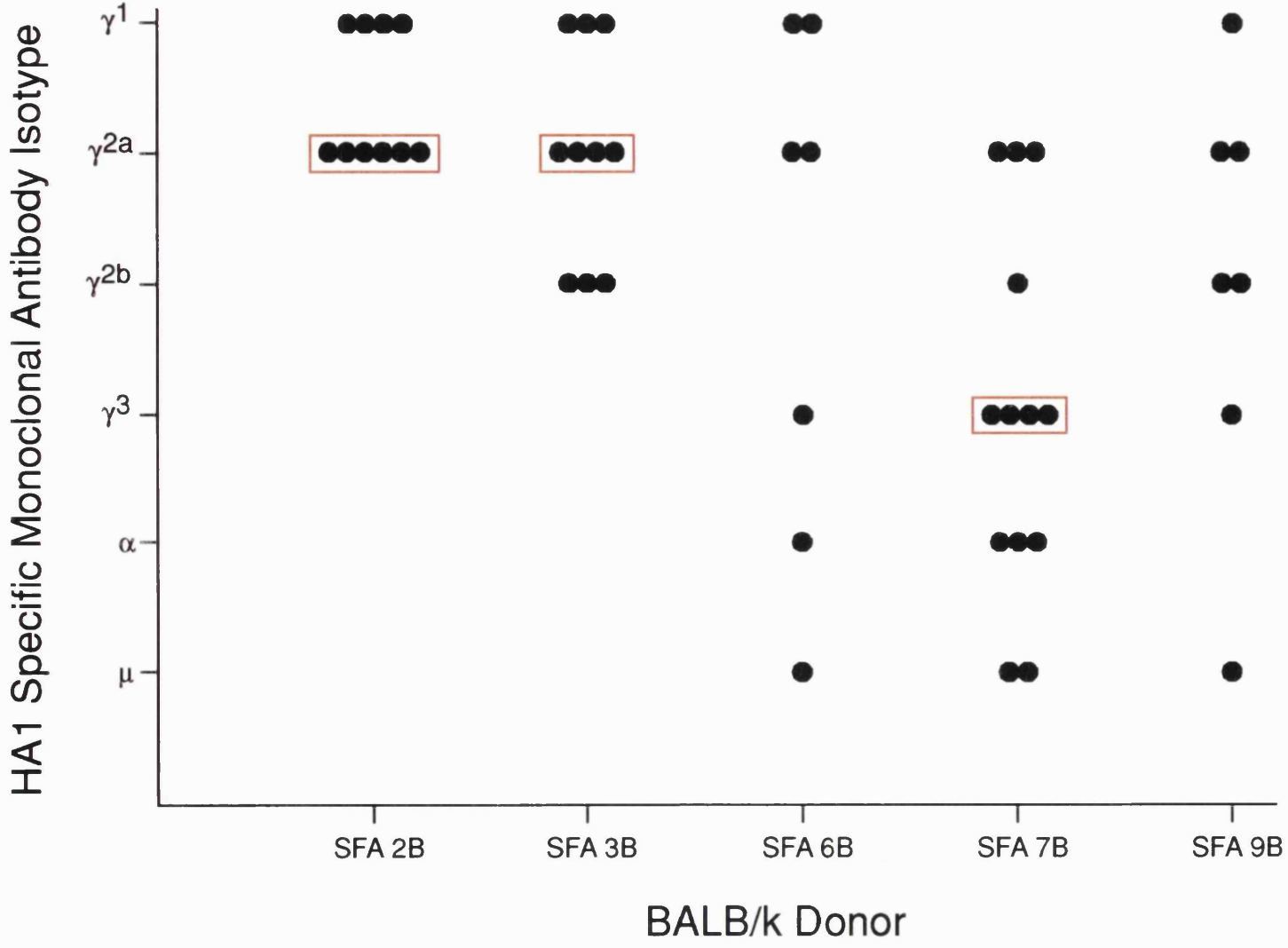
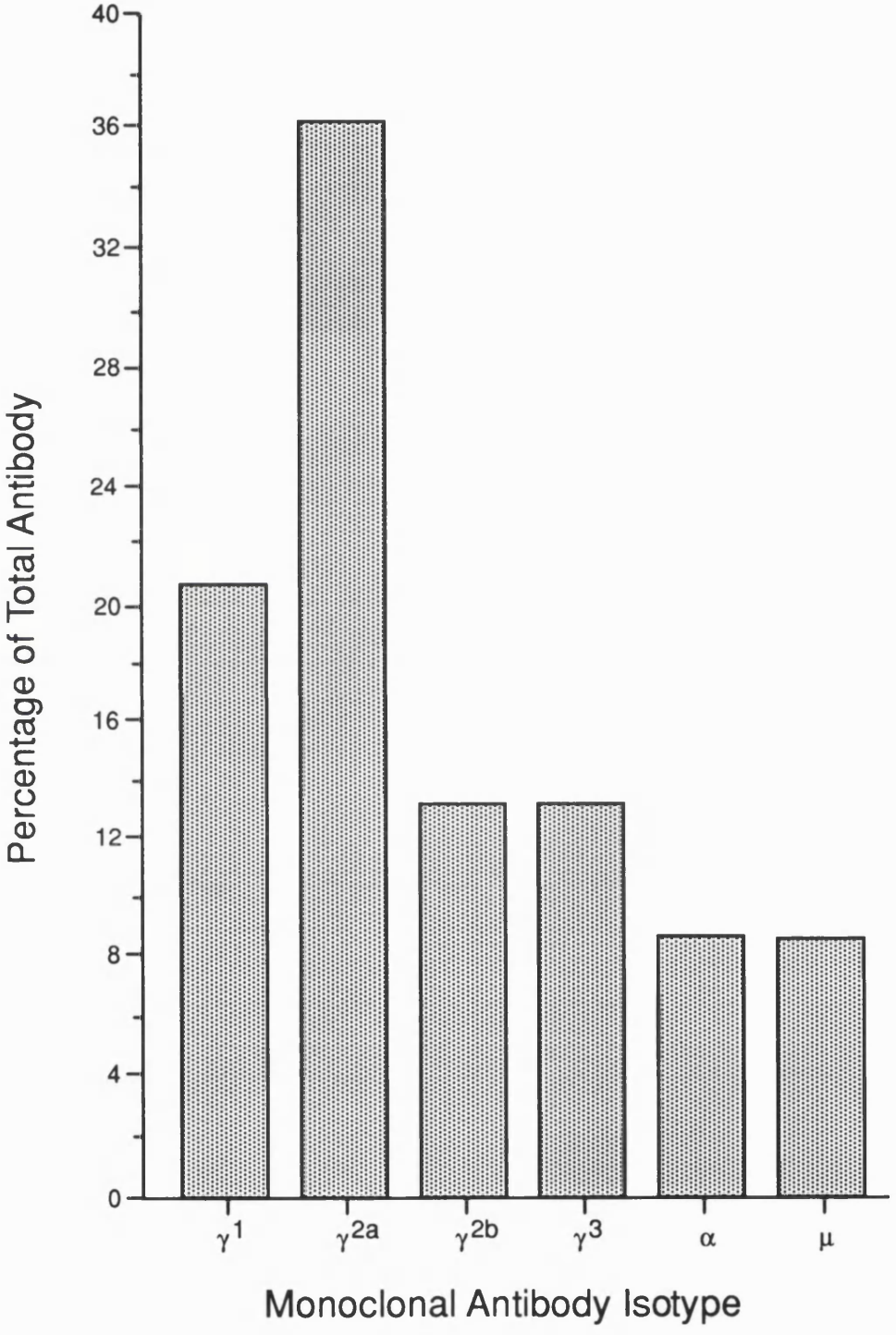

HA1 Specific Monoclonal Antibody Isotype
γ1
γ2a
γ2b
γ3
α
μ
SFA 2B
SFA 3B
SFA 6B
SFA 7B
SFA 9B
BALB/k Donor

Figure 11: Histogram of distribution of Mab isotype overall from fifty secondary response HA1-specific Mab from five BALB/k donors.


Percentage of Total Antibody
40
36
32
28
24
20
16
12
8
4
0
γ1
γ2a
γ2b
γ3
α
μ
Monoclonal Antibody Isotype

C.2.2. Definition of antibody recognition sites by *in ovo* Mab selection of neutralisation escape mutants.

The ability to propagate influenza virus *in ovo* makes definition of antibody recognition sites relatively straight-forward and accurate. Aliquots of X31 (reference virus) were incubated with Mabs and then inoculated into eggs. Mabs neutralised all viruses except those with a substitution at their specific recognition site, so any viruses recovered from the eggs three days later were neutralisation escape mutants. By sequencing the HA genes of escape mutants and comparing the sequences with that from the reference virus (X31), the antibody recognition sites can be unequivocally defined to a single base change, (hence amino acid).

To determine the specificity of the neutralizing antibody response of these BALB/k mice following natural infection with X31, fifty mutants were generated and named the BAM series (1-50) where BAM 5, 48 and 49 were duplicate selections as indicated in the results tables. The HA regions of these mutants were subsequently sequenced since this method usually results in a single base change (or two at most, as reported by Wiley *et al*, 1981) in the HA1 sequence. Hence definitive assignment of the antibody binding site can be achieved for each Mab, to a single amino acid substitution (Figures 12 and 13, Tables 6-10).

The most common occurring mutation was in residue HA1 198(A→E) accounting for 42.5% of clones sequenced here, with HA1 158 being found in 23.4% and the remainder scattered throughout the HA1 molecule, in site A (16.67%) or site E (4%) with one of the 47 in site C at residue 53, Table 11.

C2.3. Structural analysis of BALB/k Mab recognition sites.

Previous studies have identified five antigenic regions on HA1 (Wiley *et al.*, 1981; Wilson *et al.*, 1981). Immunodominance of antibody rcognition of a single amino acid have also previously been demonstrated in the CBA/Ca secondary antibody response, directed against HA1 158. It is evident from this investigation that overall, all five antigenic sites are seen by BALB/k antibodies, but there is also evidence of an immunodominant antigenic site, both in the collective study and at the individual level.

Figure 12: Dot plot representation of Mab specificity for HA1 by amino acid substitutions in Mab selected neutralisation escape mutant viruses from five individual BALB/k donors.

Each dot represents one Mab. The predominant recognition sites per donor are indicated.

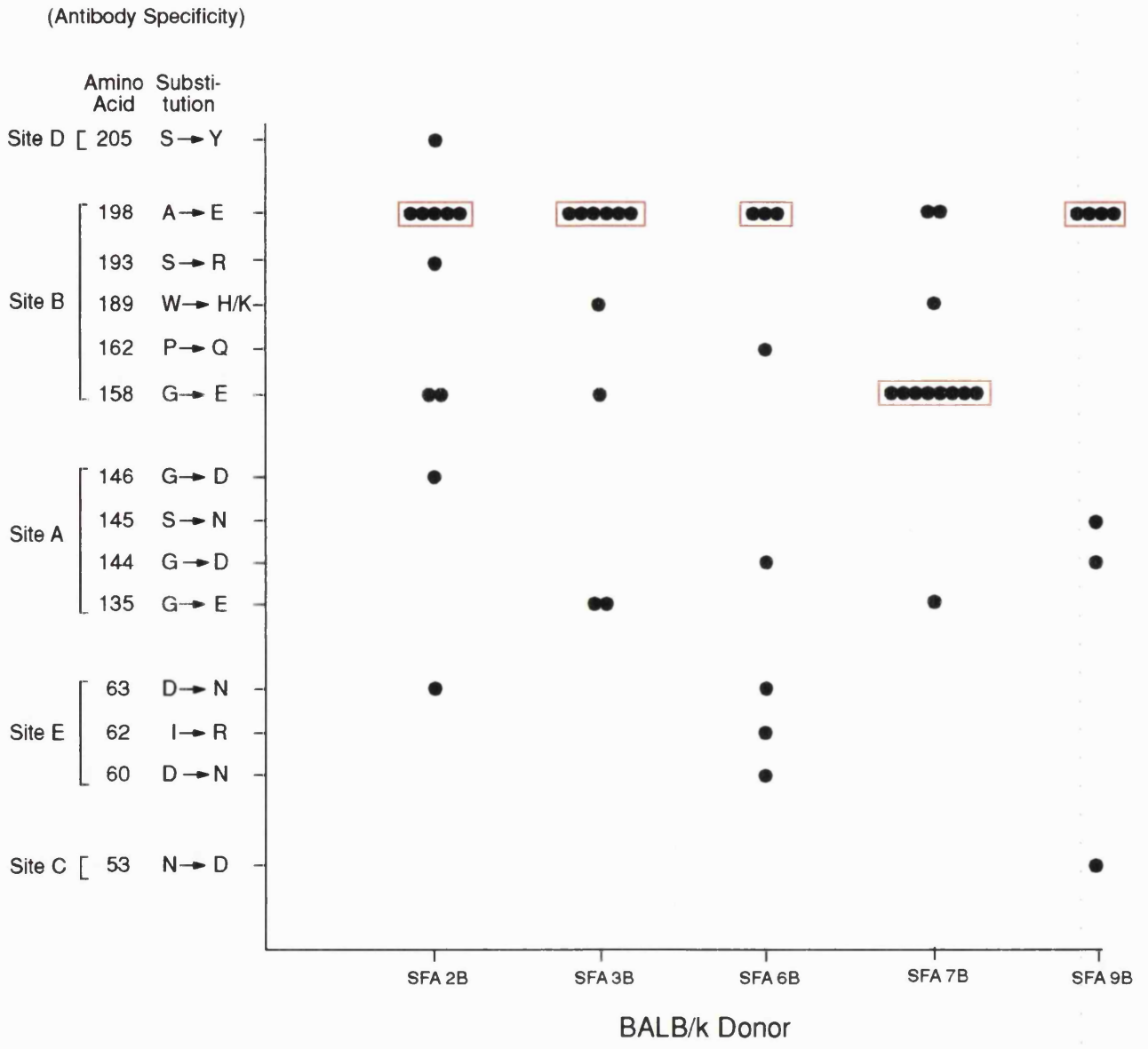

(Antibody Specificity)
Amino Acid
Substi-tution
Site D
205
S→Y
Site B
198
A→E
193
S→R
189
W→H/K
162
P→Q
158
G→E
Site A
146
G→D
145
S→N
144
G→D
135
G→E
Site E
63
D→N
62
I→R
60
D→N
Site C
53
N→D
SFA 2B
SFA 3B
SFA 6B
SFA 7B
SFA 9B
BALB/k Donor

Figure 13: Schematic diagram of the 3D structure of HA1 monomer indicating sites identified in this study as recognised by BALB/k (arrows),immunodominant sites, and residues that have featured in antigenic drift of the H3 subtype viruses (dots).

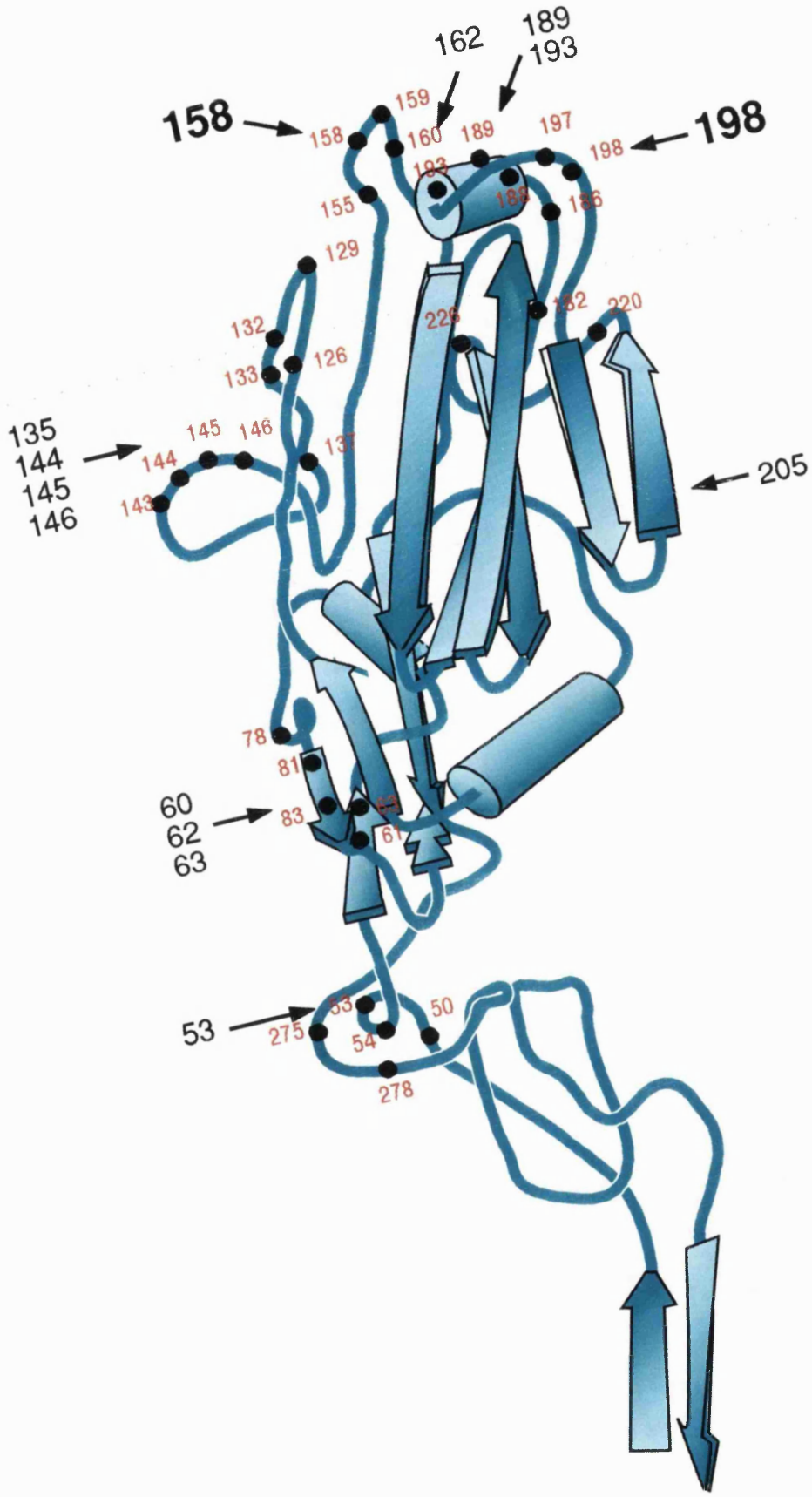

158
162
189
193
198
135
144
145
146
205
60
62
63
53
159
158
160
189
197
198
193
188
186
155
129
132
133
126
226
182
220
145
146
144
143
137
78
81
83
61
50
275
54
278

Table 6: Recognition specificities of Mab from BALB/k donor SFA 2B as defined by nucleotide and amino acid substitutions defined by RNA sequencing of the HA gene of Mab selected neutralisation escape mutant viruses (BAM) *in ovo*.

Selection of a second BAM mutant by the same antibody is indicated in brackets.

Influenza HA1 site	B-cell hybridoma clone (Mab) SFA 2B	Mab selected mutant virus BAM series	Recognised amino acid (HA1)	Amino acid substitution in escape mutant virus	Nucleotide number in HA gene	Nucleotide substitution to escape neutralisation
site E	10.2	29 (48)	63	D→N	264	G→A
site A site B	6.1	18	146 158	G→D G→V	514 550	G→A G→T
site B	2.1	8	158	G→E	550	G→A
	4.2	9	193	S→R	656	C→A
	1.1	7	198	A→E	670	C→A
	7.2	19	198	A→E	670	C→A
	8.1	30	198	A→E	670	C→A
	9.1	24	198	A→E	670	C→A
	3.1	1	198	A→E	670	C→A
site D	5.2	2	205	S→Y	691	C→A

Table 7: Recognition specificities of Mab from BALB/k donor SFA 3B as defined by nucleotide and amino acid substitutions defined by RNA sequencing of the HA gene of Mab selected neutralisation escape mutant viruses (BAM) *in ovo*.

Selection of a second BAM mutant by the same antibody is indicated in brackets.

Influenza HA1 site	B-cell hybridoma clone (Mab) SFA 3B	Mab selected mutant virus BAM series	Recognised amino acid (HA1)	Amino acid substitution in escape mutant virus	Nucleotide number in HA gene	Nucleotide substitution to escape neutralisation
site A	6.1	20	135	G→E	481	G→A
	9.1	31	135	G→E	481	G→A
site B	4.1	3	158	G→E	550	G→A
	1.1	10	189	W→H	644	A→T
	2.1	35	198	A→E	670	C→A
	3.1	12	198	A→E	670	C→A
	7.1	21	198	A→E	670	C→A
	10.1	36	198	A→E	670	C→A
	12.1	23	198	A→E	670	C→A
	14.1	22	198	A→E	670	C→A

Table 8: Recognition specificities of Mab from BALB/k donor SFA 6B as defined by nucleotide and amino acid substitutions defined by RNA sequencing of the HA gene of Mab selected neutralisation escape mutant viruses (BAM) *in ovo*.

Selection of a second BAM mutant by the same antibody is indicated in brackets.

Influenza HA1 site	B-cell hybridoma clone (Mab) SFA 6B	Mab selected mutant virus BAM series	Recognised amino acid (HA1)	Amino acid substitution in escape mutant virus	Nucleotide number in HA gene	Nucleotide substitution to escape neutralisation
site E	3.1	4	60 63	D→N D→N	255 264	G→A G→A
	8.1	34	62	I→R	262	C→G
site A	6.2	11	144	G→D	508	G→A
site B	7.1	33	162	P→Q	562	C→A
	1.1	32	198	A→E	670	C→A
	4.2	41	198	A→E	670	C→A
	5.1	13	198	A→E	670	C→A

Table 9: Recognition specificities of Mab from BALB/k donor SFA 7B as defined by nucleotide and amino acid substitutions defined by RNA sequencing of the HA gene of Mab selected neutralisation escape mutant viruses (BAM) *in ovo*.

Selection of a second BAM mutant by the same antibody is indicated in brackets.

Influenza HA1 site	B-cell hybridoma clone (Mab) SFA 7B	Mab selected mutant virus BAM series	Recognised amino acid (HA1)	Amino acid substitution in escape mutant virus	Nucleotide number in HA gene	Nucleotide substitution to escape neutralisation
site A	6.1	50	135	G→E	481	G→A
site B	2.1	25	158	G→E	550	G→A
	3.1	26	158	G→E	550	G→A
	5.1	14	158	G→E	550	G→A
	8.1	27	158	G→E	550	G→A
	10.2	16	158	G→E	550	G→A
	11.1	28	158	G→E	550	G→A
	15.1	40	158	G→E	550	G→A
	19.1	42	158	G→E	550	G→A
	9.1	38	189	W→K	642	C→A
	1.2	37	198	A→E	670	C→A
	7.1	15	198	A→E	670	C→A

Table 10: Recognition specificities of Mab from BALB/k donor SFA 9B as defined by nucleotide and amino acid substitutions defined by RNA sequencing of the HA gene of Mab selected neutralisation escape mutant viruses (BAM) *in ovo*.

Selection of a second BAM mutant by the same antibody is indicated in brackets.

Influenza HA1 site	B-cell hybridoma clone (Mab) SFA 9B	Mab selected mutant virus BAM series	Recognised amino acid (HA1)	Amino acid substitution in escape mutant virus	Nucleotide number in HA gene	Nucleotide substitution to escape neutralisation
site C	2.1	44(49)	53 125	N→D F→F	234 452	A→G C→T
site A	5.1	46	108 145	L→L S→N	401 511	T→A G→A
	4.1	45	144	G→D	508	G→A
site B	1.1	43	198	A→E	670	C→A
	3.1	17	198	A→E	670	C→A
	6.1	6	198	A→E	670	C→A
	8.1	47	198	A→E	670	C→A

Table 11: The amino acid substitutions and frequency in escape mutant viruses selected *in ovo* by Mab derived from individual BALB/k donor mice.

a) double mutant of 146(G→D) with 158(G→V)

b) double mutant of 145(S→N) with silent mutation 108(L→L)

c) double mutant of 53(N→D) with silent mutation 125(G→V)

d) double mutant of 60(D→N) with 63(D→N)

BALB/k Donor mice	HA1 amino acid substitutions in monoclonal antibody selected escape mutant viruses															
	53 N→D	60 D→N	62 I→R	63 D→N	108 L→L	125 F→F	135 G→E	144 G→D	145 S→N	146 G→D	**158** G→E	162 P→Q	189 W→H/K	193 S→R	**198** A→E	205 S→Y
SFA 2B				1						1[a]	2			1	5	1
SFA 3B							2				1		1		6	
SFA 6B		1[d]	1	1				1				1			3	
SFA 7B							1				8		1		2	
SFA 9B	1				1[b]	1[c]		1	1						4	
Total Frequency (%)	2.18	2.18	2.18	4.36	2.18	2.18	6.54	4.36	2.18	2.18	**23.98**	2.18	4.36	2.18	**43.6**	2.18

C2.3 (a). Immunodominant antigenic site HA1 198 (A→E).

In four out of five donor mice, for which antibody specificity was defined, (donors 2B, 3B, 6B and 9B), the majority of antibodies (greater than 50% per donor) focused on HA1 198_{Ala} as deduced from the single base change at nucleotide 670 C→A. Substitution at 198 also accounted for 42.5% of the total antibody from all five donors, with minor specificities for the other sites present for each individual. HA1 198 is in site B, which has previously been reported as the predominant site recognised by BALB/c mice (Figure 14).

C2.3 (b). Immunodominant antigenic site HA1 158 (G→E) for donor 7B.

Antibodies recognising HA1 158 represent the second most frequently recognised site, accounting for 23.4% of total antibody overall. Eight of the twelve HA1 158-specific antibodies from all five donors, are from donor SFA 7B, equivalent to 75% of antibodies generated from this donor. Laboratory mutants with amino acid subsitutions at this postion are frequently obtained in the CBA/Ca H-2^k antibody response to X31 by this laboratory (Smith *et al.*, 1991).

C2.3 (c). Isolation of four minor antigenic variants (HA1 62, 162, 205 and 53).

Mab SFA 6B 8.1 defined a *novel* antigenic specificity at HA1 62 (I-R), within site E. This substitution has not been reported in natural variants of H3 subtype to date, although a similar change has been reported for avian H3 isolates (Webster *et al.*, 1992).

A second novel laboratory mutant with a change at position HA1 162 (P-Q) was also isolated from donor 6B (clone 7.1). This substitution has not been previously reported for either laboratory mutants or natural variants of H3 subtype.

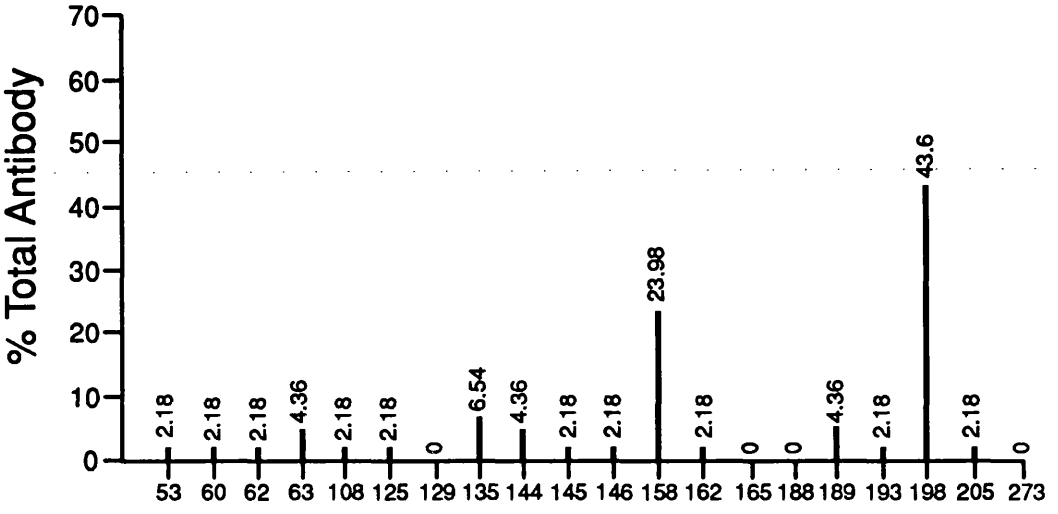
The third antigenic mutant was selected by antibody from donor 2B 5.2, with a substitution at position 205 (S-Y). This substitution has not been reported in natural variants of the H3 subtype, but laboratory mutants have been isolated previously (Underwood, 1984; Webster *et al.*, 1992).

Figure 14: Line graph of the Mab recognition specificities as a percentage of the total response. A comparison of the overall secondary response of BALB/k and CBA/Ca mice to X31 influenza virus HA1 and immunodominant recognition sites.

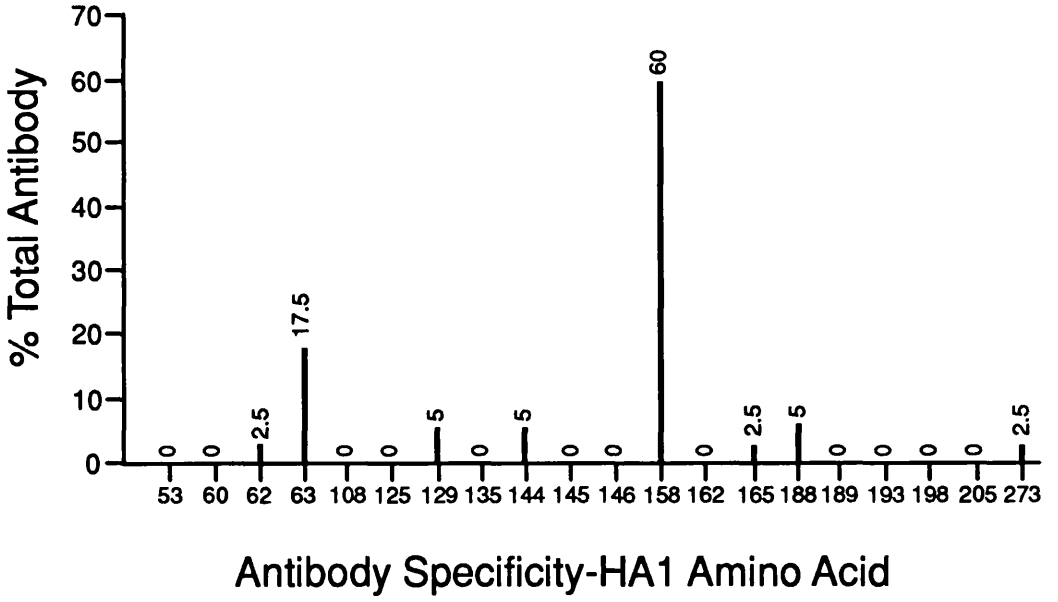
a) The overall BALB/k Mab secondary response. HA1 198 is the immunodominant recognition site.

b) The overall CBA/Ca Mab secondary response. HA1 158 is the immunodominant recognition site.

a) BALB/k



b) CBA/Ca



C2.3 (d). A novel laboratory mutant for antigenic site C at HA1 53.

In the 3D structure of the HA monomer two loop regions to the bottom of the HA1 subunit represent a major antigenic site, denoted antigenic site C on the basis of sequence variation in natural variant viruses of the H3 subtype, with substitutions at HA1 50, 53 and 54 and HA 1 275 and 278. However the antigenic status of the region had not been confirmed by the isolation of laboratory mutants (using without exception BALB/c derived Mabs). This laboratory has recently isolated a novel mutant virus of X31, HA1 273, using Mab from CBA/Ca (H-2^k) mice thereby confirming the antigenicity of the region for neutralizing antibody responses.

I have now obtained a further example by the isolation of a mutant virus HA1 53(Asp→Asn) using Mab from donor 9B. Interestingly, earlier work from this laboratory (Graham *et al.*, 1989) had shown that A^k- restricted T-cell clones recognize a synthetic peptide HA1 48-67 and were sensitive to the HA1 53 change in both natural variants (e.g. A/Tex/77 or A/BK/79) and a synthetic peptide analogue. This illustrates the focusing of B and T-cell responses to common antigenic sites within a given haplotype.

C2.3 (e). Minor components of the BALB/k response.

Other mutants obtained in this study contain single substitutions at positions within antigenic site A (with the exception of BAM 18 which has mutations at both HA1 146(G→D) and 158(G→V)) at HA1 135(G→E), 144(G→D) and 145(S→N), and site B at residues HA1 189(E→H) and 205(S→Y) representing a minor component of the BALB/k repertoire. Mutations at these positions have been reported elsewhere for X31 variants selected by Mab from BALB/c mice.

This provides evidence of antigenicity for all five antigenic sites. The collective data for all five donors shows that each of the five sites are recognised by at least one antibody from the forty-seven generated in this study, but also emphasises the importance of undertaking such a study for individual mice due to the differences in specificities per donor and the observed immunodominance, particularly with reference to donor SFA 7B.

C3. CONCLUSION

Many studies defining antigenic regions of proteins use western blotting and peptide based ELISA assays, and although some epitopes may thus be defined, the majority are overlooked due to lack of native protein conformation in these assay systems, hence results may be misleading and incomplete. Influenza virus offers a unique, definitive method in which antibody recognition is established using the virus itself, i.e. whole, native antigen, by Mab-selection of neutralisation escape mutants *in ovo* and sequence comparison of the mutant HA gene with that of wild type reference virus (X31), for definition of the antibody recognition sites, not just to a single amino acid, but to a single base change which when substituted, alters antigenicity of the protein (Brand & Skehel, 1972; Knossow *et al.*, 1984). The validity of this approach has been confirmed by crystallographic data for escape mutants and high resolution EM studies of antibody-antigen complexes (Wiley *et al.*, 1981; Wilson *et al.*, 1981; Amit *et al.*, 1985, 1986; Wilson & Cox, 1990; Wilson & Stanfield, 1993).

This technique has been extensively used to determine antigenic sites on influenza HA (Gerhard *et al.*, 1981; Caton *et al.*, 1982; Underwood, 1982; Staudt & Gerhard, 1983) and most laboratory mutants are located within sites which have featured in antigenic drift, confirming immune selection pressure for change (Wiley *et al.*, 1981).

This study has confirmed antigenicity of all five previously designated sites by the presence of antibodies from five donors which recognise sites scattered all over the HA1 ectodomain, but mapping to these five sites in concordance with previous studies (Gerhard *et al.*, 1981; Staudt & Gerhard, 1983). In addition however, repertoire analysis at the level of the individual donor has highlighted the significance of immunodominance. Whilst a generalisation can be made for diversity in the humoral immune response, on closer inspection, it is evident that not all five sites are seen by each individual. For example, donor 3B has eight out of ten antibodies directed against site B (HA1 (158), 189 and 198) and two against HA1 135 in site A, but none against sites C, D or E, which are represented when antibody specificities for the other four donors are examined, (e.g. donor 9B, HA1 53 in site C, donor 2B, 205 in site D and donors 2B and 6B, 60-63 in site E). Similarly, donor 2B recognises site B (198, (158)), site D (205) and site E (63), donor 6B with antibodies to

site A (144) and site E (60, 62 and 63), donor 7B to site A (135) and site B ((158), 189 and 198) and donor 9B to site A (144 and 145) and site B (198).

This study has identified three novel antigenic mutants, HA1 62(I→R), only reported previously in avian isolates and CBA/Ca Mab-selected mutants, HA1 162(P→Q) in site B and 53(N→D) in site C, which have been found in natural variants, but not isolated in laboratory mutants before.

Some bias to the IgG2a isotype, was also observed in this investigation, representing 36% of the total. This isotype has been shown by Coutelier (1987) to be prevalent in most viral infections of the mouse using a variety of viruses, inoculation protocols and strains of mice (as described previously), and its importance in the clearance of virus demonstrated by Reale in 1990. The properties of this isotype which make it important in elimination of virus are efficient activation of complement (Burton, 1985), enhanced expression of IgG2a FcR on activated macrophages (Tsuru *et al.*, 1987) and cytotoxic activity mediated *via* the FcR for γ2a (Nuchtern *et al.*, 1990). Prevalence of IgG2a is a probable result of selective recruitment of the T_h1 type $CD4^+$ T-cells, hence secretion of IFN-γ and TNF-β, both of which mediate antiviral activities of other cells as described above.

Another striking finding in this study is immunodominance. While the immune response has the potential to make antibodies to all five antigenic sites as demonstrated by sequencing of natural variants, approximately half of the BALB/k antibodies generated overall are focused on HA1 198 and represent greater than 50% of antibodies per individual for four donors, (75% of antibodies from donor 7B are directed against HA1 158), contrary to the previously held belief of great diversity in the antibody response to influenza HA (Staudt & Gerhard, 1983). Immunodominance has also been reported in a similar investigation of CBA/Ca antibody recognition specificities in this laboratory. What is common to these two studies, but different to all previous studies on the antigenicity of influenza HA is the route of inoculation. Antibodies were elicited by natural infection in these two studies, whereas *all* previous work has been by immunisation, so while certain regions of the protein are intrinsically antigenic, the antibody response is biased to only one of the potential sites.

In addition, it is also apparent that the immunodominant site differs between strains. CBA/Ca ($H\text{-}2^k$) mice were previously shown by this laboratory to focus on HA1 158, and

BALB/c (H-2^d) to site B HA1 198. In this study, the BALB/k response focused on 198 (a BALB/c-like response) in four out of five donors, and 158 in the fifth donor 7B (a CBA/Ca-like response). It is interesting to note that while the BALB/k response shows dominance of a BALB/c antibody recognition site (43%), the second most frequently recognised site is HA1 158 (23%), the CBA/Ca dominant site, suggesting genetic control, possibly by both MHC and non-MHC genes, on the selected repertoire.

Previous studies have demonstrated both MHC and non-MHC effects on antibody responses to proteins such as staphylococcal nuclease (Lozner *et al.*, 1974), insulin (Berzofsky, 1980), ferredoxin (Sikora & Lery, 1980) and many others exhibiting differences, not in the specific antibody site recognised, but in the level of antibody response. For example, in the response to group a and subtype specific group d determinants of HBsAg, mice of H-2^q haplotype were found to be high responders, (i.e. to rapidly generate high titres of specific IgG antibody), H-$2^{a,\ b,\ d\ and\ h}$ intermediate responders and H-$2^{s\ and\ f}$, low to non-responders (Milich & Chisari, 1982). These were shown to be due to MHC effects by use of H-2 congenic and recombinant strains.

It is evident that for both the CBA/Ca secondary antibody response, and BALB/k secondary antibody response to influenza virus infection, the majority of antibodies select the same nucleotide and amino acid change. The question of a possible structural basis for the observed immunodominance arises, and this is addressed in the following chapters by analysis of antibody V-region gene usage.

Chapter 2

Antibody gene usage by BALB/k HA1-specific Mab from five individual donors with immunodominant recognition specificity for HA1 198 (or HA1 158).

D1. INTRODUCTION

In chapter 1, I have shown that the protective antibody response of BALB/k mice (H-2^k) elicited by natural infection with influenza virus X31 exhibited immunodominance of an epitope centred on residue HA1 198 (or HA1 158 for donor 7B), as had others in this laboratory (CBA/Ca mice (H-2^k) immunodominant site, HA1 158, and BALB/c mice (H-2^d), immunodominant site HA1 198) previously. Minor specificities for all of these recognised sites scattered around the HA1 molecule, but mapping to previously defined antigenic sites.

Interestingly, the secondary antibody response of five BALB/k mice illustrated a difference in recognition specificities and immunodominant site per individual donor. Over 50% of antibodies from each of donors 2B, 3B, 6B and 9B recognised HA1 198, suggesting genetic restriction of the selected repertoire by background (non-MHC) genes for these four donors. However, over 75% of antibodies from donor 7B focused on HA1 158, similar to CBA/Ca mice of the same haplotype, suggesting a possible influence of MHC on repertoire selection.

Given the potential diversity of the B-cell response to foreign proteins, the previously reported 1500 paratypically distinct antibodies against HA, and that almost the entire exposed surface of a protein (not homologous to self) is considered immunogenic to some extent (Benjamin *et al.*, 1984), why is such a restricted response observed in this study? Could there be a structural basis for immunodominance? There are potentially tens of millions of different antibodies that can form by combination of V-(D)-J genes and heavy and light chain pairing. Preferential usage of a limited number of antibody genes might be able to direct the response to a limited number of potentially immunogenic sites.

A possible explanation might be that, during infection, the neutralising epitopes of HA1 behaved as haptenic determinants eliciting an extremely narrow window of antibody responses. In which case, there would be a structural basis for the observed immunodominance of HA1 198 and HA1 158, and the occurence of Mab specificities that select novel laboratory mutants (HA1 53, 62, 158, 162, 205 and 273). This could be due to

some selective processing and presentation of HA1 at the site of viral replication to the immune system. Alternatively there may be restricted usage of germline inherited V region genes as has been found for certain anti-haptenic responses. I have addressed this question by undertaking an extensive structural analysis of $(VDJ)_H$ and $(VJ)_L$ gene usage in the antibody response to HA1 198.

A large family of HA1 198 (and HA1 158) specific Mabs were generated during this study from five individual (BALB/k) donors of different antibody isotypes (μ, α, $\gamma 1$, $\gamma 2a$, $\gamma 2b$ and $\gamma 3$). I sequenced the entire $(VDJ)_{H/L}$ genes of representative Mabs from the same individual (different isotypes) and different individuals (same isotype) to determine whether the same progenitor (μ^+) cells generated a single specificity and whether there was germline dominance of V_H gene usage.

D2. RESULTS

Twenty of the forty-six monoclonal antibodies generated from five individual BALB/k donors, for which antibody recognition specificity was determined in this study recognise residue HA1 198(A→E). These 198-specific Mab originate mainly from four of the five donors, and from which most isotypes are represented. Eleven of the 46 Mab are HA1 158 specific, of which eight are from donor SFA 7B.

Given the potential diversity of the response and the observed immunodominance, of specificity for one amino acid residue, particularly at the individual level, (either HA1 198 or 158), it may be possible that these antibodies in each individual's response arose by selective recruitment and expansion of one or a few high affinity, highly specific neutralising antibody-bearing progenitor cells which now dominate the memory response.

To determine whether this was the case, Mab of different isotypes from each donor with the same specificity were selected, and V_H and V_L genes sequenced for each. In total, eighteen 198-specific Mab were sequenced, from five donors, with three different isotypes per donor, representing isotypes α, μ, $\gamma 1$, $\gamma 2a$, $\gamma 2b$ and $\gamma 3$ overall.

To determine whether there was a correlation between antibody V gene usage and isotype, Mab were selected such that antibodies of a given isotype were sequenced from at least three individual donors, e.g. $\gamma 1$ present in four donors and $\gamma 2a$ in three donors.

The selected antibodies are shown in Table 12, and include four from donor SFA 2B, six from donor SFA 3B, three from donor SFA 6B, two from donor SFA 7B and three from donor SFA 9B. All eight HA1 158-specific Mab from donor SFA 7B (immunodominant for HA1 158) were also sequenced to determine whether the difference in immunodominant site for this donor can be explained by different antibody gene usage to a) 198-specific Mab from different BALB/k donors, and b) 198-specific Mab from this donor.

V_H gene usage was determined by direct sequencing of mRNA from total cellular RNA (in triplicate) using C_H specific primers. V_L gene usage was determined by cDNA synthesis from total cellular RNA, followed by PCR amplification and direct sequencing of light chain region genes using a C_κ specific primer and eleven different degenerate L-chain leader sequence specific primers.

Table 12: Monoclonal antibodies of immunodominant recognition specificity from five BALB/k donors selected for immunoglobulin gene sequencing and assignment of V_H and V_L families.

BALB/k Donor	B-cell hybridoma clone	Antibody specificity X31 HA(H3N2)	Isotype
SFA 2B	1.1	HA1 198 A→E	γ1
	7.2		γ1
	8.1		γ1
	3.1		γ2a
SFA 3B	3.1	HA1 198 A→E	γ1
	12.1		γ1
	2.1		γ2a
	10.1		γ2a
	7.1		γ2b
	14.1		γ2b
SFA 9B	8.1	HA1 198 A→E	γ1
	3.1		γ2a
	6.1		γ2b
SFA 6B	1.1	HA1 198 A→E	γ1
	4.2		μ
	5.1		α
SFA 7B	1.2	HA1 198 A→E	α
	7.1	HA1 198 A→E	α
	10.2	HA1 158 G→E	α
	2.1		μ
	15.1		μ
	19.1		γ2a
	11.1		γ2b
	3.1		γ3
	5.1		γ3
	8.1		γ3

V_L mRNA was not sequenced directly due to endogenous expression of a κ-chain by the myeloma cell line used in the hybridoma fusions (Jones & Bendig, 1991). The amplified PCR products of the myeloma κ-chain were eliminated by gel purification of the PCR products. When samples from all eleven PCR reactions were run on an agarose gel, two products of the correct size for one V_κ, one J_κ and partial C_κ (approximately 400bp in total) were usually detectable for each antibody sequenced, from two different primers. The myeloma κ-chain sequence was always amplified by primer MKV-2, and this was present on each gel for each of the thirty-seven Mab amplified (in triplicate). This band was ignored, and bands of approximately 400bp present in other lanes i.e. from amplification by MKV-1, or MKV-3 to 11, were considered to be expressed by the B-cell, and thus were extracted for purification and sequencing. Where more than one band (excluding MKV-2) was present, due to the degeneracy of the primers, all bands of the correct size were purified and sequenced.

PCR amplifications were performed in triplicate, in different combinations of Mab at a time, and on different days. Both strands of all PCR dsDNA products were sequenced to eliminate sequence discrepencies due to Taq polymerase errors (Tindall & Kunkel, 1988). Some of the extra bands were found to contain stop codons when sequenced, therefore were non-productive. These were not analysed further. Productively rearranged L-chains were present for each B-cell hybridoma as detected by sequencing, and as also confirmed by detection of antibody in ELISA when isotyping and in virus neutralisation assays (HI). V_L families and genes were assigned for all of these by EMBL database comparison for identity.

D2.1. Antibody gene usage in the response to influenza HA by individual BALB/k donor mice.

Where assignment of true designated V_H or V_L gene names proved impossible from the literature and computer database searches, a letter was allocated in this study to indicate same or different genes within a family as marked by an asterisk " * " and prime " ' " to indicate the same gene usage *via* database searching, but nucleotide sequence homology of

Table 13: Immunoglobulin gene family usage by Mab specific for influenza X31 HA1 198 immunodominant epitope from BALB/k donor SFA 2B.

* indicates letters assigned for undocumented genes in the literature (see text) i.e. no gene name given for the closest matched gene in the article.

Immunoglobulin gene usage by donor BALB/k SFA 2B HA1 198-specific monoclonal antibodies									
Clone	V_L family	V_L gene*	J_κ gene	V_H family	V_H gene	D gene	CDR3H amino acid length	J_H gene	C_H isotype
1.1	$V_\kappa 8$	α	5	VGam 3.8	VGK 7	DSP 2.2	9	4	γ1
7.2	$V_\kappa 8$	α	5	VGam 3.8	VGK 7	DSP 2.2	9	4	γ1
8.1	$V_\kappa 12/13$	ω	2	VGam 3.8	VGK 7	DSP 2.2	10	2	γ1
3.1	$V_\kappa 8$	β	1	VGam 3.8	V75/Vmu1	DSP 2.2	10	4	γ2a

just below 90%. Antibodies are thought to derive from the same gene if sequence homology exceeds 90%, and the same family at 80% and above. Less than 70% homology is indicative of a different family.

D2.1 (a). HA1-198 specific Mab gene usage by B-cell hybridoma clones from BALB/k donor SFA 2B.

Five of the ten Mab from donor SFA 2B, for which antibody specificity was determined recognised HA1 198. Four of these five Mab were of the γ1 isotype, and the fifth γ2a. Mab from hybridoma SFA 2B 1.1, 7.2 and 8.1 (γ1), and SFA 2B 3.1 (γ2a) were sequenced, and results are summarised in Table 13.

All four Mab utilise V_H genes from the same V_H family, VGam3.8. SFA 2B 1.1, 7.2 and 8.1 use gene VGK-7, and 2B 3.1 uses gene V75/Vmu-1 from this family. SFA 2B 1.1 and 7.2 also use the same V_L gene (termed "α" in this study) from the $V_\kappa 8$ family. All other genes used by these two clones are also identical, i.e. $J_\kappa 5$, $J_H 4$, $C_H \gamma 1$ and DSP2.2. CDR3H length (9 amino acids) and sequences are also almost identical, i.e. at the junction of V-D-J joining, suggesting that these two clones originated from the same progenitor cell. The other two clones, 2B 3.1 and 8.1, use genes from the L-chain families $V_\kappa 19$ and $V_\kappa 12/13$ respectively, also using different J_κ (-1 and -2) and J_H (-4 and -2) genes. CDR3H regions are both ten amino acids long, but of different sequences, although the closest database D gene match is still DSP2.2 for both clones. Although 2B 8.1 is of the same isotype as 2B 1.1 and 7.2, it only shares V_H family and V_κ gene VGK 7 usage with these, therefore originated from a different progenitor cell.

D2.1 (b). HA1-198 specific Mab gene usage by B-cell hybridoma clones from BALB/k donor SFA 3B.

Table 14

Six out of ten Mab from donor SFA 3B recognised HA1 198, two each of γ1, γ2a and γ2b isotype. All six Mab were sequenced from this donor, i.e. 3B 2.1, 10.1 (γ1), 3.1,

Table 14: Immunoglobulin gene family usage by Mab specific for influenza X31 HA1 198 immunodominant epitope from BALB/k donor SFA 3B.

* indicates letters assigned for undocumented genes in the literature (see text) i.e. no gene name given for the closest matched gene in the article.

Immunoglobulin gene usage by donor BALB/k SFA 3B HA1 198-specific monoclonal antibodies									
Clone	V_L family	V_L gene*	J_κ gene	V_H family	V_H gene	D gene	CDR3H amino acid length	J_H gene	C_H isotype
2.1	V_κ12/13	ω	1	VGam 3.8	3B*	DFL16.1	11	3	γ2a
3.1	V_κ12/13	ω	1	VGam 3.8	3B'*	DFL16.1	11	3	γ1
10.1	V_κ12/13	ω	1	VGam 3.8	V_HL6'	DSP2.3/4	12	4	γ2a
14.1	V_κ12/13	ω	1	VGam 3.8	T9-1	DSP2.3/4	12	4	γ2b
7.1	V_κ19	δ	5	V_H 7183	.10	DSP2.5/7/8	9	1	γ2b
12.1	V_κ19	δ	5	V_H 7183	.10	DSP2.5/7/8	9	1	γ1

Table 15: Immunoglobulin gene family usage by Mab specific for influenza X31 HA1 198 immunodominant epitope from BALB/k donor SFA 9B.

* indicates letters assigned for undocumented genes in the literature (see text) i.e. no gene name given for the closest matched gene in the article.

Immunoglobulin gene usage by donor BALB/k SFA 9B HA1 198-specific monoclonal antibodies									
Clone	V_L family	V_L gene	J_κ gene	V_H family	V_H gene*	D gene	CDR3H amino acid length	J_H gene	C_H isotype
3.1	$V_\kappa 19$	β^*	1	VGam 3.8	Vmu-1b	DQ52	11	4	$\gamma 2a$
6.1	$V_\kappa 4$	.68	4	VGam 3.8	Vmu-1c	DQ52	9	4	$\gamma 2b$
8.1	$V_\kappa 12/13$	ω^*	2	VGam 3.8	Vmu-1d	DQ52	10	4	$\gamma 1$

12.1 (γ2a), 7.1 and 14.1 (γ2b). Clones 3B 7.1 and 12.1 use the same V_H gene and family, V_H7183.10, same D gene DSP2.5/7/8 (CDR3H = 9 amino acids), J_H1, J_κ5 and V_L family Vk19 gene δ, but are of different isotypes (7.1=γ2b and 12.1=γ1).

The other four clones, 3B 2.1, 3.1, 10.1 and 14.1 use identical V_H genes from the VGam3.8 family. Clones 3B 2.1 and 3.1 use identical antibody genes V_κ12/13 ω, J_κ1, VGam3.8 3B, J_H3, DFL16.1 (CDR3H=11 amino acids), but have different isotypes (C_H genes). Clones 3B 10.1 and 14.1 share the same V_L 12/13 ω and J_κ1 genes as 2.1 and 3.1. Both 3B 10.1 and 14.1 use different V_H genes from VGam3.8, VHL6' and T9-1 respectively, but the same J_H4 and D_H gene, DSP2.3/4 (different to the other DSP2 genes used by donor 2B) and CDR3H region is similar, although these also have different isotypes. So, all six Mab use VGam3.8 V_H family, and four of the six use V_L family V_κ12/13, gene ω and J_κ1. The six Mab therefore originate from three progenitor cells.

D2.1 (c). HA1-198 specific Mab gene usage by B-cell hybridoma clones from BALB/k donor SFA 9B.

Table 15

The four out of seven Mab from this donor that recognised HA1 198 were sequenced. These were clones 9B 3.1 (γ2b), 6.1 (γ2b) and 8.1 (γ1). All three clones utilise Vmu-1 gene of the VGam3.8 family and J_H4. CDR3H lengths vary but sequences are similar, belonging to the DQ52 D gene group, but with an insertion of three bases (GTC) in 9B3.1 and a deletion of six bases in 6.1 (GGC and TTC) (Figure 18, chapter 3). These therefore have the same V-D-J heavy chain rearrangment, but use entirely different L-chain genes, V_κ19 β, J_κ1 in 9B 3.1, V_κ4.68, J_κ4 in 9B6.1 and V_H 12/13 ω, J_κ2 in 9B8.1.

D2.1 (d). HA1-198 specific Mab gene usage by B-cell hybridoma clones from BALB/k donor SFA 6B.

Table 16.

The three out of seven Mab from this donor which were 198-specific were sequenced. These were 6B 1.1 (γ1), 4.2 (μ) and 5.1 (α). 6B 1.1 uses V_H family VGam3.8,

Table 16: Immunoglobulin gene family usage by Mab specific for influenza X31 HA1 198 immunodominant epitope from BALB/k donor SFA 6B.

* indicates letters assigned for undocumented genes in the literature (see text) i.e. no gene name given for the closest matched gene in the article.

' indicates antibodies using the same gene as defined by database comparison, but having less than 90% homology.

Immunoglobulin gene usage by donor BALB/k SFA 6B HA1 198-specific monoclonal antibodies									
Clone	V_L family	V_L gene	J_κ gene	V_H family	V_H gene	D gene	CDR3H amino acid length	J_H gene	C_H isotype
1.1	$V_\kappa 21$	E	2	VGam 3.8	Vmu-1c*	DQ52	11	4	γ1
4.2	$V_\kappa 19$	β*	1	J558	concensus DBA-1a	DSP2.2	12	1	μ
5.1	$V_\kappa 19$	β*	1	J558	concensus DBA-1a'	DQ52	11	4	α

gene Vmu-1, and DQ52, J_H4 as do most other BALB/k 198-specific Mab. This is paired with $V_\kappa 21E$ and $J_\kappa 2$. Clones 6B 4.2 (μ) and 5.1 (α) use the same L-chain genes, $V_\kappa 19$ β and $J_\kappa 1$, and H-chain J558 concensus DBA/1 gene from a different family, $V_H J558$, to 9B 3.1 and the other BALB/k 198-specific Mab. 6B 4.2 uses $J_H 1$ and DSP2.2 whereas 6B 5.1 uses $J_H 4$ and DQ52, but all CDR3H lengths and sequences vary widely between the three Mab.

D2.1 (e). HA1-198 specific Mab gene usage by B-cell hybridoma clones from BALB/k donor SFA 7B.

Table 17

Only two out of twelve Mab from donor 7B recognised HA1 198, clones 1.2 and 7.1. This donor predominantly focused on HA1 158 in eight out of twelve Mab, which will be discussed later. Both of the HA1 198-specific Mab were sequenced and both showed identical antibody gene usage throughout. That is, both used $J_\kappa 1$, $V_\kappa 19$ β, $J_H 4$ containing the same C→G mutation within this gene, V_H family J558 consensus gene DBA/1b, $C_H\alpha$ and DQ52. CDR3H regions were both 10 amino acids long and of identical sequence, therefore N region addition. V_L genes were 100% homologous, and V_H genes were 96.15% homologous, therefore these two B-cell hybridoma clones originated from the same progenitor cell, and possibly the same B-cell and myeloma fusion product.

Interestingly, these also use the seldomly observed (in this study) family and gene J558-DBA/1 and V_L from $V_\kappa 19$ β, $J_\kappa 1$ and $J_H 4$ as did the α and μ isotype B-cell hybridoma clones from donor SFA 6B, 4.2 and 5.1, although CDR3H sequences and lengths vary between Mab from these two individual donors, and 6B 5.1 lacks the C→G substitution in the $J_H 4$ gene of 7B 1.2 and 7.1.

D2.2. Antibody gene usage by the BALB/k strain in the influenza HA specific memory response: the overall picture.

In total, eighteen HA1 198-specific Mab were sequenced from five individual donors. Three V_H families were utilised in this response with $^{12}/_{18}$ (66.6%) using genes from VGam3.8, and all μ and α Mab using V_H genes from $V_H J558$ family. Twelve out of the

Table 17: Immunoglobulin gene family usage by Mab from BALB/k donor SFA 7B specific for influenza X31 HA1 198 (immunodominant epitope in the BALB/k strain) and HA1 158 (immunodominant in this donor).

* indicates letters assigned for undocumented genes in the literature (see text) i.e. no gene name given for the closest matched gene in the article.

' indicates antibodies using the same gene as defined by database comparison, but having less than 90% homology.

Mab specificity	Immunoglobulin gene usage in donor BALB/k SFA7B HA1 specific monoclonal antibodies									
	Clone	V_L family	V_L gene	J_κ gene	V_H family	V_H gene	D gene	CDR3H amino acid length	J_H gene	C_H isotype
HA1 198	1.2	V_κ19	β*	1	J558	concensus DBA-1b*	DQ52	10	C-G 4	α
	7.1	V_κ19	β*	1	J558	concensus DBA-1b*	DQ52	10	C-G 4	α
HA1 158	10.2	V_κ9	.42	1	J558	y*	DFL16.1	8	2	α
	2.1	V_κ1A	K5.1	5	J558	Dns-B	DSP2.9	6	2	μ
	15.1	V_κ21	σ*	1	J558	V_H50	DFL16.1	8	4	μ
	3.1	V_κ19	β*	1	J558	concensus DBA-1c*	DQ52	10	4	γ3
	5.1	V_κ21	A	1	J558	V_H186.1	DFL16.1[a] DSP2.2 DL16.2	11	4	γ3
	8.1	V_κ19	β*	1	J558	x*	DQ52	10	C-G 4	γ3
	19.1	V_κ9	.42	1	J558	V_H186.2	DFL16.1	8	2	γ2a
	11.1	V_κ21	E	2	J558	V_H186.2'	DFL16.1	7	4	γ2b

eighteen (66.6%) used J_H4, but only nine of these with $V_HVGam3.8$, the rest with J558. CDR3H lengths were variable, hence D gene usage. Five V_L families were utilised. The most commonly used V_κ family was $V_\kappa19$ ($^8/_{18}$) 44.4%, then $V_\kappa12/13$ ($^6/_{18}$). Ten out of eighteen Mab used $J_\kappa1$, all with either $V_\kappa19$ or $V_\kappa12/13$, followed by $J_\kappa5$ used by $^5/_{18}$.

This study illustrates a very diverse antibody V_L gene usage with a relatively less diverse V_H gene usage in the BALB/k strain against influenza HA1 198 following natural infection.

At the individual level, donor 2B uses one V_H family and two V_L families, and the repertoire originates from three progenitor cells. Donor 3B uses two V_L and V_H families, and also originates from three progenitors. Donor 6B also uses two V_L and V_H families and originates from two progenitors. The two Mab from donor 7B are identical and Donor 9B uses genes from three different V_L families, but a single V_H family. These results thereby demonstrate an overall more restricted V_H gene family usage than V_L in the BALB/k HA1 198-specific antibody repertoire.

D2.3. Antibody gene usage by donor SFA 7B: the anomaly.

Table 17.

Donor 7B is the exception to the rule in this study because it recognises predominantly HA1 158. We have seen that its two 198-specific Mab are of the α isotype, both from the same progenitor cell and use a different V_H family to most other BALB/k antibodies with the exception of the μ and α Mab of donor 6B. It was of interest therefore to determine whether the majority of donor 7B Mab, of different specificity were i) all using the same V_H family, ii) if that family was different to that used by 198-specific Mab from this donor, and iii) different to all families used by the other BALB/k donor antibodies, i.e. a V_H family not used by 198-specific Mab which may therefore be directing the response to HA1 158 since V_H were more retricted than V_L usage in the BALB/k response.

I therefore sequenced all eight 158-specific Mab from donor SFA 7B, 2.1, 3.1, 5.1, 8.1, 10.2, 11.1, 15.1 and 19.1, representing isotypes μ, α, $\gamma2a$, $\gamma2b$ and $\gamma3$. $\gamma3$ was not observed in the 198-specific response, reciprocally $\gamma1$ which is common in the 198-specific response is not present in the 158-specific response.

Interestingly, *all* eight Mab were utilising the same V_H family J558, as were the two donor 7B 198-specific Mab. Seven different genes are used from this family, one of which, DBA/1 from clone 3.1, is also used by the two 198-specific antibodies from this donor based on database comparison results, not sequence homology. This clone, 3.1, also has the same CDR3H length (10 amino acids) and sequence, therefore D gene (DQ52), J_H4 (without the C→G substitution), $J_\kappa1$ and $V_\kappa19$ β genes as 7B 1.2 and 7B7.1, differing only in isotype (α versus γ3). This is also true for 7B 8.1 (also γ3) but V_H gene is unassigned, therefore named "x", but with less than 90% homology with DBA/1c in 7B 3.1, and CDR3H sequence differs slightly. 7B 8.1 does however have the C→G substitution in J_H4.

Of the SFA 7B 158-specific Mab, 7B 10.2 and 7B 19.1 use the same V_κ 9.42 gene, with $J_\kappa1$, J_H2 and DFL16.1, differing only in V_H gene usage (same family) and isotype (α and γ2a). 7B 10.2 is of the α isotype but differs in all but $J_\kappa1$ usage from the other two α Mab from donor 7B (1.2 and 7.1).

The two μ Mab, 2.1 and 15.1 differ in all respects (except V_HJ558), from each other and the other μ BALB/k Mab, 6B 4.2. Three out of eight Mab use V_HJ558 V186 genes, one using V186.1, the other two using V186.2, and two of these share $V_\kappa21$ (A and E) family usage, and J_H4, but have nothing else in common. $V_\kappa9$ and $V_\kappa1$ families present here in $^3/_8$ clones are not used in the BALB/k 198-specific response.

In conclusion, all Mab fom donor 7B utilise genes from family J558. Seven out of ten Mab use J_H4, the others J_H2, $^8/_{10}$ use $J_\kappa1$, $^4/_{10}$ use $V_\kappa19$ β, $^3/_{10}$ use $V_\kappa21$ family, $^2/_{10}$ $V_\kappa9$ and $^1/_{10}$ $V_\kappa1$A K5.1. However, all 7B 158-specific Mab have less than 60% homology to each other, but come up as belonging to the same family J558 *via* a GCG, EMBL database search. This is not surprising since V_HJ558 is the largest of the V_H families with over 1000 members (Brodeur & Riblet, 1984; Dildrop, 1984), comprising around 45% of the potential repertoire of an individual mouse, with as much as 60% variation between members at extreme ends of the family.

D3. CONCLUSION

While antibody responses to polysaccharides and haptens are often oligoclonal (Hansberg *et al.*, 1976; Reth *et al.*, 1979), responses to protein antigens are generally more heterogeneous (Zanetti *et al.*, 1983). Previous studies on the immune response to influenza virus have found extensive diversity in the antibody repertoire as previously discussed. Some of these groups have also gone on to study antibody gene usage by these HA specific antibodies. The majority of antibody gene usage studies in the immune response to influenza have been performed by the same (or a few) groups (Gerhard *et al*) using hybridomas and Mab from the same few donor mice, and the conclusions have been variable depending on the criteria set by the investigator in the selection of Mab to sequence and assign V families for, such as idiotype or specificity.

An idiotope is defined as any antigenic determinant that is characteristic of the immunoglobulin produced by a single clone, or a small minority of clones of cells, and is believed to be confined to the variable region, and therefore in most cases, the antigen binding domain of the immunoglobulin (Herbert *et al.*, 1985). Idiotype (Id) is a set of one or more idiotopes by which a clone of immunoglobulin forming cells can be distinguished from other clones (e.g. serologically). An idiotype can be either individual, or cross-reactive and inherited (e.g. the inherited Id T15 in mice). An idiotype can therefore be encoded by antibody V genes, and indeed, there are many examples of either V_H or V_L specified Id, some of which are discussed later. Presumably then, antibodies sharing an idiotype may thus also be using the same antibody genes.

Brown and Sealy (1991) used such an idiotype based approach in the analysis of antibody gene usage and found restricted V_H (J558) family and V_L (V_κ21) family gene usage amongst influenza HA-specific antibodies expressing the PY206 Id, which is a dominant component of the immune response to HA in BALB/c and C57BL/6J mice. This idiotype is also found in the human response to influenza vaccination in $^{12}/_{26}$ antibodies, and also in mice against PR8, X31 and other influenza viruses (Sigal *et al.*, 1987). This was further confirmed by Moran (1987) who found this Id to be derived from V_HJ558 and located

exclusively on the heavy chain, independent of the $V_\kappa 21$ light chain. Conclusions drawn from these studies were that idiotype was defined by antibody genes. It is therefore interesting to note that general conclusions regarding antibody gene usage against influenza HA are drawn from studies based on a minority of antibodies sharing an idiotype, e.g. the C4 idiotype, which comprise only 10-15% of the secondary response (Clarke *et al.*, 1990b).

Staudt and Gerhard in 1983 found a diverse repertoire of 1500 paratopes in the BALB/c antibody response to PR8 immunisation. Mab used in these studies were derived from fourteen individual donor mice, and all subsequent antibody gene studies have been on antibodies from three of these mice, H35 (site Cb (=E in H3)), H36 (Sb (=site B in H3)) and H37 (Sb), therefore all investigations have been on the antibody repertoire generated by immunisation of BALB/c mice with an H1 virus, PR8. Seven of the thirteen Mab from H36 are Sb-specific, with different reactivity patterns, hence fine specificities, but all use V_L genes from the $V_\kappa 21C$ group (McKean *et al.*, 1984). The V_H families of the same seven Mab were then assigned by another worker in the same group (Clarke *et al.*, 1985) who found genes from V_H 7183 and V_H S107 were used for these and also two Mab from donor H37 (with 90% homology), and V_H36-60 in a third Mab from H37. All Mab from donor H37 were also using genes from the $V_\kappa 21C$ group. Kavaler (1990) sequenced antibodies from donor H35, and found six V_H families (17 genes, but mostly of the J558 family) and six V_L families (11 genes) used by 24 primary and 4 secondary response antibodies.

Clarke (1990b) also sequenced V genes from donor H35, but only those of the 10% of antibodies carrying the C4 idiotype. Restricted V_L ($V_\kappa 8$) and diverse V_H family usage was observed, but excluding the J558 family found by Kavaler in the previous study on antibodies from the same donor. Clarke concluded from these results that $V_\kappa 8$ directs the response to site Sb and V_H determines fine specificity within this site, since these antibodies had different reactivity patterns to natural variant viruses, however, antibody specificities were detected by RIA hence antibodies selected in this stidy are not necessarily neutralising.

Other idiotype based studies on the same group of mice include $Id45^+$ antibodies from donor H37, H35 and others (Caton *et al.*, 1986) showing resticted V_H usage of V_H7183, but with $V_\kappa 24$ usage in $Id45^+$ antibodies and $V_\kappa 1$ usage in $Id45^-$ antibodies, showing two different reactivity patterns for these two groups of antibodies. Again, the light chain is implicated in directing the response to a site, and heavy chain usage affecting

fine specificity. The role of the light chain in directing the antibody response was studied to see if this alone was enough to determine antbody specificity (Carmack *et al.*, 1991) as described below.

Twenty-five percent of the primary response to influenza immunisation was thought to be directed against PR8 HA site Sb, and 85% of these antibodies have the C4 idiotype which is encoded exclusively by $V_\kappa 8$ (Clarke *et al.*, 1990b). This suggested that the response is directed by the antibody light chain. This hypothesis was examined by Carmack *et al* (1991) using $V_\kappa 8$ as a transgene, which, if this was the case, would direct the total response to site Sb since the transgene should theoretically be functionally expressed in all B-cells, thus switch off assembly of other V_κ genes. The transgene was found to be expressed exclusively in the lymphoid lineage and to influence the rate of endogenous rearrangement at the κ locus, but not the mechanism and final outcome of rearrangement. On LPS stimulation of splenic B-cells, no anti-HA activity was detected even though the transgene was expressed, but following primary PR8 immunisation, HA specific $V_\kappa 8$ Mabs were detected, therefore the transgene was functional, but only formed HA specific antibodies with a more restricted set of heavy chains than previously hypothesised, indicating specificity was not determined by the light chain alone.

The light chain associated Id 23-1 found on antibodies from donor H35 primary response, but which disappear in the late primary, and secondary response, also show restricted V_κ (4/5) usage, but with use of 2 genes, C12.1 and C12.2 from the a single V_H family J558. This change in dominant idiotypes, hence V_L/V_H gene usage between primary and secondary repertoires was also seen in the BALB/c response to phOx (where the domain $V_\kappa Ox1$-$V_H Ox1$ is less frequent in the secondary response) (Berek *et al.*, 1985), and also PC (Stenzel-Poore *et al.*, 1987), NP (Allen *et al.*, 1987) and HA site Sb (Clarke *et al.*, 1985, 1990b).

The Id68 idiotype predominant in antibodies from donor mouse H37 (Clarke *et al.*, 1990a) use a single $V_H 36$-60, a variety of D and J_H genes, show restricted CDR3H length and sequence, but use diverse V_κ genes form $V_\kappa 21C$, 21E, 9 and 12/13. Id68 is therefore a heavy chain associated idiotype, so it is not surprising that these antibodies all use the same V_H family.

These studies clearly demonstrate how general conclusions for antibody gene usage within a donor for antibodies of the same specificity for HA have altered from diverse V_H and V_L usage to restricted V_L or V_H or both, due to change in antibody selection criteria, yet eight out of every ten investigations of antibody gene usage in the anti-HA response have used antibodies of a given idiotype. The conclusions drawn from such studies before 1985 suggested restricted $V_\kappa 21$ and diverse V_H usage in the HA-specific response (McKean *et al.*, 1984; Clarke *et al.*, 1985; Reale *et al.*, 1986), but after site specific studies from 1990 onwards, antibody gene usage in the same response from the same three mice was considered to be much more diverse (Clarke *et al.*, 1990a; Caton *et al.*, 1991; Kavaler *et al.*, 1991).

In this study, of an unusually focused antibody repertoire against influenza HA, following natural infection of the BALB/k strain, exhibiting immunodominance, analysis of Ig V region gene usage of Mab, specific for the same amino acid residue, also revealed relatively diverse usage of both V_H and V_L genes, for HA1 198-specific Mab.

Eighteen Mab from five individual donors were analysed and found to use 9 genes from 3 different V_H families and 6 genes from 5 V_L families, although the majority ($^{12}/_{18}$) of Mab all used the VGam3.8 heavy family, and generally divided into two groups for the light chain, using either $V_\kappa 19$ ($^{8}/_{18}$) or $V_\kappa 12/13$ ($^{6}/_{18}$) families. In donors where greater than 50% of the response is directed to the same single amino acid, such as in SFA 2B, 3B and 7B, V gene restriction is observed, but where the immunodominant epitope accounts for less than 50% of the response, e.g. $^{3}/_{7}$ in donors SFA 6B and 9B, diverse V gene usage is found. The response in these donors is probably stochastic in comparison to the other three donors.

All V_L and V_H families found in this study have occurred in previous anti-HA antibody repertoires. Following an extensive literature survey, I have found the most frequently used V_H family by BALB/c mice against HA to be $V_H 7183$, followed by J558, although a large proportion of antibodies also used $V_H S107$. The most frequently used V_L family was $V_\kappa 21$ (C or E), followed by $V_\kappa 4/5$ and $V_\kappa 19$. The majority of Mab from my 5 BALB/k donors of the same specificity use VGam3.8, and $V_\kappa 19$ or 12/13, all of which have been observed in the response to influenza previously, but only constituting minor components of the overall response.

At the individual level, a more restricted usage of V_H becomes apparent. For example, all 4 Mab from donor SFA 2B use VGam3.8, but use three different V_κ families: V_κ8, 9 and 12/13. Donor SFA 3B can be segregated into two groups, one group of $^4/_6$ Mab using VGam3.8 with V_κ12/13, the other ($^2/_6$) using V_H7183 with V_κ19. All three Mab from donor SFA 9B use VGam3.8, but all have different V_L families, using V_κ12/13, 19, and V_κ4 which is exclusively used by BALB/k SFA 9B6.1.

Mab 1.1 from donor SFA 6B also uses VGam3.8, but with V_κ21E, not used by any other BALB/k Mab. The other two Mab from this donor, 6B4.2 (μ) and 6B5.1 (α) both utilise V_HJ558 with V_κ19, as do Mab 7B1.2 (α) and 7B7.1 (α) from donor SFA 7B. Interestingly, this V_H family is only used by 198-specific Mab of either α or μ isotype, and all μ and α Mab use the same V_H /V_L combination. V_HJ558 is the largest of the V_H families with over 1000 members (45% of the total potential repertoire) (Livant *et al.*, 1986), and is often disproportionally represented in the early immune response due to random stoichiometric rearrangements, together with the most D-J proximal family (V_H7183 in BALB/c and CBA/Ca, therefore presumably also in BALB/k mice). For example, 45% of antibodies from C57BL/6 foetal liver use J558, but in BALB/c mice 22% of antibodies from foetal liver and primary adult B-cells use the J proximal family V_H7183 and 17% use J558 (Wu, G. E. & Paige, 1986).

To establish whether specific use of a particular V_H or V_L gene can determine the fine specificity of an antibody, i.e. the residue within a site recognised, e.g. HA1 158 or 198, both in site B, V region genes of Mab specific for HA1 158 from donor SFA 7B were also analysed and assigned families. HA1 158 is the second most common specificity, a site which CBA/Ca mice of the same haplotype, H-2^k, as BALB/k mice also focus on predominantly, representing 24% ($^{11}/_{46}$) of the total BALB/k antibody response to HA. Most of these HA1 158-specific Mab ($^8/_{11}$) were derived from a single donor mouse, SFA 7B.

BALB/k SFA 7B Mab specific for HA1 158 all utilise the V_HJ558 family, with four different V_L families, most of which also use V_κ21 ($^3/_8$), the most common V_H and V_L families observed in the BALB/c response to HA. The two V_L families, V_κ1 and V_κ9 also used by this group of Mab are not observed in the BALB/k HA1 198-specific response. Donor 7B has an unexpected response to a CBA/Ca (H-2^k) predominant site, but antibody V_H family usage suggests a possible intervention of MHC redirecting what would have been

a specific selection of V_H family to what otherwise turns out to be stoichiometric, using genes from the largest family instead, but only in this donor.

Overall, 10 Mab of two different specificities were sequenced for donor SFA 7B, all of which use a restricted V_H family, V_HJ558, and diverse V_L families (V_κ19, 21, 9 and 1). It has been suggested that V_L directs antibodies to a region of a molecule and V_H determines fine specificity within this region (McKean *et al.*, 1984; Clarke *et al.*, 1985; Caton *et al.*, 1986), based on common usage of V_κ21C and widely diverse V_H usage in anti-HA antibodies to site Sb but with different reactivity patterns, hence fine specificity. I have found Mab that conform to this hypothesis, in that 6B1.1 using V_κ21E and VGam3.8 recognises HA1 198, however, 7B11.1 also using V_κ21E but with V_HJ558 V186.2 recognises HA1 158, and similarly 3B7.1, 3B12.1 and 9B3.1 (V_κ19δ: V_H7183, V_H7183 and VGam3.8) all recognise HA1 198, but 7B3.1 and 7B8.1 (both also V_κ19, but with V_HJ558) recognise HA1 158. However, it is also evident from this study that Mab specific for the same region (site B), but different sites within this region (158 or 198) from clones from two individual donors, 6B4.2, 6B5.1, 7B1.2, 7B7.1, 7B3.1 and 7B8.1 all use the same V_H/V_L pair, V_HJ558/V_κ19 differing only in CDR3H length and sequence, hence it cannot therefore be concluded that antibody specificity is defined solely by V_H/V_L gene usage.

Since donor 7B uses V_H and V_L families not common in the BALB/k anti-198 response, there is a possibility that this "odd" mouse, which behaves like the CBA/Ca strain in its recognition specificity distribution (immunodominance of 158) could have more in common with antibodies from CBA/Ca mice than the other BALB/k mice, such as V families, D and J genes, and CDR3H regions. It is also possible, by extrapolating from this data, that CBA/Ca mice might also use very restricted V_H and diverse V_L families, directing the response to 158 in preference to 198, and MHC does play a role in antibody repertoire selection, reflected in V region gene family usage. This hypothesis was also investigated, and is discussed in chapter 3.

Chapter 3

Comparison of antibody gene usage by CBA/Ca Mab and BALB/k Mab specific for influenza virus HA1 158: Is there correlation of antibody gene usage with recognition specificity?

E1. INTRODUCTION

Antibody gene usage was determined for eighteen BALB/k Mab from five donors specific for HA1 198, and found to consist of diverse V_L family usage (six genes from six families), but a more restricted V_H family usage (66.6% using genes from VGam3.8). Mab from donor SFA 7B, of which $^{8}/_{12}$ recognise HA1 158, were also sequenced to determine whether fine specificity could be attributed to antibody structure, i.e. antibody V region gene usage. All eight HA1 158-specific SFA 7B Mab were found to utilise genes from several different V_L families, common to the HA1 198-specific antibody repertoire, but had very restricted V_H family usage. Seven different genes from V_HJ558 were used by the eight Mab, a family only utilised by Mab from this donor, including two anti-198 Mab, (both α isotype) and Mab of α and μ isotype from donor 6B.

Donor 7B differs from the other four BALB/k donors, in that a possible influence of MHC on antibody repertoire selection is suggested by its predominantly CBA/Ca H-2^k-like anti-158 response, compared with the anti-198 BALB/c H-2^d-like response of the other four donors. Moreover, this donor uses a V_H family not commonly used in the anti-198 repertoire, and although it shares V_L family usage with other BALB/k antibodies, it also utilises two new families, $V_\kappa 1$ and $V_\kappa 9$.

The antibody repertoire of this donor appears to be a composite of the BALB/k response, and possibly the CBA/Ca H-2^k response. It was therefore of interest to determine antibody gene usage by CBA/Ca Mab of the same specificity, HA1 158, and to establish a) whether these Mab utilise the same V gene families as BALB/k anti-158 Mab, and b) whether CBA/Ca antibodies were also restricted in V_H family usage, that is, V gene usage of particular families directing the response.

There have been previous reports of anti-influenza HA Mab V_L directing the response to a region of a protein, and V_H determining fine specificity within this region and this would appear to be so in the BALB/k response to two sites, HA1 158 and 198, within the same region, site B. If this is the case, then we would expect CBA/Ca antibodies to utilise the same V_H family, J558, as BALB/k SFA 7B, and to share use of V_L families present in the BALB/k anti-158 response, but not the BALB/k anti-198 response, i.e. $V_\kappa 1$

and $V_{\kappa}9$. Since the BALB/k and CBA/Ca strains only have the MHC ($H\text{-}2^k$) locus in common, it may then be possible that MHC exerts an influence on the selection of antibody repertoire, to use V genes or families not otherwise observed in the BALB/k (predominantly 198) response, *via*, for example, T-cell help. Eight Mab specific for HA1 158, generated in exactly the same way, from four individual CBA/Ca donors, following *intranasal* infection, of different isotypes were thus sequenced and V gene families assigned.

E2. RESULTS

E2.1 Comparison of antibody gene usage by CBA/Ca Mab and BALB/k Mab specific for HA1 158.

E2.1 (a). Antibody gene usage by mice of the CBA/Ca strain in the memory response to influenza HA1 158 following natural infection.

Eleven CBA/Ca derived Mab specific for HA1 158(Gly→Glu) were sequenced (Table 18), as described for BALB/k Mab, from four individual donors to determine V_H/V_L gene usage in comparison with V gene usage of BALB/k antibodies of the same specificity. Results are shown in Table 19. Mab were selected of the γ2a isotype from each donor (for direct comparison to each other), and of γ1, γ2b and γ3 isotypes from donors JCB 4B and donor LD6 for comparison with Mab of these isotypes from BALB/k donor SFA 7B.

Ten out of eleven Mab from these four donors use L-chain V_κ1A K5.1 gene. The other Mab, JCB 2B 29.1 uses V_κ19β. Mab from donor JCB 2B use J_κ4, whilst all the other Mab, with the exception of JCB 4B3.1 (J_κ2) use J_κ1, demonstrating *very restricted* light chain gene usage.

Heavy chain gene usage is more diverse, but donor specific. Mab JCB 2B5.1.1 used the V_H3609n gene, whilst the other antibody from this donor uses the V_HJ606 "germline" gene. These two antibodies also use different J_H genes, J_κ2, and 1 respectively, and CDR3H lengths and sequences vary despite both Mab using D_H genes most closely related to DSP2.9. Mab from all of the other CBA/Ca donors, JCB 3B, 4B, and LD6 also use J_H2 and DSP2.9, and have CDR3H lengths (six amino acids) equal to that of JCB 2B5.1.1, therefore JCB 2B 29.1 appears to be the exception to the rule in this cohort.

V_H family gene usage varied between donors. The two Mab from donor JCB 3B, 6.1 and 8.2, both used V_HJ606 NZA 3 gene, and were identical in all respects, thus originating from the same μ^+ progenitor cell. Mab 2.1 from donor JCB 4B used V_H10, a relatively new family with only two members. The other three Mab from this donor used the V_H7183.14 gene, irrespective of isotype, as deduced *via* database search and

Table 18: Monoclonal antibodies of immunodominant recognition specificity from four CBA/Ca donors selected for immunoglobulin gene sequencing and assignment of V_H and V_L families.

CBA/Ca Donor	B-cell hybridoma clone	Antibody specificity X31 HA(H3N2)	Isotype
JCB 2B	5.1.1 29.1	HA1 158 G→E	γ2a γ2a
JCB 3B	6.1 8.2	HA1 158 G→E	γ2a γ2a
JCB 4B	2.1 3.1 4.3 40.7	HA1 158 G→E	γ2a γ2a γ1 γ3
LD6	D9.1 A7.6 A12.1	HA1 158 G→E	γ2a γ2b γ2b

Table 19: Immunoglobulin gene usage by monoclonal antibodies from four CBA/Ca donors specific for HA1 158 CBA/Ca immunodominant epitope.

* indicates letters assigned for undocumented genes in the literature (see text) i.e. no gene name given for the closest matched gene in the article.

' indicates antibodies using the same gene as defined by database comparison, but having less than 90% homology.

Donor	Immunoglobulin gene usage by CBA/Ca HA1 158-specific monoclonal antibodies from four donors									
	Clone	V_L family	V_L gene	J_κ gene	V_H family	V_H gene	D gene	CDR3H amino acid length	J_H gene	C_H isotype
JCB 2B	5.1.1	V_κ1A	K5.1	4	V_H3609	n	DSP2.9	6	2	γ2a
	29.1	V_κ19	β*	4	V_HJ606	germline	DSP2.9	11	1	γ2a
JCB 3B	6.1	V_κ1A	K5.1	1	V_HJ606	NZA3	DSP2.9	6	2	γ2a
	8.2	V_κ1A	K5.1	1	V_HJ606	NZA3	DSP2.9	6	2	γ2a
JCB 4B	2.1	V_κ1A	K5.1	1	V_H10	2 genes	DSP2.9	6	2	γ2a
	3.1	V_κ1A	K5.1	2	V_H7183	.14'	DSP2.9	6	2	γ2a
	4.3	V_κ1A	K5.1	1	V_H7183	.14'	DSP2.9	6	2	γ1
	40.7	V_κ1A	K5.1	1	V_H7183	.14'	DSP2.9	6	2	γ3
LD6	A7.6	V_κ1A	K5.1	1	V_HS107	V β6.5 NZA6'	DSP2.9	6	2	γ2b
	A12.1	V_κ1A	K5.1	1	V_HS107	V β6.5 NZA6'	DSP2.9	6	2	γ2b
	D9.1	V_κ1A	K5.1	1	V_HS107	V β6.5 NZA6'	DSP2.9	6	2	γ2a

comparison, but had less than 90% identity by nucleotide sequence homology. All Mab from donor LD6, of either γ2a or γ2b isotype all use the V_HS107 VB6.5 (NZA6) gene (of the T15 group), but were less than 88% homologous by sequence comparison.

Although overall V_H family usage may appear diverse at first glance, it is worth noting that the V_H families utilised by these 11 Mab, V_HJ606, V_H7183, V_HS107 and V_H10, all have over 70%, but less than 80% homology, hence their classification as different families. At the individual level, V_H gene family usage is extremely restricted. It can therefore be concluded that both V_H family and V_L gene usage in this strain is restricted in the response against influenza HA1 158.

E2.1 (b). Direct comparison of CBA/Ca and BALB/k V gene usage in HA1 158-specific Mab.

Since all CBA/Ca Mab that selected the same amino acid residue change, HA1 158 (and nucleotide base) use the same V_L gene and V_H family, (and D_H gene, J_H2, and J_κ to some extent), this may provide evidence of a structural basis for immunodominance and recognition of this determinant epitope by the four individual donors.

In Chapter 2, I found that BALB/k Mab that selected variant viruses with the HA1 158 change differed in their V_H/V_L gene usage to BALB/k Mab selecting HA1 198 variants from the same strain and donor (although antibody gene usage was not mutually exclusive between these two groups). Mab from BALB/k donor SFA 7B utilised seven different genes from the V_HJ558 family, and four different V_L families, also seen in the BALB/k anti-198 repertoire.

The J558 family is not utilised by CBA/Ca Mab, and reciprocally, the families used by CBA/Ca Mab are not represented in the BALB/k repertoire. The most commonly used light chain gene, V_κ1A K5.1, in $^{10}/_{11}$ CBA/Ca Mab, is only used by one (of eight) BALB/k Mab, but the "odd one out" of the CBA/Ca Mab, JCB 2B 29.1, uses V_κ19, present in two of the eight BALB/k Mab. So, while both strains select for the same nucleotide change in HA1, they utilise different antibody genes to encode the antigen binding domain to do this. V_H family usage can be considered as mutually exclusive for these two groups, as can V_L

family usage, albeit to a lesser extent, (with the single exceptions for each strain of SFA 7B2.1 and JCB 2B29.1), and BALB/k anti-158 Mab have restricted V_H usage, whereas CBA/Ca anti-158 Mab have restricted V_L gene usage (and V_H of over 70% homology).

E2.2. Strain specific antibody gene usage.

Since mice of different strains appear to use different antibody gene families, either against the same, or different epitopes, it may be possible that certain antibody V gene families are relatively not well represented in the different strains, and that presence of germline antibody genes and/or expression of these differs accordingly (with the mouse strain). Antibody family usage in the response to influenza HA of BALB/k and CBA/Ca mice are shown in Figures 15 (a) and 15 (b).

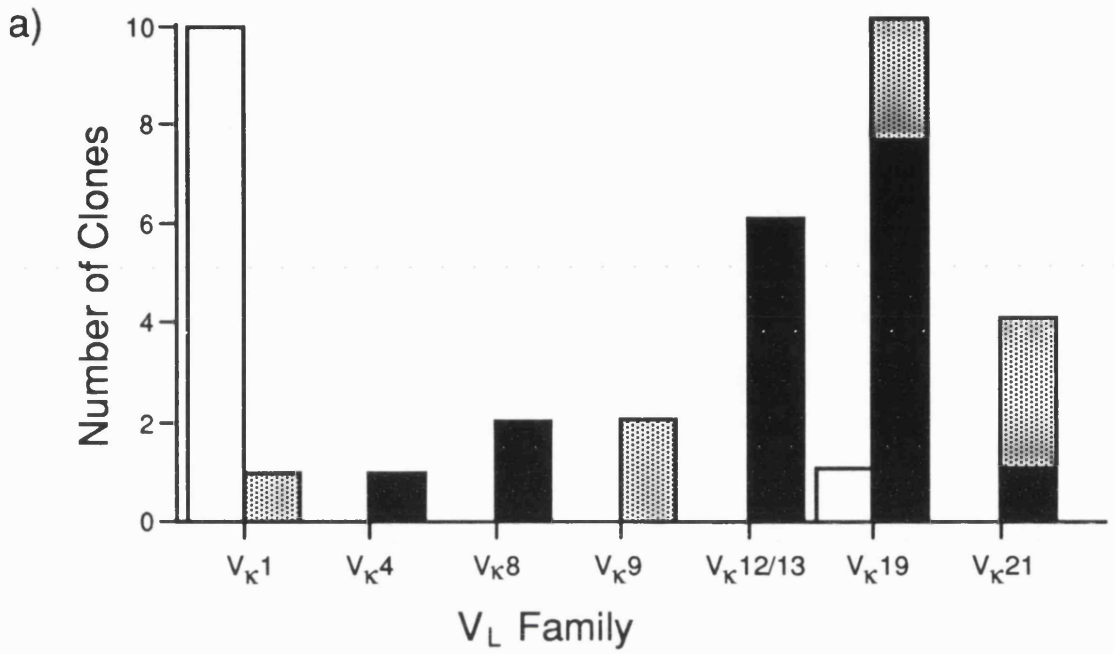
From this investigation, I have demonstrated that $^{10}/_{11}$ Mab from the CBA/Ca strain utilise V_κ1A K5.1 gene, and one of a group of closely related V_H families (J3609, J606, S107, 7183 or V_H10). The V_κ1 family is only used by one out of the 26 BALB/k Mab, and then only in a Mab of the same specificity as CBA/Ca Mab, whilst only the V_H7183 family (different gene) used by CBA/Ca Mab is also used by $^2/_{26}$ BALB/k Mab of HA1 198 specificity.

The BALB/k strain also uses antibody V gene families not observed in the CBA/Ca repertoire (with the exception of JCB 2B29.1). The BALB/k anti-198 response uses predominantly V_H VGam3.8, whereas all eight 158-specific Mab utilise the V_HJ558 family (representing $^{12}/_{26}$ of all BALB/k Mab sequenced), neither of which are present in the CBA/Ca anti-HA repertoire. In the overall BALB/k response a large variety of V_L families are used, but predominantly V_κ19 ($^{10}/_{26}$), followed by V_κ12/13 ($^6/_{26}$) and V_κ1, 4, 8, 9 and 21. There is a single example of V_κ19 usage in the CBA/Ca response (JCB 2B29.1), (just as there is only a single example of CBA/Ca predominant V_κ1 usage in BALB/k). These results therefore suggest a strain-biased, rather than specificity-biased V gene usage.

E2.3. Random stoichiometric or (D)J proximal V gene utilisation.

The most 3', J proximal V_H gene family in BALB/c, C57BL/6 and CBA/J, therefore presumably also in BALB/k and CBA/Ca mice is V_H7183 (Alt *et al.*, 1987). Genes from

Figure 15: Histograms of a) V_L family usage, and b) V_H family usage by BALB/k anti-158 (shaded) and anti-198 (**black**) Mab and CBA/Ca (**white**) anti-158 Mab.


a)
Number of Clones
0
2
4
6
8
10
V_K1
V_K4
V_K8
V_K9
$V_K12/13$
V_K19
V_K21
V_L Family

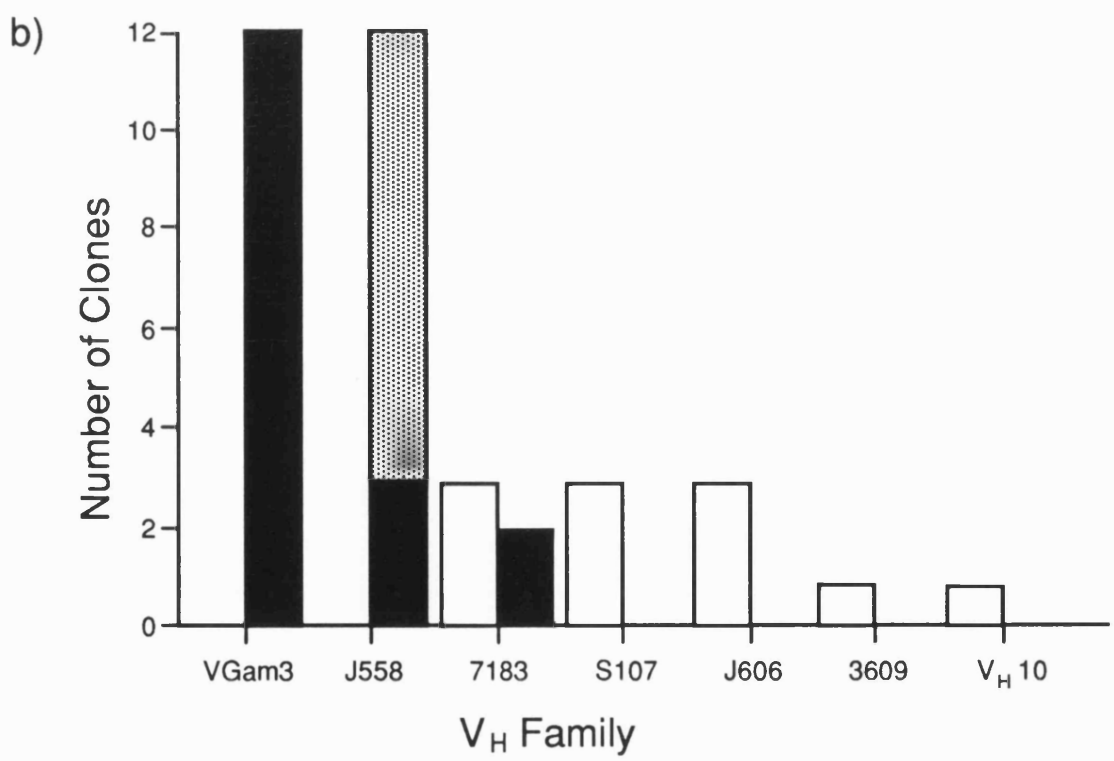
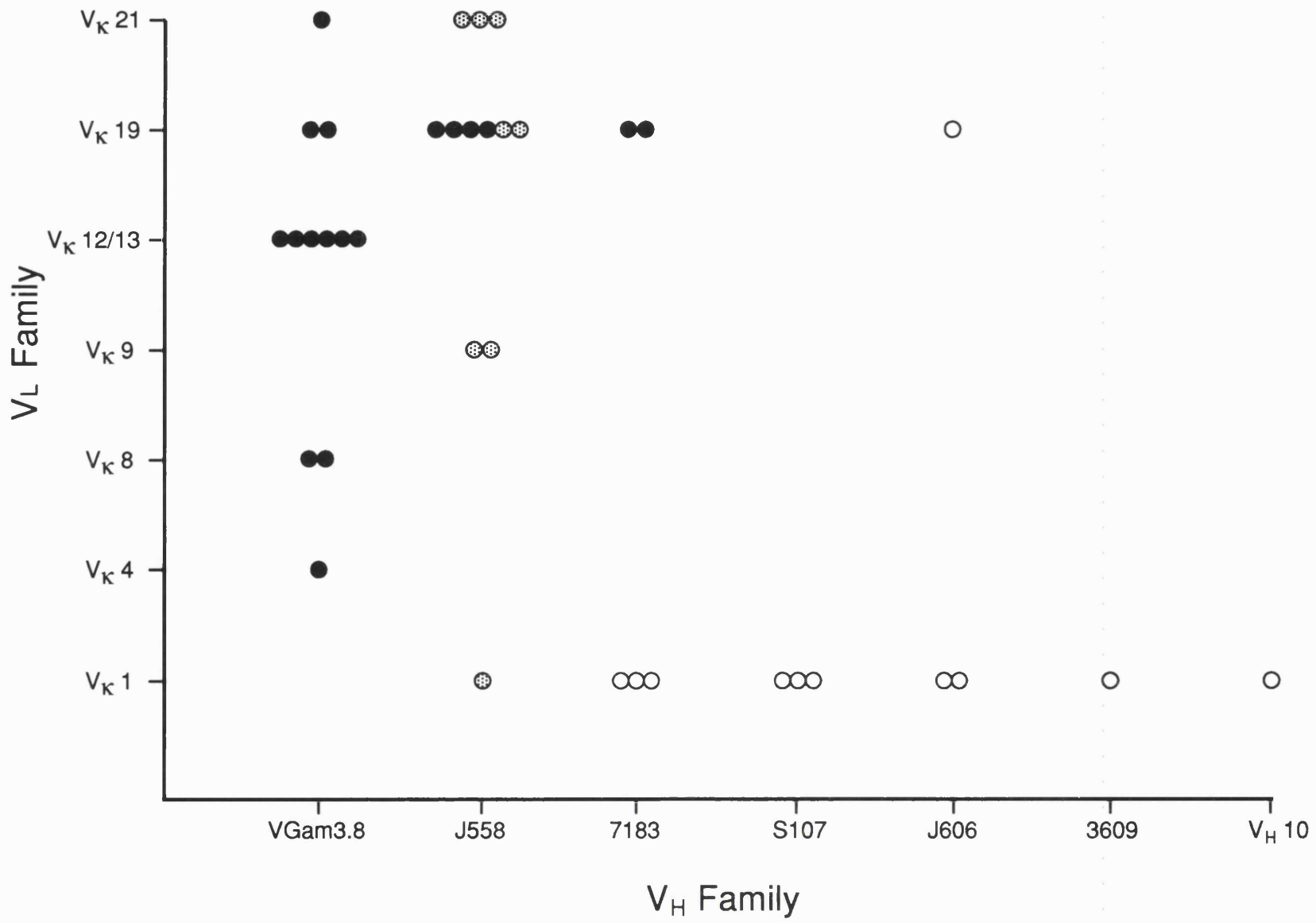

b)
Number of Clones
0
2
4
6
8
10
12
VGam3
J558
7183
S107
J606
3609
V_H 10
V_H Family

Figure 16: Dot plot of preferential pairing by immunoglobulin V_H and V_L gene families.

Each dot represents one Mab using this V_H/V_L combination from BALB/k anti-158 (shaded) and anti-198 (black) Mab and CBA/Ca (white) anti-158 Mab.


V_K 21
V_K 19
V_K 12/13
V_K 9
V_K 8
V_K 4
V_K 1
V_L Family
VGam3.8
J558
7183
S107
J606
3609
V_H 10
V_H Family

this family are not predominantly used by either BALB/k, where it is utilised by only two out of twenty-six antibodies, or CBA/Ca, where it is used by three of eleven antibodies. The largest family, V_HJ558 is absent from the CBA/Ca response, and is poorly represented in four of the five BALB/k donors, encoding only two out of twenty-six Mab. Interestingly, it constitutes the entire repertoire of the anomalous (with regards to specificity) BALB/k donor SFA 7B, suggesting selection and rearrangement of antibody genes in this study is not random.

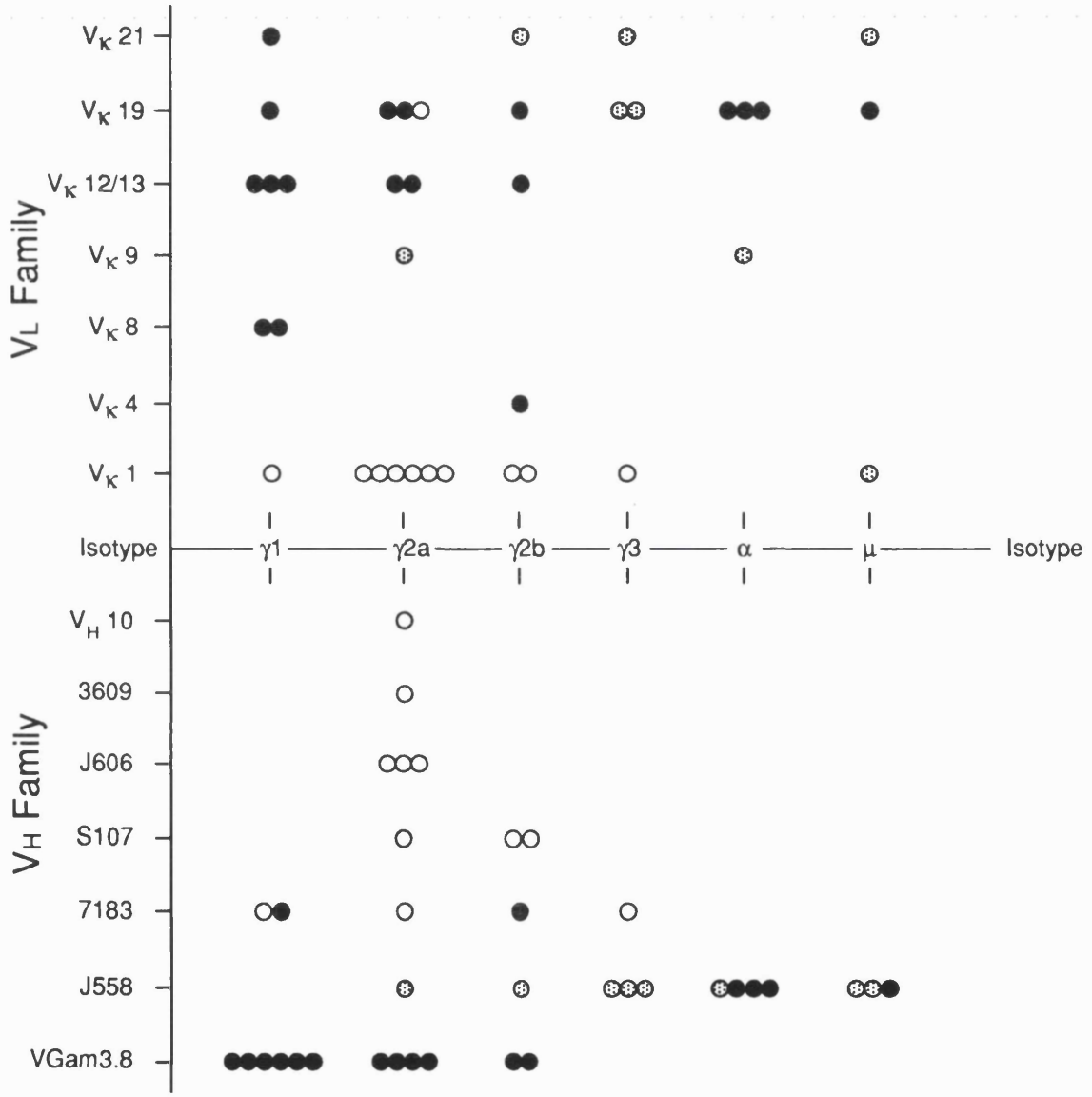
E2.4. Is there preferential pairing of V_H and V_L chains within a donor or strain?

The overall results of V_H/V_L pairing of all 37 Mab analysed in this study are shown in Figure 16. Seven V_H and seven V_L families are used in total. The most frequently used V_H families are VGam3.8 and J558 ($^{12}/_{37}$ each), and the most frequently used V_L families are V_κ1 and 19 ($^{12}/_{37}$ each), but these V_H and V_L families do not always pair with each other. The most common pairings are VGam3.8 with V_κ12/13 ($^{6}/_{37}$), and V_HJ558 with V_κ19 ($^{6}/_{37}$), but VGam3.8 is also found paired with V_κ4, 8, 19 and 21, and J558 with V_κ1, 9 and 21. V_κ19 is found paired with VGam3.8, 7183 and J606, but all antibodies possessing a V_κ12/13 L-chain pair only with VGam3.8, all of which represent a very minor component of the total repertoires (i.e. 1 or 2 out of 37 Mab). This is also found with the V_H10 used by a single CBA/Ca antibody which only pairs with V_κ1, as do the majority of CBA/Ca Mab, but this is inconclusive since only one Mab uses V_H10.

E2.5. Isotype specific V region gene usage.

At the general level, there appears to be no correlation between isotype and V_L gene usage, as shown in Figure 17. Most antibodies of different isotypes use genes from at least three different V_L families, and V_H usage also appears not to correlate with isotype for IgG antibodies (γ1, γ2a, γ2b and γ3). Mab of α or μ isotype however, all utilise genes from the V_H family J558 exclusively, represented by antibodies from two donors, BALB/k SFA 6B and 7B, and of two different specificities, HA1 158 and 198 from donor SFA 7B.

Figure 17: Dot plot of immunoglobulin gene family usage by BALB/k anti-158 (shaded) and anti-198 (black) Mab and CBA/Ca (white) anti-158 Mab per isotype.


V_L Family
V_K 21
V_K 19
V_K 12/13
V_K 9
V_K 8
V_K 4
V_K 1
Isotype
γ1
γ2a
γ2b
γ3
α
μ
Isotype
V_H Family
V_H 10
3609
J606
S107
7183
J558
VGam3.8

It is thought that α or μ isotype antibodies are generally present in the early primary response, and do not usually carry through in great numbers to the secondary response (Alt *et al.*, 1982; Berek & Ziegner, 1993) which has usually class switched to the γ isotypes at this stage. The secondary, memory response was studied here, but four α Mab and three μ Mab (equivalent to 19% of the response) have been detected here. This may be due to immortalisation (i.e. hybridoma formation) of B-cells that are engaged in a primary response to the secondary boost. Antibodies from foetal liver and early primary B-cells have been found to generally utilise either the largest V_H family (J558) or the most J-proximal (7183) (Alt *et al.*, 1987), as indeed they do in this study. High affinity antibody forming B-cells generally retain their specific V_H/V_L genes after class switching to the γ isotypes. The specified gene within a family in the primary response may, however, be considered to be different in the secondary immune response due to accumulation of point mutations during affinity maturation (Berek & Milstein, 1987), based on a "cut-off" of over 90% homology defining a single gene. It is therefore assumed that these cells would have retained usage of the V_HJ558 family if they did undergo further class switching, or mutation, since J558 is the largest V_H family and has many members, and would thus still be utilising the same V_H family as the γ isotype antibodies from the same donor.

Reciprocally, the extent of somatic hypermutation might be such that, by these criteria an antibody may alter sufficiently to be classified as a member of a different family, (less than 80% homology). Two of the α isotype Mab are from donor SFA 6B. The third Mab from this donor, 6B1.1 utilises a gene from the VGam3.8 family, as do most other BALB/k HA1 198-specific Mab. It may thus be possible that the α Mab V gene usage will shift to VGam3.8 with maturation of the response, or alternatively remain J558, inferring that V region gene usage does not correlate with isotype here.

V_L family usage is shown at the top of the plot and V_H at the bottom of Figure 17. Each dot represents one Mab.

In conclusion, it is suggested that all early antibodies use V_HJ558, but alter V_H family usage with maturation and class switching. Alternatively, antibodies which arise from the same progenitor cell (μ^+) can class switch independently to different isotypes, and still retain the same V region genes.

E2.6. The immunodominant B-cell response of an individual originates from clonal expansion of one, or a few progenitor cells.

The initial intention of analysing V gene usage with respect to isotype was to determine whether immunodominance could be explained by expansion of a single μ^+ progenitor cell, thus all B-cells of the same specificity from an individual donor would be using the same V genes prior to class switching. This was found in donor SFA 2B in two out of its four Mab, both of $\gamma 1$ isotype. Donor 2B has three Mab of the $\gamma 1$ isotype, two of which (1.1 and 7.2) use exactly the same antibody genes throughout (VGam3.8 VGK7, $V_\kappa 8\alpha$, $J_H 4$, $J_\kappa 5$ and DSP2.2), therefore originate from the same progenitor cell. The third $\gamma 1$ Mab from this donor uses the same V_L gene, but differs at all the other loci, and the fourth Mab uses completely different V-D-J families, indicating that not all of the $\gamma 1$ Mab in this donor originate from the same progenitor, nor identical clones from the same B-cell-myeloma fusion.

In donor SFA 3B, clones 2.1 and 3.1 utilise the same V-D-J genes, 7.1 and 12.1 are identical, and so are 10.1 and 14.1, but these three sets of clones differ in isotype within a pair. That is, 2.1 is $\gamma 2a$ and 3.1 is $\gamma 1$, 7.1 is $\gamma 2b$ and 12.1 is $\gamma 1$, and 10.1 is $\gamma 2a$ and 14.1 is $\gamma 2b$, therefore the six HA1 198-Mab from donor SF 3B originated from three different μ^+ progenitors.

Hybridoma clones SFA 7B 1.2 and 7.1 both utilise identical V region genes, but since these are of the same isotype (α) and share complete identity in their V_L gene and 96.15% homology in their V_H gene, and also have the same C→G substitution in their $J_H 4$ gene, as well as almost identical CDR3H regions, it is more realistic to assume they are products of the same B-cell fusion (hybridoma), although hybridoma clone 8.1, with a different recognition specificity, HA1 158, also has the same C→G substitution in $J_H 4$, and uses the same genes (by database assignment, but sequence homology in V_HJ558 DBA/1 is less than 90%), and is of the $\gamma 3$ isotype (also true for 3.1, but which lacks the $J_H 4$ substitution). 7B 8.1 also differs in CDR3H sequence, the most diverse region in the antibody molecule, which may alone account for the difference in recognition specificity. It can then be concluded that 1.2, 7.1, 3.1 and 8.1 all originated but diversified from the same progenitor cell. Three of these four clones (excluding 8.1) have almost identical

CDR3H sequences, but 3.1 has an AT→CG substitution different to 1.2 and 7.1, leading to an N→Y change in amino acid sequence at the junction of D-J joining. V_H gene assignment by BLASTN database comparison indicates all of these clones are using gene DBA/1, but sequence homology between these clones is less than 50% using the Distances program, therefore I have named them DBA/1b for 1.2 and 7.1, and DBA/1c for 3.1. This difference in V_H DBA/1 genes may thereby explain the differences in fine specificities of these clones. Mab 10.2 and 19.1 from this donor are also identical, even in CDR3H sequence, with the exception of V_H gene usage (y and 186.2' respectively), and isotype (α and γ2a), thus are from the same progenitor.

Tentative assignments of V_H families and genes were necessary for donor 7B, HA1 158-specific Mab since the two different methods used to compare them to each other gave two different answers. BLASTN comparison of these sequences for closest match to those already in the EMBL database results in assignment of genes from the V_HJ558 family for all eight Mab, whereas by the criteria set by many workers, to segregate V_H genes into related family groups of identity by sequence homology of greater than 90% defining a gene, and greater than 80% defining a family, these would all be in different families as they share less than 60% homology with each other. However, as they show less than 40% homology to all other Mab sequenced, they do not belong to the same families as those either.

There are also marked differences in the start of the sequences i.e. the 5' region of V_H gene (FR1 to CDR2) due to ^{32}P sequencing of these genes. The sequences at the ends of gels were often unclear due to the extended running time for band separation in this region. Sequences were often "fuzzy" and rarely extended beyond the end of CDR1 from the constant region primer. Ideally, I would have preferred to repeat the sequencing of V_H genes following the same protocol as for V_L genes, using a reverse primer with the constant region primer and ^{35}S sequencing of DNA, to confirm the ends of the sequence. Identity and family assignment by BLASTN for V_L genes are more closely related due to the clarity of ^{35}S sequencing gels, and complete sequence by use of two primers, hence sequencing from both ends of the template.

In the CBA/Ca repertoire, both Mab from donor JCB 3B, 6.1 and 8.2 are identical, with 92.5% identity of V_H and 97.3% identity of V_L genes, therefore are from the same cell

or fusion product. In donor JCB 4B, clones 3.1, 4.3 and 40.7 use the same V region genes, but 3.1 uses a different J_κ gene (2) and differs in CDR3H region therefore originate from one progenitor cell, and in donor LD6, all 3 Mab are identical, except in isotype, similar to Mab from donor 4B, also from one μ^+ progenitor cell.

E2.7. CDR3H analysis: Diversity region genes and V-D-J joining: If V_L genes direct the antibody to a region of a protein, and V_H to a specific site within this region, can the CDR3H region of an antibody confer fine specificity?

Figures 18 a) and b).

Due to the extent of diversity in this region, and N region addition, assignment of D genes was difficult. All three D families are represented, and segregate the antibodies with respect to donor. That is, donor 2B uses DSP2.2, donors 6B and 9B use DQ52, donor 3B uses DSP2 mostly, with $^2/_6$ Mab using DFL16.1. Donor 7B uses DQ52 with both 198-specific Mab, but also with 158-specific 3.1 and 8.1, and DFL16.1 in five of the other 158-specific Mab (7B 2.1 uses DSP2.9), showing no correlation of D region gene usage or N addition with specificity or VH gene usage. Despite use of the same D family per donor, CDR3H sequences and amino acid lengths differ per clone, within a donor.

CDR3H regions of ten of the eleven CBA/Ca Mab sequenced are six amino acids long and all eleven use DSP2.9. Sequences of clones 2B5.1.1, 3B6.1, 3B8.2, 4B2.1, 4B4.3 and 4B40.7 are almost identical. LD6 Mab CDR3H sequences are identical and similar to sequences of the aforementioned clones, particularly at the amino acid level. They are however, very different to those of the BALB/k SFA 7B Mab of the same specificity. Only SFA 7B 2.1 has similar CDR3H sequence and length to a CBA/Ca Mab, 2B5.1.1. Mab SFA 7B3.1 surprisingly has a similar CDR3H region to Mab 1.2 and 7.1 from this donor, but of different specificity (198). CDR3H regions of all SFA 198-specific Mab also vary widely, therefore specificity cannot be attributed to this region alone.

Figure 18 a): Nucleotide sequences of immunoglobulin CDR3H regions at the junctions of D_H-J_H joining of 18 BALB/k anti-198 Mab from five donors, 8 BALB/k anti-158 Mab (donor SFA 7B) and 11 CBA/Ca anti-158 Mab from four donors.

```
 Frames      ------FR3-end->------------------------CDR3-----------------------<-FR4-start---JH-
 BALB/k 198
SFA 2B1.1    TATCTCTGTGCTAGA---------------------GGAGACCATGGT---TACGTTATGGACTAC-TGGGGTCAAGGAACCTC
SFA 2B3.1    TATTTCTGTGGTAGG---------------------GGGAACTATGGTAACTATGTTATGGACTAC-TGGGGTCAAGGAACCTC
SFA 2B7.2    TATTTCTGTGCTAGA---------------------GGAGACTATGGT---TACGCTATGGACTAC-TGGGGTCAAGGAACCTC
SFA 2B8.1    TATTTCTGTGCTAGA---------------GGCCATGGTAACTACTTTCACTTT------GACTAT-TGGGGTCAAGGCACCAC
SFA 3B2.1    TATTTCTGTGCAAGA-GAGAGGGATTACTACGGTAGTAACTGTGGGTTTCCT-----------TAC-TGGGGCCAAGGGACTCT
SFA 3B3.1    TTTTTCTGTGCAAGA-GAGAGGGATTACTACGGTAGTAACTGTGGATTTCCT-----------TAC-TGGGGCCAAGGGACTCT
SFA 3B7.1    TATTACTGTGCACGA-------------GAGGGAATCGACTGG---TACTTCGATGTC----------TGGGGTGCAGGGACAAC
SFA 3B10.1   TATTTCTGTGCAAGA----CAGGCTACGGTAGCC------GATTAC------TATGCTCTGAACTAC-TGGGGTCAAGGAACCTC
SFA 3B12.1   TATTACTGTGCAAGA-------------GAGGGAATCGACTGG---TACTTCGATGTC----------TGGGGCGCCGGGACCAC
SFA 3B14.1   TATTTCTGTACAAGA----GTGGCTACGGTTGCC------GATTAC------TTTGCTCTGGGCTAC-TGGGGTCAAGGAACCTC
SFA 6B1.1    TATTTCTGTGCTAAC----------GGTTATGAGGAGGTCTAC---------TATGCTATGGACTAC-TGGGGTCAAGGAAACTT
SFA 6B4.2    TATTACTGTACAGAG-------------AAGATGATGGTTACGACTACTCTGTTG---ATGGACTCC-TGGGGTCTGAGGAGCAC
SFA 6B5.1    TATTACTGTGCAAGA---------------GATGATGATTACCCATAC---TCTGCTATGGACTCC-TGGGGTCAAGGATT
SFA 7B1.2    TATTACTGTACAAGA---------------GACTATGGT---ACCTAC---TATGTTATGGATAAC-TGGGGTCAAGGAAC
SFA 7B7.1    TATTACTGTACCAGA---------------GACTATGGT---ACCTAC---TATGTTATGGATAAC-TGGGGTAAAGGAC
SFA 9B3.1    TATTTCTGTGCTAAG----------GGTTATGATTACGTC---TTCTAT---TCT---ATGGACTAC-TGGGGTCAAGGAACCTC
SFA 9B6.1    TATTTCTGTGCTGCC-------------TATGATTACGAC---------GATGCT---ATGGACTAT-TGGGGTCAAGGAACCTC
SFA 9B8.1    TATTTCTGTGCTAGA----------GGCTTTGGTCCC---------TACTATTCT---ATGGACTGC-TGGGGTCAAGGAACCTC
 BALB/k 158
SFA 7B2.1    TATTACTGTGCATTG---------------------GGTGACTACTTT-----------GACTAC-TGGGGTCTAAGGCAC
SFA 7B3.1    TATTACTGTACAAGA---------------GACTATGGT---ACCTAC---TATGTTATGGACGAC-TGGGGTCAAGGAGCCTC
SFA 7B5.1    TACTACTGTGGGAAG-------GATAGACGCTCGGGTTTCGGTGCTTTG-----------GACTAC-TGGGGTCAAGGAA
SFA 7B8.1    TATTTCTGTGCAAAA---------------TGGGATGGT------TACCTCTATGTTATGGACTAC-TGGGGTCAAGGAACCTC
SFA 7B10.2   TATTACTGTGCAGTG---------------TGGTACCCTGAGAGG-----------TTGAACTAC-TGGGGTCAAGGCAC
SFA 7B11.1   CATTACTGTACAAGA---------------TGGGACTATGCT---------------ATGGACAAC-TGGGGTCAAGGAGACAC
SFA 7B15.1   TATTACTGTGTAAGG-------------TCTGGGTCCTATGCG---------------AAGGACTAC-TGGGGTACAGGWM
SFA 7B19.1   TATTATTGTGCAGTA---------------TGGTACCCTGAGAGG-----------TTGAACTAC-TGGGGTCAAGGCACTAC
 CBA/Ca 158
JCB 2B5.1.1  TATTATTGTAGTATT---------------------GGTGACTACCTT-----------GACTAC-TGGGGCCAAGGCACCAC
JCB 2B29.1   TATTACTGTGCAAGA----GTCTACTATGATTACGACAGG---TTCTTCGATGTC-------------TGGGGCGCAGGGACCAC
JCB 3B6.1    TATTACTGTCCGTAT---------------------GGTGAATTTCTT-----------GACTAC-TGGGGTCAAGGCACNGC
JCB 3B8.2    TATTACTGTACCTAT---------------------GGTGAATTTCTT-----------GACTAC-TGGGGTCAAGGCACCAC
JCB 4B2.1    TATTACTGTGTTTGT---------------------GGTAACTTCTTT-----------GACTAC-TGGGGTCAAGGCACCAC
JCB 4B3.1    TATTACTGTGCCTTA---------------------AGCGACTTCTTT-----------GACTAC-TGGGGCCAAGGCACCAC
JCB 4B4.3    TATTACTGTTCAATT---------------GGT------GACTAC---CAT---------GACTAC-TGGGGCCAAGGCACCAC
JCB 4B40.7   TATTACTGTTCAATT---------------GCT------GACTAT---CAT---------GACTAC-TGGGGCAAGGCGCCGCT
LD6 A7.6     TATCACTGTACAATA---------------------TCGTATTTCTTT-----------GACTAC-TGGGGCCAAGGCACCAC
LD6 A12.2    TATTACTGTGCAATT---------------------TCGTACTACTTT-----------GACTAC-TGGGGCCAAGGCACCAC
LD6 D9.1     TATCACTGTACAATA---------------------TCGTATTTTTTT-----------GACTAC-TGGGGCCAAGGCACCAC
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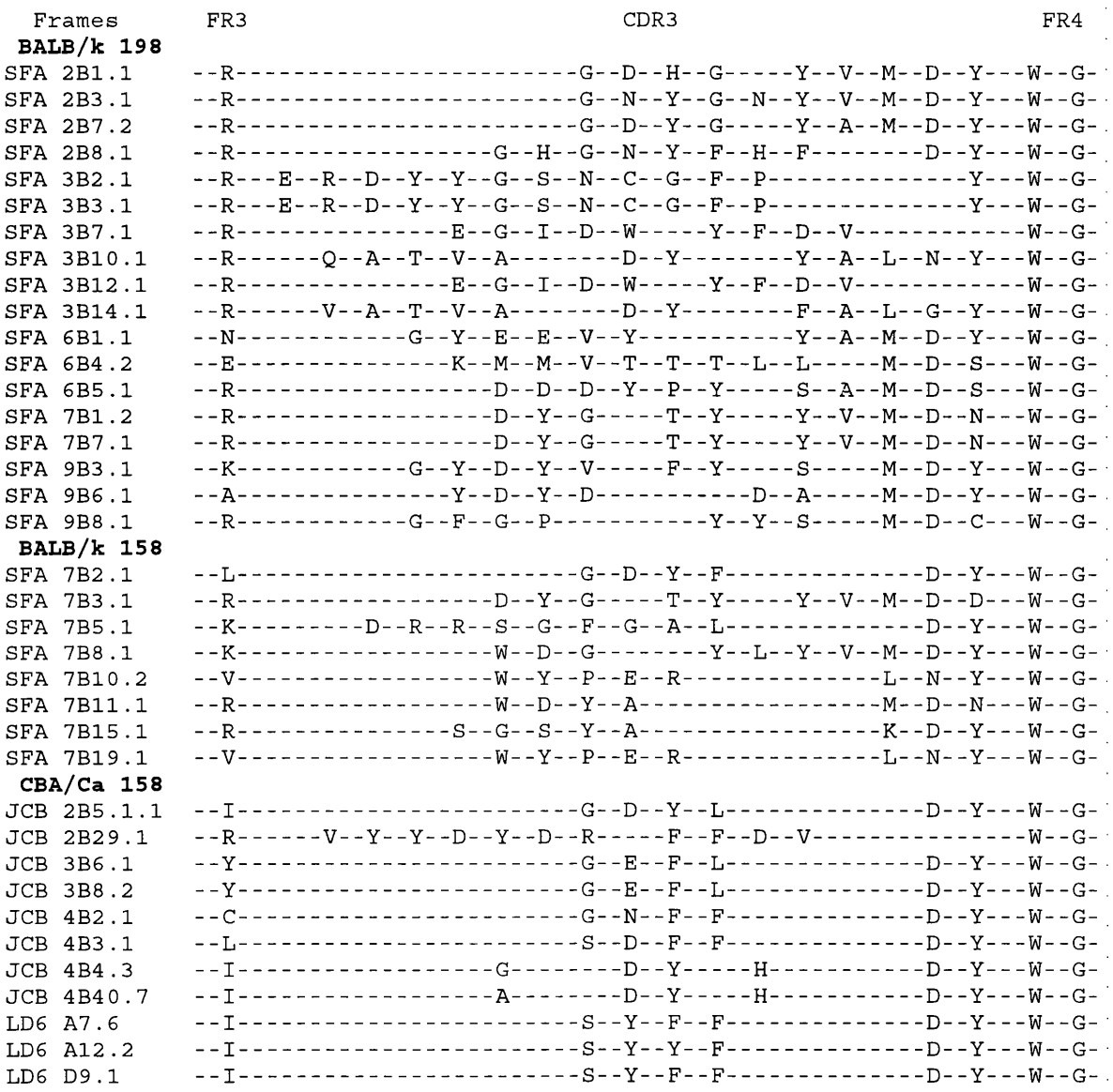
Figure 18 b): Amino acid sequences of immunoglobulin CDR3H regions at the junctions of D_H-J_H joining of 18 BALB/k anti-198 Mab from five donors, 8 BALB/k anti-158 Mab (donor SFA 7B) and 11 CBA/Ca anti-158 Mab from four donors.


Frames
FR3
CDR3
FR4
BALB/k 198
SFA 2B1.1 --R----------G--D--H--G-----Y--V--M--D--Y---W--G-
SFA 2B3.1 --R----------G--N--Y--G--N--Y--V--M--D--Y---W--G-
SFA 2B7.2 --R----------G--D--Y--G-----Y--A--M--D--Y---W--G-
SFA 2B8.1 --R----------G--H--G--N--Y--F--H--F--------D--Y---W--G-
SFA 3B2.1 --R---E--R--D--Y--Y--G--S--N--C--G--F--P----------Y---W--G-
SFA 3B3.1 --R---E--R--D--Y--Y--G--S--N--C--G--F--P----------Y---W--G-
SFA 3B7.1 --R----------E--G--I--D--W-----Y--F--D--V----------W--G-
SFA 3B10.1 --R------Q--A--T--V--A--------D--Y--------Y--A--L--N--Y---W--G-
SFA 3B12.1 --R----------E--G--I--D--W-----Y--F--D--V----------W--G-
SFA 3B14.1 --R------V--A--T--V--A--------D--Y--------F--A--L--G--Y---W--G-
SFA 6B1.1 --N----------G--Y--E--E--V--Y----------Y--A--M--D--Y---W--G-
SFA 6B4.2 --E----------K--M--M--V--T--T--T--L--L-----M--D--S---W--G-
SFA 6B5.1 --R----------D--D--D--Y--P--Y-----S--A--M--D--S---W--G-
SFA 7B1.2 --R----------D--Y--G-----T--Y-----Y--V--M--D--N---W--G-
SFA 7B7.1 --R----------D--Y--G-----T--Y-----Y--V--M--D--N---W--G-
SFA 9B3.1 --K----------G--Y--D--Y--V-----F--Y-----S-----M--D--Y---W--G-
SFA 9B6.1 --A----------Y--D--Y--D----------D--A-----M--D--Y---W--G-
SFA 9B8.1 --R----------G--F--G--P----------Y--Y--S-----M--D--C---W--G-
BALB/k 158
SFA 7B2.1 --L----------G--D--Y--F----------D--Y---W--G-
SFA 7B3.1 --R----------D--Y--G-----T--Y-----Y--V--M--D--D---W--G-
SFA 7B5.1 --K---------D--R--R--S--G--F--G--A--L----------D--Y---W--G-
SFA 7B8.1 --K----------W--D--G--------Y--L--Y--V--M--D--Y---W--G-
SFA 7B10.2 --V----------W--Y--P--E--R----------L--N--Y---W--G-
SFA 7B11.1 --R----------W--D--Y--A----------M--D--N---W--G-
SFA 7B15.1 --R----------S--G--S--Y--A----------K--D--Y---W--G-
SFA 7B19.1 --V----------W--Y--P--E--R----------L--N--Y---W--G-
CBA/Ca 158
JCB 2B5.1.1 --I----------G--D--Y--L----------D--Y---W--G-
JCB 2B29.1 --R------V--Y--Y--D--Y--D--R-----F--F--D--V----------W--G-
JCB 3B6.1 --Y----------G--E--F--L----------D--Y---W--G-
JCB 3B8.2 --Y----------G--E--F--L----------D--Y---W--G-
JCB 4B2.1 --C----------G--N--F--F----------D--Y---W--G-
JCB 4B3.1 --L----------S--D--F--F----------D--Y---W--G-
JCB 4B4.3 --I----------G--------D--Y-----H----------D--Y---W--G-
JCB 4B40.7 --I----------A--------D--Y-----H----------D--Y---W--G-
LD6 A7.6 --I----------S--Y--F--F----------D--Y---W--G-
LD6 A12.2 --I----------S--Y--Y--F----------D--Y---W--G-
LD6 D9.1 --I----------S--Y--F--F----------D--Y---W--G-

E3. CONCLUSION

Antibody gene usage in the CBA/Ca anti-HA1 158 response is extremely restricted. Of 11 Mab from four donors, 10 use the same V_L gene, and although five V_H families are used, these are all greater than 70%, but less than 80% homologous. It can be concluded from the CBA/Ca antibody gene usage results that V_κ1A K5.1 together with any of the V_H families, 3609, S107, J606, 7183 or V_H10 encode antibodies that are able to recognise the distal globular head region of the HA monomer, and specifically select for variant virus, HA1 158(Gly→Glu). Had the CBA/Ca study been performed alone, it would have been concluded that antibody gene usage specified a structural basis for immunodominance but, in conjunction with the data from chapter 2 on the BALB/k 158 and 198-specific response, this does not appear to be the whole story. This general conclusion for immunodominance in CBA/Ca cannot be extended to other strains. Both V_κ gene families V_κ19 and V_κ1 are used by BALB/k mice, but V_κ1 is only used the the BALB/k anti-158 repertoire.

All V_L and V_H families except V_HJ606, used by three Mab from two CBA/Ca donors, have previously been reported in the BALB/c anti-influenza HA response. Of the V_H families utilised by CBA/Ca, only V_H7183 is used by BALB/k Mab in the response to HA1 198 (different gene), but it is paired with a different V_κ family. The same D gene family, DSP2 is used by both strains, but again different genes. V_HJ558 family is used by all BALB/k anti-158 Mab, but not at all by CBA/Ca Mab of the same specificity.

It is not possible to conclude that antibody gene usage determines specificity, or immunodominance in the light of these two sets of results taken together, since a) different families are represented by antibodies of both BALB/k and CBA/Ca of the same specificity, b) these families are mutually exclusive and c) examples of both V_L and V_H genes and families (and $J_{H/L}$-D) are available in the BALB/k anti-198 repertoire. For example, BALB/k SFA 7B3.1 and 8.1 (158) and BALB/k SFA 6B5.1 and 7B1.2 and 7.1 (198) all use V_κ19β, J_κ1, V_HJ558 DBA/1, DQ52 and J_H4, that is, the *same* antibody gene combinations encoding antibodies which recognise two *different* sites, from two different donors.

Segregation of V_H/V_L families in this study suggest strain-restricted rather than specificity-biased V gene usage. V gene usage characteristic of the mouse strain has been

previously demonstrated (Wu, G. E. & Paige, 1986) for foetal liver and preB cells. The most frequently used V_H family in BALB/c mice was found to be V_H7183 (22%), then J558 (17%), but in the C57BL/6 mice J558 represented 45% of total antibody V_H family usage. The size of each family is thought to be constant between strains (Brodeur & Riblet, 1984) but there is evidence for strain dependent expression of V_H families, particularly J558 and 7183 (Winter *et al.*, 1985; Wu, G. E. & Paige, 1986).

In a study of Ig gene expression in LPS activated B-cells *in vitro* in six different strains of mice (Jeong *et al.*, 1988), differences were observed in the frequency of use of particular families. The largest family, J558 was used most by all strains including BALB/c and CBA/CaJ, with the singular exception of C.AL20 which used 7183 more frequently (35.9% compared with 32.9% of J558). BALB/c and CBA/CaJ mice were thought to use genes from J558, 7183 and Q52 in that order. However strains of Igh e allotype showed three times higher expression of 7183 than other strains and higher expression of 7183 than the larger Q52 family. MHC background had no real effect on Ig family expression since it did not affect expression of any family. BALB/c, C.AL20 and B10.D2/oSnJ, all H-2^d haplotype but with different allotypes, had different expression patterns, but BALB/c (H-2^d) and CBA/CaJ (H-2^k), both of the same allotype, a, had the same pattern.

Patterns of expression are usually explained by family size and proximity to the J_H locus, i.e. the largest family and most J proximal family are favoured. J558 is the largest family, and 7183 the most J proximal, in both CBA/Ca and BALB/c, therefore BALB/k, yet both of these families are poorly represented in this investigation, in both strains. J558 is present in $^2/_{27}$ Mab, from eight donors, excluding SFA 7B, and 7183 in $^5/_{37}$ Mab from nine donors, although J558 dominates the SFA 7B repsonse ($^{10}/_{10}$), regardless of specificity.

Strain specific Ig V_H expression is evident in this study by the exclusivity of V family usage. V_H J3609, J606, S107 and V_H10 are only expressed by CBA/Ca antibodies, and VGam3.8 and J558 by BALB/k antibodies. This also applies, to some extent, to Ig V_L family expression, in that CBA/Ca express predominantly V_κ1, only found in $^1/_{26}$ BALB/k Mab, whereas V_κ4, 8, 9, 12/13, 21 and 19, (with the exception of JCB 2B29.1), are only expressed by the BALB/k strain. This does not appear to be a consequence of selection of antibody of a given specificity since BALB/k anti-158 Mab use V_H and V_L families and genes also used in the BALB/k anti-198 response.

Reports of the most utilised V_H families in the spleen of different strains (Jeong *et al.*, 1988) suggest the order of use in BALB/c to be J558 (largest), Q52 (second largest), 36-60, 7183, J606 and S107, but J558, Q52, J606, 7183, S107 and 36-60 in C57BL/6 mice. These orders neither correlate with family size (J558>Q52>7183>J606>36-60), nor order on the chromosome (3609-J558-J606-VGam3.8-3660-X24-S107-Q52-7183-DJ in BALB/c) (Rathburn *et al.*, 1989). D proximal genes from V_H7183 and Q52 3' families are over-represented early in ontogeny in the emerging B-cell pool, and rearranging in preB-cell lines (Atkinson *et al.*, 1993) from BALB/c mice infected with Abelson murine leukaemia virus, but BALB/c uses 3' families more than C57BL/6. However, the most 3' genes of these families are not preferentially used, therefore the mechanism of selection of V_H family is not proximity to D_H. Generally, 5' families were used by Mab in this study, and the 3' family, Q52 is not represented at all. There is, to my knowledge, no evidence of either stoichiometric or J proximal utilisation of V_L families to date.

There is no real evidence for preferential pairing of the expressed V_H and V_L families from this study, with the possible exception of V_κ12/13 and VGam3.8 (although VGam3.8 also pairs with other V_κ families). There have been several conflicting reports in the literature on preferential pairing. For example, Nadel *et al* (1992) found V_L λ3 repeatedly paired with V_H Q52 in the available repertoire of BALB/c mice elicited by immunisation with rabbit anti-λ antibody coupled with LPS, following polyclonal stimulation of λ B-cells, but λ2/V_H pairing varied per individual mouse (V_H family distribution differed from one λ isotype to another). This is in sharp contrast to the data of Kaushik *et al* (1990) who suggested that the frequency of a given V_H/V_L pairing was the direct product of the individual V_H and V_L frequency in C57BL/6 mice, i.e. proportional to family size but biased to 3' exons in neonatal/ foetal mice, and V_κ were neither biased to 3' in neonates or stoichiometric in adults. The differences were attibuted to several points discussed below. First, extensive studies have shown that there are large variations in the acquisition of V_H repertoires between different strains of mice (Jeong *et al.*, 1988; Yancopoulos *et al.*, 1988; Freitas *et al.*, 1990), that is, strain dependent expression of V_H and regulation of gene usage by a locus external to the V_H region (Wu, G. E. & Paige, 1988). Furthermore, two main mechanisms have been suggested for preferential association of light and heavy chains. The first could imply conformation and thermodynamic

constraints on H and L chain assembly, and the second could act after expression of assembled (H/L) antibody on the B-cell surface by selection of the same V_H/V_L pairs.

Investigation of V region gene usage within individual BALB/k donors has revealed that immunodominance of a single specificity was generated by a single, or small group of progenitor cells. This is evident in SFA 2B, where clones 1.1 and 7.2 utilise the same V region genes, and CDR3H regions are almost identical ($^{25}/_{27}$ nucleotide bases), therefore N region addition at the V-D-J junction. The six Mab from donor 3B originate from three progenitor cells. Clones 2.1 and 3.1 show 94.74% homology in V_L and 82.46% homology in V_H, and share identical J and D genes, but differ in CDR3H region due to somatic hypermutation during maturation, and also in isotype (C_H). Since class switching occurs after V-D-J rearrangement, it is clear that these two clones derive from the same progenitor. This is also true for 7.1 and 12.1 (V_L=95.44%, V_H=88.9% homologous) and 10.1 and 14.1 (V_L=97.19%, V_H=59.45% homologous), both pairs of which show minor CDR3H sequence differences (identical in 7.1 and 12.1, and $^{6}/_{36}$ different bases in 10.1 and 14.1) as well as different isotypes.

The two identical 198-specific Mab from donor 7B, 1.2 and 7.1, are in my opinion products of the same fusion since they show 100% homology in V_L and 96.15% in V_H, as well as identical CDR3H regions.

This has also been observed in CBA/Ca Mab 3B6.1 and 8.2 (92.5%V_H, 97.3%V_L), 4B4.3 and 40.7 (58.34%V_H, 97.67%V_L), 4B3.1 and 4.3 (82.56%V_H, 93.6%V_L but different J_κ and CDR3H region in 3.1) and all three Mab from donor LD6. Surprisingly, this phenemenon is not found in the eight BALB/k SFA 7B 158-specific Mab which are all generated from independent progenitor cells.

A common observation in the response to influenza is the appearance of identical somatic mutations in independent hybridoma lines derived from a single immunised mouse, often attributed to divergent variants of a single stimulated clone (McKean *et al.*, 1984; Sablitzky *et al.*, 1985; Cleary *et al.*, 1986). However identical mutations are also observed in antibodies derived from different animals, or in hybridoma lines considered to be of independent clonal origin based on differences in germline fragments utilised to express the antibody. Mutation hotspots occur mainly in CDRs, but they can also occur in framework residues, as long as the residue is not structurally critical (Berek & Milstein, 1987).

This study thus provides evidence to support the hypothesis that a single specificity was generated by selective expansion of an individual, or a few high affinity antibody forming progenitors, but also that other independent cells can share this specificity without common V region gene usage, i.e. a wide range of V genes can encode an antibody structure able to recognise a single determinant.

Dominant V_H gene usage has been found in the BALB/k anti-158 and anti-198 responses, and a restricted V_L response overall, (and V_L and V_H restriction in the individual) in the 158-specific CBA/Ca response to influenza, following natural infection. The response of BALB/k MHC congenic mice appears to be a composite of both BALB/c elements and CBA/Ca elements in terms of antibody gene usage, as deduced from BALB/k anti-198 antibody gene usage, and from the common exclusive V_L gene usage by BALB/k anti-158 antibodies and CBA/Ca anti-158 antibodies.

DISCUSSION

In general, the observed immune response to foreign proteins is diverse, mapping to regions effectively spanning all potential external immunogenic sites of the protein, whereas the immune response to haptens is extremely restricted, to the single antigenic determinant available. In this, and a previous investigation in this laboratory, we have found that the immune response to influenza HA, following natural infection elicits an extremely narrow window of antibody specificities, and immunodominance is observed in two different strains of mice, CBA/Ca (Smith *et al.*, 1991), and BALB/k in this study.

To reiterate, a hapten is described as "a substance, usually a small molecule of under 1000 molecular weight, that can carry only one antigenic determinant, that can combine with antibody, but cannot initiate an immune response unless it is bound to a carrier before introduction into the body" (Herbert *et al.*, 1985), as demonstrated by Mitchison (1971) in the hapten-carrier system. The hapten can elicit a specific immune response when conjugated to a larger "immuno-visible" carrier molecule, such as DNP or NIP conjugated to BSA, HSA, ovalbumin, KLH etc. which enables the immune system to elicit a specific secondary response to the hapten alone on subsequent challenge with the hapten, on an irrelevant carrier, plus carrier specific T-cell help. The hapten-carrier complex is necessary for immune recognition, yet the immune response is still capable of generating an extremely specific response to a very minor portion of the whole immunogen.

Could regions of the HA protein that are neutralising epitopes then be behaving as haptenic determinants? Could this be the mechanism whereby immunodominance of certain regions arises? But then, given the potentially immunogenic nature of the entire surface protein, why are these specific sites repeatedly selected over any other sites in individual donors? How is it possible for the immune system to "ignore" certain regions of the protein and focus almost exclusively on a single residue, as I have demonstrated in this study. Could immunodominance merely be artefactual and a consequence of the method by which we are selecting HA specific antibodies?

Whilst other regions of HA may be recognised, these may not neutralise infective virus. We are indeed specifically selecting for neutralising antibodies by the method used

to select HA-specific antibodies (i.e. by haemagglutination inhibition assays (HI), thus neutralisation of viral agglutination of erythrocytes, hence infectivity). I have demonstrated strain specific immunodominance in the BALB/k antibody response to influenza HA, but all previous studies on the antibody response to influenza HA have shown extensive diversity despite detection of HA-specific antibodies by HI, as I have, so there must be other factors determining such an outcome. There are several ways in which our methodology differs from previous studies on the antibody response to HA.

All previous work has involved the immunisation of BALB/c mice, often with an H1 subtype virus (PR8), which generated a diverse response (Cancro *et al.*, 1978; Gerhard *et al.*, 1981; Caton *et al.*, 1982; Underwood, 1982; Staudt & Gerhard, 1983; Brown, L. E. *et al.*, 1990), or at best, recognition of one or two sites, e.g. site B (Underwood, 1984). Investigation of the response (in this laboratory) following natural infection revealed immunodominance of a single recognition site and selection of the same variant virus. This was the case for mice of two other strains, CBA/Ca, and also for BALB/k mice in this study, suggesting that immunodominance was due to either different haplotypes, as CBA/Ca and BALB/k are both H-2^k and BALB/c are H-2^d, but BALB/k and BALB/c are theoretically identical in every other respect, or immunodominance was due to the route of inoculation since it is observed in the response to natural infection, but not to immunisation. The latter was confirmed by the observation of immunodominance in the BALB/c response following natural infection (C.Smith:personal communication).

Most of these studies had also defined specificity of anti-HA antibodies by reactivity patterns to natural variant viruses and by HI or radioactive immunosorbent assays (RIA) which allowed serological assignment of recognition sites rather than specific residues at the molecular level, but still exhibiting extensive diversity, e.g. 12 different specificities in 15 antibodies (Brown, L. E. *et al.*, 1990), 104 distinct reactivity patterns (RP) for 125 Mab (Staudt & Gerhard, 1983) or 10 different reactivity groups specific for residues in sites A, B or D (Underwood, 1982). Caton *et al* (1982) and Underwood (1984) then narrowed this down to site B by mapping fine specificity of antibodies by selection of escape mutants and sequencing of mutant viral RNA encoding HA and comparing it with that of the wild type virus, but these results defined several residues within a site. Our studies, employing the same procedure for definition of antibody recognition sites, but differing in the route of

inoculation (natural infection), and strain used have mapped fine specificty down to a single immunodominant residue.

There have been few studies directly comparing the antibody response following natural infection or immunisation, nonetheless, differences have been noted. One of the earliest studies (Sabin, 1959) reports differences in the level of antibody response, persistence of antibody, resistance to infection of the alimentary canal, the site of infection, and duration of immunity, to poliomyelitis elicited by natural infection, immunisation with killed virus or immunisation with live attenuated virus. Most other studies document differences in isotype selection between immunisation and infection. Examples of which include investigations by Coutelier *et al* (1988), who showed that CBA/Rij mice infected with LCMV, LDV, adenovirus, MHV, sendai or reovirus 3 all elicit a predominantly γ2a response, but in excess of 90% of total serum γ2a antibodies produced during the course of infection were not virus specific, suggesting viruses could influence the switch of irrelevant Ig to γ2a during an infection. Furthermore, when mice immunised with soluble protein such as tetanus toxoid, which should elicit a γ1 response (Rosenberg & Chiller, 1979) were infected with virus at the same time, isotype distribution was modified with a preferential production of γ2a, and was also observed with concurrent KLH-transferrin immunisation and LDV infection. However, this occurred only with viruses which induced hypergammaglobulinaemia, such as LCMV and LDV. The isotype profiles were not modified by, for example, polyoma virus, which does not induce hypergammaglobulinaemia. These findings suggest that this isotype bias in antiviral antibody, leading to predominance of γ2a, was not due to particular properties of viral antigen, and regulatory mechanisms triggered by viruses, but due to other factors such as release of soluble factors by infected cells which may play a role in selection of γ2a antibody, T_h1 selection, IFN-γ production, etc. Antibodies bearing the γ2a isotype have different functional properties to other isotypes, such as activation of complement (Klaus *et al.*, 1979), which are necessary in defence against viruses. Preferential production of γ2a could therefore correspond to a more efficacious response of the infected organism to the virus.

Balkovic *et al* (1987) suggested 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 γ1. Infection of CBA/CaJ or C3H/HeN mice with virulent or non-virulent influenza virus elicited high γ2a, low γ1/γ2b and very low γ3 serum antibody levels typical of antiviral responses. Surprisingly, immunisation with inactivated virus also gave high γ2a, moderate γ2b and very low γ1/γ3 profiles, whereas immunisation with HA/NA protein gave a typical protein isotype profile of high γ1, low γ2a and very low γ2b/γ3 serum antibody.

Hocart *et al* (1989a) found γ2a to be the predominant subclass produced to H3 in mice irrespective of route of inoculation, but the magnitude of response varied with the route and schedule of inoculation and virus preparation and dose. The *intravenous* and *intramuscular* routes produced the largest response after two doses. The response to *intranasally* administered HA is much higher than i.p., suggesting the i.n. route can give better protection from influenza than the i.p. route due to amplification of antigen during virus growth or induction of secretory IgA (Coutelier *et al.*, 1987, 1988; Hocart *et al.*, 1989b). *Intraperitoneal* 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. In BALB/c mice this order was γ1>γ2a>γ2b. An explaination of why these profiles are found could be resistance to fragmentation by pepsin and proteases present on mucosal surfaces (Parham, 1983) since the order of resistance is reflected in serum titre of antibody, and also that γ2a is the most effective antibody against H3N2 due to its biochemical propreties (Tao, 1987).

It has been suggested that the subclass response may be strain specific (Perlmutter *et al.*, 1978; Moreno & Esdaile, 1983), in that the thymus dependent response to dextran T10-ovalbumin conjugate, which is a carbohydrate antigen and should elicit predominantly γ3 antibodies, in BALB/c gives a γ1:γ3 ratio of 9:1, but in CBA/Ca this is 1:1.

The γ2a isotype predominates regardless of route of inoculation of influenza (i.n. versus i.m.) in serum and lungs of CBA/CaH mice. However, the response to

non-replicating antigen, e.g. H3 consists of all four γ isotypes and varies between strains (Hocart *et al.*, 1989b). BALB/c has higher titres of γ1/γ2a than C57BL/6 which has 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 2b are more effective than γ1 and γ3, and in CBA/Ca mice, γ2a has twice the neutralising activity of γ2b.

The hypothesis that virus can increase γ2a production of the concomitant antibody response by acting on T_H lymphocytes fits well with the observation by Coutelier (1991) that polyclonal production of γ2a after viral infection is a T-cell dependent phenomenon. The existence of T_H preferentially inducing an *in vivo* γ2a response has been reported and it is therefore conceivable that viruses could selectively stimulate this type of cell (T_h1, producing IFN-γ, which stimulates class switching to γ2a). Immunisation with protein elicits a non-protective γ3 response not seen in immunisation with whole influenza virus which is predominantly γ1, therefore the subsets of T lymphocytes stimulated by influenza virus infection are different to those stimulated by non-replicating agents (Leung & Ada, 1982; Mills *et al.*, 1986a; Morrison *et al.*, 1986).

It has been suggested that isotype induced by T-cell dependent and independent antigen in different strains may be controlled by Ig-allotype linked (non-MHC) genes (Slack, 1987), in that a higher γ1 and low γ2a response to TNP has been linked to the BALB/c Ig allotype a^1, and the converse linked to the a^2 allotype in, for example, C57BL/6.

The BALB/k response to HA following natural infection, studied here, was predominantly of the γ2a isotype, although in one of the five donors γ3 was present in equal proportion to γ2a and α ($^3/_{12}$ Mab each). IFN-γ secreted by T_h1 cells, associated with anti-viral activity, usually stimulates class switching to γ2a, and as γ2a levels increase, so γ1,γ2b, γ3 and ε levels decline (Snapper *et al.*, 1988), but there have been examples of IFN-γ stimulation of γ3 (Snapper *et al.*, 1992) by T-cell dependent type 2 (TI2) antigens (exemplified by haptenated polysaccharides typically abundant in bacterial cell walls) and this may also be the case in this instance.

While magnitude of responses and isotype profiles have been studied with respect to route of inoculation, to my knowledge none of the previous studies have considered possible differences in antibody fine specificity to the same antigen administered by

different routes. Here, I have shown a very focused antibody response from BALB/k mice to influenza HA following natural infection, (also shown for CBA/Ca mice (Smith *et al.*, 1991) in this laboratory), contrary to the great diversity in the response shown in all previous studies, but elicited by immunisation. I therefore conclude that the route of inoculation is extremely important in definition of antibody repertoire, particularly in relation to infectious agents such as influenza, since this is a neutralising response which will be elicited during the course of a natural infection in the population. Where a response was assumed to be diverse, or focused, one should consider carefully the route of inoculation, nature of the antigen and selection of positive antibodies before generalities can be made about responses to a particular antigen.

The original aim of this investigation was to determine whether MHC haplotype exerted an effect on antibody repertoire selection. The antibody repertoire of CBA/Ca mice of the H-2^k haplotype, and BALB/c mice of H-2^d had been defined previously in this laboratory and a difference in recognition sites observed, thus by defining the antibody repertoire of the MHC congenic strain BALB/k (H-2^k), this could be determined.

The outcome of this investigation was equivocal in some respects. Four out of five mice resembled BALB/c (with identical background genes) in their immunodominant response to HA1 198. The fifth donor surprisingly behaved like CBA/Ca (similar only at the MHC locus), responding predominantly to HA1 158. This suggested that MHC haplotype may have a role in directing selection of the antibody repertoire, presumably *via* T-cell recruitment, although this may be dependent on a number of other non-genetic factors such as environment, antigenic challenge and timing of Ig^+ B-cell release from the bone marrow progenitor-pool, processing and presentation of proteolytic peptide fragments and availability of specific T_H-cells, since only one in five mice appears to be "influenced" by MHC.

There are other examples of apparent MHC restriction in the literature, such as the immune response of mice to immunisation with Mycobacterium leprae (Adeleye *et al.*, 1991), where different antibody specificities were found for different strains of mice with the same haplotype. The structure and number of different epitopes recognised was attributed to non-MHC genes (e.g. B10 responded to fewer peptides than the BALB series). An MHC effect was also demonstrated in B10 congenic mice differing at the H-2 locus

(haplotypes a, h4, k, d, q, f, s) which responded to different molecular weight proteins of M.leprae. This was also demonstrated with mice of k/k haplotype, but differing in allotype (Teuscher *et al.*, 1985). Mice immunised with whole dead M.leprae of k/k haplotype and either a/a or b/b allotype were all intermediate responders, but mice of d/d haplotype and a/a or c/c were low responders, and in BALB congenics H-$2^{q, b \text{ or } k}$ mice were high to intermediate responders, and H-$2^{s \text{ or } d}$ low responders, therefore responder status is MHC-directed, irrespective of allotype in this case. Similar responder status studies have also been performed with Brugia malayi (Kwanlim & Maizels, 1990), Ascaris (Tomlinson *et al.*, 1989), Staphylococcal nuclease (Lozner *et al.*, 1974), and hepatitis B surface antigen (Milich & Chisari, 1982).

The antibody repertoire can be considered at different levels at a given point in the lifetime of an adult mouse. 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 (Tonegawa, 1983; Berek *et al.*, 1985). The emergent repertoire (precursor compartment/pool) that is, the cohort of V-domains expressed by preB and B-cells produced daily in the bone marrow is approximately $3\text{-}5 \times 10^7$ different clones (Opstelten & Osmond, 1983). The available repertoire (immunocompetent pool), i.e. the number of domains currently expressed, is limited to a maximum of 10^8 specificities (Freitas *et al.*, 1986a), and the actual repertoire (effector cell pool) which is the V-domains used by effector Ig-secreting cells are about 10^6 cells (Benner *et al.*, 1982; Coutinho *et al.*, 1984). 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 (Freitas *et al.*, 1986b). In steady state conditions, there is a continuous passage of cells from the precursor compartment to the immunocompetent cell pool to the effector cell pool. Size of different repertoires and the dynamic properties of lymphocytes suggest 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 it is therefore important to establish whether these are dictated by random choice, or are due to oriented selection of specificities.

It is generally believed that in adults, B-cells undergo random V gene rearrangement and emerge from bone marrow prior to exposure to antigen, therefore the population of B-cells available to respond could vary at different times and in different animals. Changing responsiveness could result from change in frequency of T_H or B-cell precursors during the critical time of virus exposure, or fluctuations in immunoregulatory influences e.g. tolerance. Therefore, it is unlikely that five individual mice would all be at the same time stage in B-cell development, hence recruitment of the same antibody specificity for a single amino acid.

Could there be a stochastic element to immunodominance, or can it be explained by preferential antibody gene usage? The second part of my thesis was concerned with sequencing and identifying the antibody genes of Mab with the same specificity from different individual donors and this revealed several interesting points.

While antibody responses to polysaccharides and haptens are often oligoclonal, antibody responses to proteins are generally more heterogeneous, and this is generally reflected in antibody gene usage by B-cells producing Ig against these epitopes.

There have been a multitude of studies concerning antibody gene usage for haptens. Early studies on 2-phenyl-5-oxazalone (phOx) (Berek & Milstein, 1987) demonstrated extreme restriction in V gene usage. Primary response Mab from immunised BALB/c mice against this hapten used V_HOx1 gene and V_κOx1, with strong D/J restrictions. By day 14 mutations had accumulated as affinity increased, and following an i.v. boost secondary response Mab at 6-8 weeks had shifted in V_H gene family usage to a gene with 60% homology to V_HOx1, but retained the same D-J restriction. Nevertheless, light chain genes V_κOx1 and V_κ45.1, and heavy chain genes V_HOx1 and V_HM21 (MOPC-21-like gene) were repeatedly used by phOx specific secondary and tertiary Mab (Kaartinen *et al.*, 1989; Milstein *et al.*, 1992; Solin *et al.*, 1992). Four allelic forms of V_HOx1 (Q52 family) with assumed common ancestry have been found in nine strains of mice. PhOx specific Mab generated by immunisation of mice of a, e or g allotype have identical V_H segment sequences and idiotype to previously sequenced BALB/c anti-phOx Mab (e.g. Id 495, 350 or 260). Mice of allotype c and f have one difference each, and allotype j have four nucleotide differences. All V_κ sequences were identical (Kaartinen *et al.*, 1989). It is suggested that characteristics of antibodies in a specific primary response are inherited to

a considerable extent since the same V_H has a role to play in the anti-phOx responses in nine strains, thus termed a "public antibody". This public antibody also shares additional characteristics per strain i.e. V_κOx1-J_κ5 pairing (Milstein *et al.*, 1992) and an unusually short D-N segment (2 residues). As a result, Mab from different strains sometimes have identical amino acid sequence in the entire V_H and V_κ domains. However, the use of an alternative V_H gene from S107 family by C57BL/6 was attributed to the absence of a V_HOx1 gene in the genome, i.e. "a hole in the repertoire" (Solin *et al.*, 1992).

NP (4-hydroxy-3-nitrophenyl), another commonly studied hapten also exhibits restriction of antibody gene usage to V_H186.1, DFL16.1 and V_λ1, coding the NP^b idiotype in C57BL/6 mice (Reth *et al.*, 1978; Cumano & Rajewsky, 1986). Other examples of anti-haptenic antibodies with restricted $V_{H/L}$ gene usage include those against phthalate ($CRI_{Xmp\text{-}1}$ in C57BL/6) (Lou *et al.*, 1992), and p-azophenyl arsonate (CRI Id in A/J mice which is inherited in Mendelian fashion with the Igh-C locus) (Milner & Capra, 1982; Wysocki *et al.*, 1987).

The response to phosphoryl choline however, is more diverse in V_H gene usage. For example, eighteen different genes from three families, V_HS107, 7183 and 36-60, were used by Mab following immunisation of BALB/c mice with KLH-PC (Crews *et al.*, 1981), leading to the description of T15, M167, M511 and M603 families of antibodies. Malipiero however in 1987, described restricted V_H gene usage in the PC-specific response of V_H1 gene from the S107 family rearranged to J_H1 and paired with one of three V_κ genes from three different families:- V_κT15 from V_κ22 family, V_κ167 from the V_κ24 family and V_κM3 from the V_κ8 family, all rearranged to J_κ5.

Carbohydrate antigens also exhibit restricted V gene usage, e.g. galactan specific antibodies use a single V_κ gene with different J_κ and very diverse V_H domains from the pairing of one, or a few V_H genes to different Ds and at least 3 J_H (Rudikoff *et al.*, 1983), and also 3-fucosyllactosamine (V_H441 of the X24 family and V_κ24B) (Kimura *et al.*, 1989; Snyder *et al.*, 1994).

Protein antigens, conversely are thought to generate a more heterogeneous response, both in the number of different epitopes and diversity of antibody gene usage. Because the total immune response to even a relatively small protein can involve multiple specificities, definition of any potential relationship between protein recognition and antibody structure,

and the genetic basis thereof must be considered in terms of antibodies recognising each individual epitope. Previous studies that have shown V_H/V_L restriction associated with binding of haptens provide a working hypothesis that the immune response to a specific protein epitope will be similarly restricted to products of the same V_H and V_L genes families, whereas antibodies directed against different determinants on the same protein might use different V_H/V_L combinations. This has been demonstrated for HEL specific antibodies in BALB/c mice where two antibodies of very similar fine specificities for HEL shared V_H and V_L genes, but those with specificity for a different region of HEL expressed different genes (Smith-Gill *et al.*, 1984) generating a very diverse overall response to HEL of 18 fine specificities from 44 Mab from B10A or A/J mice and use of 7 different V_κ families and three different Kabat V_H subgroups. So, the overall picture of diverse V gene usage for a protein arose from different V gene usage by antibodies to different epitopes of that protein.

There are exceptions to the "restricted for haptens, diverse for proteins" V gene usage rule, as analysed by Czerwinski *et al* (1994). Restricted V gene usage, either of V_H or V_L or both has been reported for carbohydrate antigens including blood group antigens A and B, and 3-fucosyllactosamine, and also for the protein antigens lysozyme, human tumour associated antigen and influenza HA (Yancopoulos *et al.*, 1988). Reciprocally, diverse V gene usage has been reported in the antibody response to the haptens digoxin alprenol and DNP, the oligosaccharide antigens isomaltosyl oligosaccharide $\alpha(1\rightarrow6)$ dextrans and cancer associated ganglioside antigen, as well as many proteins including TMV protein, autologous insulin, and interestingly influenza HA (Meek *et al.*, 1989), as discussed in chapters 2 and 3.

Restricted V_H has been reported in carbohydrate induced Fc binding autoantibodies, monoclonal rheumatoid factor (MRF), only for Mab of the $\gamma 1$ isotype (V_HJ558), whereas V_H gene usage is diverse for MRF of other isotypes (Stanley *et al.*, 1992; Randen *et al.*, 1993). Differences have also been found in V gene usage of antibodies against human blood group antigens M and N which differ only at two closely spaced amino acids at positions 1 and 5. Anti-M antibodies use a variety of V_H and V_L families commonly used by antibodies against carbohydrate antigens, but anti-N antibodies had diverse V_L usage and very restricted V_H usage (Q52 family) (Czerwinski *et al.*, 1994).

Restriction of V gene usage also varies with the organism and the particular antigen used. For example, anti-dsDNA antibodies in human SLE of $3I^+$ idiotype use $V_\kappa 1$ with heterogeneous V_H usage (4 families) (Manheimer-Lory *et al.*, 1991), but anti-DNA antibodies in BALB/c mice and schleroderma target autoantigen in tight skin (TSK) mice (Kasturi *et al.*, 1993) all use V_HJ558 and diverse V_κ (Krishnan & Marion, 1993). Collagen type II induced arthritis antibodies (Mo *et al.*, 1993) and cardiolipin (CL) anti-DNA antibodies (Kita *et al.*, 1993) in mice however, have restricted V_κ (21 and 23 respectively) and restricted V_H (J558) expression.

Antibody gene usage in the response to infectious agents is also as variable as that against proteins, carbohydrates, autoantibodies and haptens. For example, the response to Haemophilus influenzae type B capsular polysaccharide (Adderson *et al.*, 1991, 1992) is dominated by a limited number of V_κ and V_λ genes and two or three V_H genes from a single family. Conversley, antibodies against hepatitis B core antigen use three different V_H and V_L families (Skrivelis *et al.*, 1993), and BALB/c anti-Abelson murine leukaemia virus antibodies (Atkinson *et al.*, 1993) and human rabies specific antibodies use three different V_H families and diverse V_L families, both κ and λ (Ikematsu *et al.*, 1993). The literature therefore gives a general impression of restriction of V gene usage in anti-haptenic responses and diversity in anti-protein responses.

Since the majority of infectious agent studies were performed by immunisation of animals either with proteins of the organism or killed samples, the responses and antibody gene usage are expected to fall into the "diverse for proteins" category, as indeed most do. The influenza story however, is a complex one. Many conflicting reports are available. What these have in common is introduction of antigen by immunisation. Definition of antibody gene usage varies between selection of antibodies specific for a particular site, of differing fine specificities (Clarke *et al.*, 1985; Caton *et al.*, 1986, 1991; Kavaler *et al.*, 1990), which fit in with other anti-protein V gene usage studies, exhibiting diverse gene usage, or selection of antibodies expressing a particular (usually V gene encoded) idiotype, which have either restricted V_L (Clarke *et al.*, 1990b) or V_H (Clarke *et al.*, 1990a) or both (Moran *et al.*, 1987; Sigal *et al.*, 1987; Brown, A. R. & Sealy, 1991). Influenza HA specific Mab of the same fine specificity patterns have also been found to utilise the same V_H and V_L families (Caton, 1990; Kavaler *et al.*, 1990, 1991), which conforms with the general hapten model for antibody gene usage.

In this study I chose to determine antibody V gene usage for Mab with identical recognition sites, i.e. selection of identical mutations in HA1 at the nucleotide level to determine whether a structural basis for recognition could be established. My results are neither compatible with the "general diversity for proteins" or "general restriction for haptens" models for antibody gene usage. Mab from one mouse strain, CBA/Ca, specific for a single residue change HA1 158, used restricted V_L gene V_κ1A K5.1, with donor restricted V_H families (all of which share greater than 70% homology). However, Mab from a different strain, BALB/k, (sharing only MHC haplotype with CBA/Ca) specific for the same residue had diverse V_L and very restricted V_H family usage. This difference was attributed to the strain, i.e. non-MHC background genes, since virus, route of inoculation and antibody specificity were identical. In the same strain, BALB/k, Mab of a different recognition specificity, HA1 198, (immunodominant in $^4/_5$ donors), exhibited both diverse V_L and relatively diverse V_H usage, although $^{12}/_{18}$ Mab utilised V_H VGam3.8, representing almost the entire anti-198 specific response from three of the donors (with only two exceptions from donor SFA 3B $^2/_6$ Mab).

In mice, H chain V segments are clustered in subgroups or families of genes sharing greater than 80% nucleotide sequence homology (Brodeur & Riblet, 1984). Twelve V_H gene families have been described with chromosomal organisation within the IgH locus which is conserved across species (Brodeur *et al.*, 1988). The actual number of different active genes per V_H family in each mouse has not been established (Dildrop, 1984; Livant *et al.*, 1986; Caton *et al.*, 1991) but there is evidence for variation in V_H gene family usage between strains (Wu, G. E. & Paige, 1986; Jeong *et al.*, 1988). V_H family expression is genetically controlled during early ontogeny (Yancopoulos *et al.*, 1984, 1988; Perlmutter *et al.*, 1985) and lymphopoiesis (Freitas *et al.*, 1990), and varies with cellular environment (Freitas *et al.*, 1989a), as well as being influenced by T-B interactions (Freitas *et al.*, 1989b). In normal non-immunised mice, peripheral B-cell repertoires are continuously renewed and selected (Freitas *et al.*, 1986a) and 2-5 x 10^2 new B-cells are formed daily (Opstelten & Osmond, 1983; Rocha *et al.*, 1990). One percent, or less, of all peripheral B-cells are Ig secreting, hence stable expression of V_H families in the context of high turnover of B-cells must result from continuous selection of peripheral B-cells, leading to strain specific, and indeed, individual-specific V_H family usage.

An unusual finding in this study is the use of the *same* V_H/V_L pairings to encode antibodies with two *different* recognition specificities in two different donors. Early observations (Moran *et al.*, 1984) demonstrated cross reactive idiotypes on Mab to different epitopes on PR8 HA (H1) and shared idiotypic determinants among antibodies to H1 and H3 HAs. This suggested that despite diversity in the clones responding to HA, in some instances, regulatory mechanisms might operate to restrict clonal population responding to particular antigenic determinants. Despite Mab having reciprocal binding properties and possessing a paratope specific for particular structures on an identical or closely related topographic region of HA (Reale *et al.*, 1986), these antibodies had different idiotypes. Three Mab of distinguishable paratopes shared cross reactive idiotypes, however, structures of V_H and V_L in both were quite dissimilar, so it is possible that a wide diversity of antibody structures exist which are directed against subtly different structures in biologically important antigens.

There are many examples of identical V_H or V_L usage specifying recognition of different antigenic determinants, as well as different V genes specifying recognition of one epitope. Kabat and Wu (1991), following an extensive database search and comparison, found that anti-β(1→6) D glycan and anti-β(2,6) fructosan antibodies both used identical V_H but different V_L genes. Conversley, antibodies against IgG1, arsonate, blood group A antigen, E.coli, DNA, phOx, colorectal carcinoma antigen and α(1→6) dextran all have identical V_L with different V_H. They suggest antibody specificity relies more on the V_H than the V_L chain since more antibody of different specificities use the same V_L than V_H and the CDR3H region is thought to contribute to antibody specificity and diversity (Chothia & Lesk, 1987) due to sequence variation from N and P addition at the V-D-J junctions. This was observed in this investigation of the response of BALB/k to HA1 198, all using very diverse V_L, but donor dependent V_H families, and strain dependent VGam3.8 family usage. CBA/Ca however had very restricted V_L gene usage.

There have also been reports to the contrary, of antibodies of completely different specificities sharing identical V_H and V_L genes, e.g. PC and DNA specific antibodies, and autoantibody, NP, phOx, PC, dextran, colorectal carcinoma antigen, dimethylamino naphthalene sulphonate, and poly(Glu60Ala30Tyr10), i.e. specific antibodies which have identical V_H1-94 and V_L1-95 regions, but extensive differences in CDR3H and FR4H

regions. This too has been demonstrated in this study in two Mab from donor SFA 7B specific for HA1 158 and 198 using the same V gene combinations (and DJ), but differing in their CDR3H regions.

Distinct roles for CDR3H have been postulated based on the following three lines of evidence. Conformation studies of all six CDR regions suggest all except CDR3 may have a relatively small repertoire of main chain configurations (canonical forms, Chothia *et al.*, 1989). Furthermore, three different antibodies that bind human tumour associated antigen GA733 were found to have five different CDRs, but identical CDR3H regions (Caton *et al.*, 1990) and finally, the finding that affinity of binding of anti-arsonate antibodies could be increased 12.9-fold by a single amino acid substitution in CDR3H, whereas substitutions in e.g. CDR2H resulted in a very minor increase and no real effect was observed by changes in CDR2L and CDR3L (Sharon, 1990a, b).

X-ray crystal structure analysis of antibody-antigen complexes have shown that one or both CDR3 loops are always in contact with the antigen (Rock *et al.*, 1994), e.g. the antibody residues in contact with lysozyme are L30 and 32 (CDR1L), L49 (FR2), L50 (CDR2), L91, 92 and 93 (CDR3), and H30 (FR1), H31, 32 (CDR1), H52,53,54 (CDR2) and H96, 97, 98 and 99 (CDR3) (Amit *et al.*, 1985, 1986). The length of CDR3 also has a profound effect on the shape of the antibody, such that a single amino acid difference can produce significant changes in the overall structure (Chothia & Lesk, 1987; Chothia *et al.*, 1989), (immunoglobulin CDR length is not altered by affinity maturation *via* somatic hypermutation as is the case in T-cell receptor peptide driven selection). This is corroborated by Snyder's work on the carbohydrate antigens 3-fucosyllactosamine (FL) and galactosylgloboside (Snyder *et al.*, 1994). Two anti-FL antibodies of different affinities both use V_H441 and V_κ24B genes but have different length CDR3 regions. On exchange of the CDR3H region of the low affinity antibody with that of the high affinity antibody and pairing of the chimera to L-chains of the high affinity antibody, an increase in affinity was observed, however this was not the case when the chimeric H-chain was paired with the low affinity L-chain, which differs at only three residues from the high affinity L-chain, therefore CDR3H alone cannot account for specificity or affinity.

Other studies to support the CDR3H role in antibody specificity include Wu *et al* (1993) who state that in general, for antibodies with the same specificity e.g. NP, CDR3H

lengths can vary (between 5-15 amino acids) but antibodies may be directed to different epitopes of the same antigen. For example, three antibodies against lysozyme of CDR3H lengths 8, 7 and 5 amino acids are specific for different epitopes of HEL (Amit *et al.*, 1986; Padlan *et al.*, 1989), but antibodies against bromelain-treated murine erythrocytes and E.coli both had CDR3H of nine residues but were found to share the same epitope, phosphorylcholine. CDR3H has been located to the centre of the antibody combining site by stereophotos of α-carbon 3D structures (Kabat *et al.*, 1991), therefore length may play an important role in fitting the conformation of the antigen. Antibodies of different specificities may however share small peptide sequences within CDR3H e.g. YYGS or SSGY at different positions in CDR3 e.g. 95-98 in anti-DNA antibodies and 97-100 in anti-NP and still not share common epitopes.

In this study, the majority of antibodies from CBA/Ca specific for HA1 158 had CDR3H length of 6 amino acids and of almost identical sequence, but one of the antibodies JCB 2B 29.1 has a CDR3H length of 11 amino acids and no sequence homology to the other 10 CBA/Ca antibodies of the same specificities. Eight out of eleven antibodies have CDR3L lengths of 9 amino acids (the remaining three are 10 amino acids long) all of very similar sequence. CDR3H sequences of BALB/k anti-158 specific antibodies are very variable, ranging from 6-11 amino acids long and varying in sequence from each other and from the CBA/Ca anti-158 antibodies. CDR3L sequences are less variable than CDR3H, but show no homology with CBA/Ca CDR3L sequences, and the eighteen BALB/k anti-198 Mab CDR3H regions are highly heterogenous. No common canonical forms can be assigned for any CDR region for all antibodies studied in this investigation due to the extreme diversity of these regions, in particular CDR3H, and diverse antibody gene usage despite a very focused response to influenza HA1 158 or 198.

To summarise, in this study, I have defined antibody fine specificity in the response of BALB/k MHC congenic mice to HA following natural infection with influenza virus (X31). I have shown, by generating antibody neutralisation escape mutant viruses and sequencing their HA genes, immunodominance in this response for amino acid residue HA1 198, site B in $^4/_5$ individual BALB/k donors, a site predominantly recognised by BALB/c mice. I have also demonstrated possible MHC effects on the selection of the antibody repertoire due to focusing of antibodies to HA1 158 in the BALB/k donor 7B, a site

predominantly recognised by CBA/Ca mice (Smith *et al.*, 1991). I have established by sequencing 26 BALB/k Mab heavy and light chains and V_H and V_L genes, eighteen of 198 specificity and eight of 158 specificity, and 11 CBA/Ca heavy and light chains of 158 specificity and comparing the three sets of antibody gene usage data, that immunodominance cannot be explained structurally, by antibody V gene usage alone. I have also shown that antibodies of the same fine specificities can utilise different V_H and V_L genes, from different donors, but also that antibodies of *different specificity* from the same donor can utilise the *same* V_H and V_L genes. Donor specific V gene restriction is evident from this study. The CBA/Ca anti-158 antibody repertoire expresses restricted V_L genes and different, but related V_H gene families per individual donor. Antibodies of the BALB/k anti-198 repertoire express diverse V_L gene families with donor restricted V_H families (VGam3.8, not often seen in the response to influenza). The BALB/k anti-158 repertoire is generated by extremely restricted expression of genes from the V_H J558 family, not represented in the CBA/Ca repertoire, and present only in α and μ antibodies of the BALB/k anti-198 repertoire in the same donor, but coupled with diverse V_L family usage which is a composite of both BALB/k and CBA/Ca repertoires.

Individual repertoires are generated from one, or a few progenitor cells per donor in the BALB/k anti-198 response and the CBA/Ca anti-158 response, but of many varied origins in the anomalous BALB/k anti-158 response.

The anti-HA response does not appear to be "stoichiometric" since the largest family, J558 is poorly represented in both BALB/k anti-198 and CBA/Ca anti-158 repertoires, nor is the most D/J proximal V_H family, 7183 favoured, with a predominance of V_H VGam3.8 in the BALB/k repertoire, and S107 and related families in the CBA/Ca repertoire. A V_L family, $V_\kappa 1$, rarely observed in the anti-HA repertoire predominates in the CBA/Ca repertoire, and $V_\kappa 12/13$ (also rare) in the BALB/k 198 repertoire. The most frequently observed $V_\kappa 21$ family is rare in this study representing only $^4/_{37}$ Mab. MHC effects are not apparent on the selection of V_L or V_H gene family expression in the BALB/k SFA 7B (anti-158 repertoire) and do not appear to restrict expression of any V_H/V_L gene families when compared to the BALB/c anti-HA repertoires recorded in the literature. If MHC can in any way be instrumental, it must be acting at a different level to focus the antibody repertoire on site HA1 158 in donor SFA 7B. Interestingly, the T-cell repertoire

of BALB/k mice focuses on HA1 peptide 226-245 (my unpublished findings), an I-E^k restricted peptide, not on the BALB/c immunodominant HA1 peptide 177-199 or CBA/Ca immunodominant HA1 peptides 118-135 and 54-63.

In conclusion, natural infection and selection of HA neutralising antibodies contribute to the immunodominance observed herein and contrast with the findings obtained following immunisation with inactivated virus. However, neither immunodominance, nor specificity of these antibodies can be attributed to V_L or V_H usage alone.

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Appendix I

Nucleotide sequences of influenza virus haemagglutinin specific secondary antibody genes following natural infection of BALB/k and CBA/Ca mice.

Nucleotide sequences of BALB/k and CBA/Ca influenza HA specific immunoglobulin heavy chain V domains: Signal, FR1 and CDR1

```
  Frames      ----------------<-FR1-start-----------------------------------------------------------------------FR1-end->-------------CDR1------
 BALB/k 198
SFA 2B1.1     ------------------------------------------------------------------------------------------------------ATC-TCTCGA-GACTATGAATGCAGN
SFA 2B3.1     ---------------------------------------------------------------------CAGACAAGATCTCCTGCAGGCTTCTGGCTATCC-TTCACA-GACTATTCAATGCAC
SFA 2B7.2     ------------------------------------------------------------------------------------------CGTCGGCTTCCT-TCACAG-ACTATCCAGCACGGC
SFA 2B8.1     ------------------------------------------------------------------------------------------NGGAAARATGAC-TCGATA-CTATCCAGGCACGGG
SFA 3B2.1     -------------------NTTGGNNNGGGTTNTNNTGGCTTCTTCGMGTTGCAAGGGWTGGACWGGCATTGTCGSCTGCTAGGCTTCTTGGTNNSCTTC-TCAAAT-TATGGAATGGCWAGG
SFA 3B3.1     ------------------------------------------------------------------------------------AGGCTTCTGGTATCCTTC-TCAAAC-TATGGAATGAACCGG
SFA 3B7.1     ---------------------------------------NGCAGTTCGCAGTGCAGCCTAGAGGTCCCTGTACTCTCCTGTGCAGCCTGCTGGCATTAC-ACTTTC-AGTAGCTACATGTCT
SFA 3B10.1    AAGCTTGCCCATTGG-CCATGCCAAGNCATNGNTGGACCTTYCGGCCGCTCGAAGAAGCCTGAGGWTAGCTCAACTCTCCTCGWTCGWGCTTMCTGTACC-TTCACA-GACTATGGAATCAAA
SFA 3B12.1    ------------------------------------------------------------------------------------------------------NNC-ANANNAGGCATGTCT
SFA 3B14.1    -------------------TGCGCTCCWRRKCCTAGCTCAATCAKKTGTACTKCTGACTTCCATCTTGAGCTGAAGCAGTCAAGTCTCCTCAGCTTCTAT-TCCTTC-ACAACTATGAATAAC
SFA 6B1.1     ------------------------------------------------------------------------------------------------------------CATTCA
SFA 6B4.2     -------------------------------NTGTCAGACCGNACNCGACTCGGACCCGTAATCACTCTNAGGGACGTTCCGAAGACCTATGTGTTTGTG-ACTGCA-TGTGTGACTCTGAAA
SFA 6B5.1     ------------------------------------------------------------------NNTGGCCCTGCTGGCCTCTGCTTC-ACATTT-CACCTTCATATGCAC
SFA 7B1.2     ----------------------------------------------------TGCATAGGCTATGGACTCAGTGAAGCTGTCCTGCAAGGCAATGGCATCACA-TTTACT-GGCAATGAAATGCAC
SFA 7B7.1     ------------------------------------------------------------------------GTCCTGCAAGGCAATGGCAACACA-TTTACT-GGCTATGAAATGCAC
SFA 9B3.1     ----------------------------------------------------NNAGTTCAGAGCCGGAGAGACAGACTAGATCTCCTGCTAAGGCTTCGTATC-CATTCA-CAGCTATTCAAGNGT
SFA 9B6.1     GCTATCTATGCAATT-CCAAAGTTCAGCCAATCATGGTCTAGATCGTACCTGACTGAAGAAGCTGAGGAACAGTCAAGATCTCCTAGCAAGCAGTTATAC-CTTCAC-AGCTATTCAATGCAC
SFA 9B8.1     GATTCGGATGCAGCG-CAAAAGTTCAGCCGATCAGTGTGTGGATCGTACGTGAGTGAAGAAGCTGAGAGACAGTCAAGATCTCCTAGCAAGCACTGGATA-CTTCAC-AGCTATTCAGTGCAC
 BALB/k 158
SFA 7B2.1     ------------------------------------------------------------------------------------------------------------------------
SFA 7B3.1     ------GCCTCTCTT-CATACTCACCATCMMGTCACTGCAGYAYYTSGCCCAGCTGYCAGCCTRCCTCAGTYAAGCTGTCCTGCAGCTTTGGCTACACAT-TTACTG-ACCTATGAAATACAC
SFA 7B5.1     ------------------------------------------------------------------------------------------------------------TGCAAC
SFA 7B8.1     CTTCATACTCTGCNC-TCCARCTSAGCTGCACGCAGCAGGACTGCAGTGTTAGCTGGGCTTCAGTGCAGTTGTCCTGCAAGGCTTCTGCTACACCTTCAC-AAGCTA-AGATATAAACTGGTG
SFA 7B10.2    GGCASAASGTGCASA-TCTCGYGRTMCAGTCTGCAACAGCTGGWCCRTGCTGTCGGTTAACTTGGACTCAGTCAGATACCCTGCATGGCTACTGATACAC-ATTCAC-TGTCTCAACATGGAC
SFA 7B11.1    TATAYGCAAAGGTGC-ACAGGCATAAGCGACTCACCTAGGMCACAGCARCAAGATGGCAAGAACCGGCMGGAASCACGGCAAACCTACGCCGATATSTTM-MTTGAC-ASGSSAMAGRACGAA
SFA 7B15.1    ----------------------------NACTACTCTACTGATACGTCTCACAGTCTGCCTARACTGGTCACCAGAGTGAGATACCCTGCCAGCTTCTGG-ATCGCA-TTCACTGCATCTCCA
SFA 7B19.1    VAGACTGCGGACTCT-CTGAGAACTGCCAAGCAAACAGTCTGGACCATGACAGGAGAAGCCTGGGCAACTCAACACAAGATCCCTGCAWWGGCTTCTGGA-TCACAC-ATCACTGCATCAACA
 CBA/Ca 158
JCB 2B5.1.1   ---------------------------------------------NATTCTCTGAACTCTCCATGTGTTACCTTGGTAATTCATTCAGTAGCACTGGAT-GKCGGC-TTTCGCGCATCCACC
JCB 2B29.1    ------------------------------------------------------------------------NNCTACTGTGTAMTGGCWAKM-TCCAGT-GCSAGATTATTATTA
JCB 3B6.1     ------------------------------------------------------------------------------------------------------------------------
JCB 3B8.2     ------------------------------GAGCTTGTCAACTGTCWCTYWGTAAACWCWCTTGTGCWKCATTTCTGGATTCACTTTTANGTGAATGCC-ATGGGA-TGGATTCTGGGRTCC
JCB 4B2.1     ----------------------------NTCCRSTKAKCWTKGGAATGCAGCCAAGGTCATGGAACTCTCATGTGCAGCCTCGGAACTCCTTCAATACCT-ATGACA-TGTACTGGATCGCCA
JCB 4B3.1     ---------------------------------------------NNCTTTGAATGCCTGGAGTCCCTAAATCTCTCCTATCAACCTCTGATCACT-TCAGCA-GCTATATCATGTCAT
JCB 4B4.3     ---------------------------------------------NGATCAANTTACCTGGAATCCCTAAYYTMCTATGGACCCTTTGCAAACACT-TTAGTA-CCTATAACATGTCAT
JCB 4B40.7    ------------------------------------------------------------------------------------------------GGT-ATCCTTCGTGTCCTC
LD6 A7.6      ---------------------------------------------------------------------ATCTCTGAGWCTCTCYKKACAGTTTCTGGATCACCM-TTCACT-GATTACGACATGACT
LD6 A12.2     ---------------------------------------------------------------------NCCTCTGAGTCTGMCKTSCTKCATTCTGGATCTTCA-TTCTCT-GATTACGACATGATT
LD6 D9.1      ------------------------------------------------------------------------------------------CGATTCGCC-TACGCT-AGTTACGACATGACT
  Frames      ------signal----<-FR1-start-----------------------------------------------------------------FR1-end->--------CDR1-1---------
```

Nucleotide sequences of BALB/k and CBA/Ca influenza HA specific immunoglobulin heavy chain V domains: FR2 and CDR2

```
  Frames      <-FR2-start----------------------FR2-end->----------------------CDR2------------------------------
 BALB/k 198
SFA 2B1.1     TGGGTGAAGNAGGCTCCAGGACAGGGGTTAAAGTGGATGGGC-TGGATAAACACTGAGACTGGTGAGCCAACATATGCAGATGACTTC------AAGGGA
SFA 2B3.1     TGGCGAAGCAGGCTCCAGCAAGGGGTAAAGTGGATGGGCTGG----ATAAACACTGAGACTGGTGAGCCGATATATGCAGATGACTTC------AGGGAA
SFA 2B7.2     TCGAAGCAGGCTCCAGCAAAGGATCTAAAGTGGATGGCCTGG----ATAAACACTGAGACTGGTGAGCCAACATATACAGATGACTTC------AAGGGA
SFA 2B8.1     TGGCAACAGGCTCCAGGAAAGGGATCAAAATGGATGGTCTGG----ATAAATACTGAGACTGGTGAACCAACACATGCAGATGACTTC------AAGGGA
SFA 3B2.1     GCGGAAGCAGGTCGACCAGGTGYGGCTACCAGGCGGATGGAC-TGGATAAACACCAACACTGGAGAGCCAACATATGTAGATGACTTC------AAGGGA
SFA 3B3.1     GTGAAGCAGGTCCTCCAGGCAAAGGGTTTTAAGTGGATGGGC-TGGATTAACACCTACACTGGAGAGCCAACATATGCTGATGACTTC------ACGGGA
SFA 3B7.1     TGGGTTCGCCAGACTCCAGCCAAGAGGCTGGGGTTGGTCGCA----ACTACTAATAATAATGGTGGTAGCACCTATTATCCAGAC---ACTATGAAGGGT
SFA 3B10.1    TGGGTAGCTACGTCTCCTGGTAAGGGTTTAAAGTGGATGGGC-TGGATAAACACCTATACTGGAGAACCATCATATGCTGATGACTTC------AAGGGA
SFA 3B12.1    TGGGTTCGCCAGACTCCAGTCAAGAGGCTGGAGTTGGTCGCA----ACTATTAATAGTAATGGTGATAGCGCCTATTATCCAGAC---AGTGTGAAGGGC
SFA 3B14.1    TGGGTCGGAGTGACTCCAGGTAAGGGTTTAAAGTGGATGGGC-TGGATAAACACCTACACTGAAGAGCCAACATATGTTGATGACTTC------AGGGAC
SFA 6B1.1     TGCACTGGTGCATCTCCAGGAAAGGGTTTAAAGTGGATGGGC-TGGATAAACACTGAGACTGGTGAGCCAACATATGCAGATGACTTC------AAGGGT
SFA 6B4.2     TGCAATGGTGAAGAAGACNCATGTGCATGGCCTGGAATGGAT-TGGACGTATCCATCCACGGAAGTGGTGGTACTGCTACAATCAGAA---GTTCAAGGA
SFA 6B5.1     TGGGTAAAGCAGCCACACAGGACATGGCCTGGGATGGATTGG-AGCCATTTATCCAGGCAATTGGTGATCTTCCTACAATCAGAAGCT------AAAGGC
SFA 7B1.2     TGGGAGAAGCAGACACACAGAGCATGGCCTGGAATGGATTGG-AGATATTCATCCAGGAAGTGGTGGTACTGCCTACAATCAGAAGTT------CAAGGC
SFA 7B7.1     TGGGAGAAGCATACACACAGAGCATGGCATGGAATGGATTGG-AGATATTCATCCAGGAAGTGGTGGTACTGCCTACAATCAGAAGTT------CAAGGA
SFA 9B3.1     TGGATCGGCATGGCTCCAGGTAAGGGTTTAACGTGGCTGGGC-TGGACTVAAACTGAGACTGGTGAGCCAACATATGCAGATGACTTC------AAGGGA
SFA 9B6.1     TGGATGAAGTCAGCTCCAGGACAGGGTTTAAAGTGGATGGGC-TGGATAAACACTGAGACTGGTGAGCTAACATATGCAGATGACTTC------AAGGGA
SFA 9B8.1     TGGTTGAAGCAGGCTCCAGGAAAGGGTTTAAAGTGGATGGCC-TGGATAAACACTGAGACTAATGAGCCAACATATGCAGATGACTTC------AAGGGA
 BALB/k 158
SFA 7B2.1     ------------------------------------NNGVVT-WTTYTTTCTTGGAGGATGGTGATCCCTAGGTACACTCGAAGTTTA------GAAGGT
SFA 7B3.1     TGGGTGAAGCAGACACCTGTGCATGGCCTGGAATGGATTGGA-GCTATTCATCCAGGAAGTGGTGGTACTGCCTACAATCAGAAGTTC------AAGGAC
SFA 7B5.1     TGGGTCATGGTATTGAATGGTTCAGGGATACTCTCAAGTATT----GGTAGCAACAATGTAATG-----------GAGAGAGGT------TTGAGG---
SFA 7B8.1     AGGCAGAGGCCTGAACAGGGTCTTGAGTGGATTGGATGGATT----CTTCCTGGAGATGAGGTAGTTCTGTACAAGAAGAAGATG---GAGGTCAGG---
SFA 7B10.2    TGGTTGAAGCAGAGCCATGGAAAGAGCTTGAGTGGATTGGAG----ATATTAATCCTTACACTGGTGGTACTGTCTACAACCAGA---AGTTCAAGG---
SFA 7B11.1    CGGAAGMCGAACASATGGTCCAAAYTGATTGCAGATGGGAGA----GATTACATCCATACAATGGTGGTACTAACTTCAATGAGA---AGTTCAAGA---
SFA 7B15.1    TGGACTGGGTGCAGCAGAGCAGTGGAAAGAGCCATTGAGTGG----ATTGGAGATATTAATCCTAACACTGGTGGTCTACATCACATAGAGGTTTAT---
SFA 7B19.1    TGGASGTGGGTSAAGCAGAGCAGWGCCAAKGGTCTTGAGTGG----ATTGGAGATATTCAATCCTTACAATGGTGGTACTATCTACAACCAGAAGTTCAA
 CBA/Ca 158
JCB 2B5.1.1   AGGGGAAGTAGGCTGAGGCTGGAGTGGATTGCTGTAATCACA----------GTCAAATCTGATAATTATGGAACAAATTATGCAGAGTCTGTGAAAGGC
JCB 2B29.1    TGGAAGTGGATTCACGDCAGTTCCAGTAAACAACTGGAATGG----ATGGGCTACATAGCTACGATGGTAGCAATAACTACAACCCATCTCTCAAAAATC
JCB 3B6.1     ----------------------------------NNTGCTGAA----ATTAGAAACAAAGCTAATAATCATGCAACATATTATCCTGAGTCTGTGAAAGGG
JCB 3B8.2     CGGCATATCTCCAGTAGAGAGGGCCTTGAGTGGGTTGCTGAA----ATTAGAAACAAGGCTAATAATCATGCAACATATTTTACTGAGTCTGTGAAAGGG
JCB 4B2.1     TGGTCTCCAGGAAAGGGTTTTGGAATGGATGCTCATATAACA----AATAAAAG------TAATAATTATGCAACAAATTATGCCGATTCAGTGAAAGAC
JCB 4B3.1     TGGGTTCGAGACTCGGCGAAGAGGCTGGTATGGGTCGCAACC----ATTAGAAG------TGGTGGTGGTGATACCTACTATCCAGACAGTGTGAAGGGC
JCB 4B4.3     TGGATCCCAGACTCGGGCAAGAGGCTGGTTGGGTACGCAACC----ATTAGTAG------TGGTGGTGATAATTCCTACTATCCAGACAGTGTGAAGGGC
JCB 4B40.7    TGCTGTATGTCAGACTCTGTCAATAGCCTGGAGTGGGTGCAA----CCATTAGTAGTGGTGGTGATAACACCAACTATCCAGACA---GGTGTGAAGGGC
LD6 A7.6      AGGTACAGCAAGCCTCTAGGAAAGCTACTTGAGTGGTTGGCT-TTGATTAGAAACAAAACTAATGGTTACACGACAGAATACAGTGCATCTGTGAAGGGT
LD6 A12.2     GGGTACGTCAAGCCTCCAGGGAAGGCACTTGAGTGGTTGGCT-TTGATTAGAAACAAAACTAATGGTTACACAACAGAGTACAGTGCATCTGTGAAGGGT
LD6 D9.1      AGGATCAGCAAGCCTCCAGGAAAGCTACTTGAGTGGTTGGTA-TTGATTAGAAACAAAACTAATGGTTACACGACAGAATACAGTGCATCTGTGAAGGGT
  Frames      <-FR2-start----------------------FR2-end->----------------------CDR2------------------------------
```

Nucleotide sequences of BALB/k and CBA/Ca influenza HA specific immunoglobulin heavy chain V domains: FR3

Frames	<-FR3-start----------FR3-end->
BALB/k 198	
SFA 2B1.1	CGATTTGCCTTCTCTTTGGAAACATCTGCGAGCACTGCCTATTTGCAGATCAACAACCTCAAAAATGAGGACACGGCTACATATCTCTGTGCTAGA
SFA 2B3.1	CGGTTTGCCTTCTCTTTGGCAACCTCTGCCAACACTGCCTATTTACAGATCAACAACCTCAAAAAAGAGGACACGGCTACATATTTCTGTGGTAGG
SFA 2B7.2	CGGTTTGCCTTCTCTTTGGAGACCTCTGCCAGCACTGCCTATTTGCAGATCAACAACCTCAATACTGAGGACAGGGCTATATATTTCTGTGCTAGA
SFA 2B8.1	CGGTTTGCCTTCTCTTTGGACACCTCTGCCAGTTCTGCCTATTTACAGATCAACAACCTCAAAAATGAGGACACGGCTACATATTTCTGTGCTAGA
SFA 3B2.1	CGGTTTGCCTTCTCTTTGGGAACCTCTGCCAGCACTGCCTATTTGCAGATCAACAACCTCAAAAATGAGGACACGGCTACATATTTCTGTGCAAGA
SFA 3B3.1	CGATTTGCCTTCTCTTTGGAACCCTCTGCCAGCACTGCCTATTTGCAGATCAACAACCTCAAAAATGAGGACACGGCTACATTTTTCTGTGCAAGA
SFA 3B7.1	CGATTCACCATCTCCAGAGACAATGCCGAGAGCACCCTGTACCTGCAAATGAGCAGTCTGAAGTCTGAGGACACAGCCATCTATTACTGTGCACGA
SFA 3B10.1	CGGTTTGCCTTCTCTTTGGAAACCTCTGCCAGCACTGCCTATTTGCAGATCAACAACCTCAAAAATGAAGACATGGCTACATATTTCTGTGCAAGA
SFA 3B12.1	CGATTCACGGTCTCCAGAGAGAATGCCAAGAACACCGTGCACCTGCACATGAGCAGTCTGAAGTCTGAGGACACAGCCATGTATTACTGTGCAAGA
SFA 3B14.1	GGTTTGCCTTCTCTTTGGATACCTCTGCTTCCACTGCCTATTTGTCGATACACCAACCTCAAAAATGAGGACATGGCTACATATTTCTGTACAAGA
SFA 6B1.1	CGGTTTGCCTTCTCTTTGGAAACCTCTGCCAACACTGCCTATTTGCAGATCTACAACCTCAAAAATGAGGACACGGCTACATATTTCTGTGCTAAC
SFA 6B4.2	SAGGCBTMTCTGACTGCAGACAAATCCTCCAGCACAGCCTACATGGAGCTCAGCAGCCTGACATCTGAGGACTCTGCTGTCTATTACTGTACAGAG
SFA 6B5.1	AAGGCAACATTGACTGCAGACAAATCCTCCAGCACAGCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGAGGTATATTACTGTGCAAGA
SFA 7B1.2	AAGGCCACACTGACTGCAGATAAAATCTCCAGCACAGCCTACATGGAGCTCAGCAGCCTGACATCTGAGGACTCTGCTGTCTATTACTGTACAAGA
SFA 7B7.1	AAGGCCACACTGACTGCAGATAAAATCTCTAGCACAGCCTACATGGAGCTCAGCAGGATGACATCTGAGGACTCTGCTGTCTATTACTGTACCAGA
SFA 9B3.1	CGGTTTGCCTTCTCTTTGGAAACCTCTGCCAACACTGCCTATTTGCAGATCTACAACCTCAAAAATGAGGACACGGCTACATATTTCTGTGCTAAG
SFA 9B6.1	CGGTTTGCCCTCTCTTTGGAAACCTCTGCCAGCACTGCCTATTTGCAGATCAACAACCTCAAAAATGAGGACACGGCTACATATTTCTGTGCTGCC
SFA 9B8.1	CGGTTTGGCTTCTCTTTGGAAACCTCTGCCAGCACTGCCTATTTCCAGATCAACACCCTCAAAAATGAGGACACGGCTACATATTTCTGTGCTAGA
BALB/k 158	
SFA 7B2.1	ATGGCCACATTGACTGCAGATCCATCCTCCAGCACAGCCTACATGCAACTCAGCAGCTTGGCNTCTGAGGACTCTGCGGTCTATTACTGTGCATTG
SFA 7B3.1	AAGGCCACACTGACTGCAGACAGGCCTCCAGCACATGCCGACATGGAGCTCAGCAGCCTGACATCTGAGGACTCTGCTGTCTATTACTGTACAAGA
SFA 7B5.1	AAAGCCAAACTGGATGCAGGCACAGTGTCCAACACAGCCTACTTGGAGCTCAACAGTCTGACATCTGAGGACTCTGCTATCTACTACTGTGGGAAG
SFA 7B8.1	CCACACGGCGACATGCACTGGTCCTCCGACACGCACAGTCAGATGCGCTCACGAAGGCTGACATCTGAGGACTCTGCTGTCTATTTCTGTGCAAAA
SFA 7B10.2	AAAGGCCACATTGACTGTAGACAAGTCCTCCAGGACAGCCTACATGGAGTCCGCAGCCTGACATCTGAGGACACTGCAGTCTATTACTGTGCAGTG
SFA 7B11.1	GGAAGGCAACACAGACTGAGACAAATCCTCCAGCACAGCATACATGCAACTCAGCAGCCTGACATCAGAGGACTCTGCGGTCATTACTGTACAAGA
SFA 7B15.1	GGCAAGGCCACATTGCAGTACGAAGTCCTCCAGCTCAGCCTACATGGACGCCGCAGCATGACATCTGAGGACACTGCAGTCTATTACTGTGTAAGG
SFA 7B19.1	GGTAAAGGCATATTGACTGTAGACAGTCCTCCAGCCAGCCTACATGGACTCCGCAGCCTGACATCTGAGGACACTGCAGTCTATTATTGTGCAGTA
CBA/Ca 158	
JCB 2B5.1.1	AGATTCGCCATTTCAAGAGATGATTCAAAAAGCAGTGTCTACCTGCAGATGAACAGATTAAGAGAGGAAGACACTGCCACTTATTATTGTAGTATT
JCB 2B29.1	GTAATCTCCATCACTCGTGACACATCTGAGAACCAGTTTTTCCTGAAGTTGAATTCTGTGACTACTGAGGACACAGCCACATATTACTGTGCAAGA
JCB 3B6.1	AGGTTCACCATCTTTAGAGATGATTCCAAAAGTACAATGTACCTTCAAATGAACAGTTTAAGAGCTGAAGACACTGGCATTTATTACTGTCCGTAT
JCB 3B8.2	AGGTTCACCATCTCAAGAGATGATTCCAAAAGTAGACTGTACCTGCAAATGAACAGCTTAAGAGTTGAAGACACTGGCATTTATTACTGTACCTAT
JCB 4B2.1	AGATTCACCATCTCCAGAGATGATTCACAAAGCATGCTCTATCTGCAAATGAACAACCTGAAAACTGAGGACACAGCCATGTATTACTGTGTTTGT
JCB 4B3.1	CGATTCACCATCTCCAGAGACAATGCCAGGAACACCCTGTATCTGCAAATGAGCAGTCTGAGGTCTGAGGACACGGCCATGTATTACTGTGCCTTA
JCB 4B4.3	CGATTCACCGTCTCCAGAGACAATGCCGGGAACACCCTGTACCTGCAAATGAGCAGTCTGAGGTCTGAGGACACGGCCATGTATTACTGTTCAATT
JCB 4B40.7	CGATTCACCGTCTCCAGTGTCAATGCCAGGAACACCCTGTACCTGCAAATGAGCAGTCTGAGGTCTGAGGACACGGCCATGTATTACTGTTCAATT
LD6 A7.6	CGGTTCACCATCTCCAGAGATAATTCCCAAAACATCCTCTATCTTCAAATGAAAGCCCTGAGAGCTGAGGACAGTGCCACTTATCACTGTACAATA
LD6 A12.2	CGGTTCACCATCTCCAGAGATAATTCCCAAAGCATCCTCTATCTTCAAATGAATGCCCTGAGAGCTGAGGACAGTGCCACTTATTACTGTGCAATT
LD6 D9.1	CGGTTCACCATCTCCAGAGATAATTCCCAGAACATCCTCTATCTTCAAATGAATGCCCTGAGAGCTGAGGACAGTGCCACTTATCACTGTACAATA
Frames	<-FR3-start----------FR3-end->

Nucleotide sequences of BALB/k and CBA/Ca influenza HA specific immunoglobulin heavy chain V domains: CDR3 and FR4

```
  Frames      ------------------------CDR3-----------------------<-FR4-start---JH--------------------
 BALB/k 198
SFA 2B1.1     ----------------------GGAGACCATGGT---TACGTTATGGACTAC-TGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGTCCA
SFA 2B3.1     ----------------------GGGAACTATGGTAACTATGTTATGGACTAC-TGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCCCA
SFA 2B7.2     ----------------------GGAGACTATGGT---TACGCTATGGACTAC-TGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCCA
SFA 2B8.1     ----------------GGCCATGGTAACTACTTTCACTTT------GACTAT-TGGGGTCAAGGCACCACTCTCACACTCTCCTCAGCCA
SFA 3B2.1     -GAGAGGGATTACTACGGTAGTAACTGTGGGTTTCCT------------TAC-TGGGGCCAAGGGACTCTGGTGACTCTCTCTGCAAG
SFA 3B3.1     -GAGAGGGATTACTACGGTAGTAACTGTGGATTTCCT------------TAC-TGGGGCCAAGGGACTCTGGTGACTGTCTCTGCAGC
SFA 3B7.1     -------------GAGGGAATCGACTGG---TACTTCGATGTC----------TGGGGTGCAGGGACAACGGTCACCGTCTCCTCAGCCA
SFA 3B10.1    ----CAGGCTACGGTAGCC------GATTAC------TATGCTCTGAACTAC-TGGGGTCAAGGAACCTCAGTCACAGTCTCCTCAGC
SFA 3B12.1    -------------GAGGGAATCGACTGG---TACTTCGATGTC----------TGGGGCGCCGGGACCACGGTCACCGTCTCCTCAGCCA
SFA 3B14.1    ----GTGGCTACGGTTGCC------GATTAC------TTTGCTCTGGGCTAC-TGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCC
SFA 6B1.1     ----------GGTTATGAGGAGGTCTAC---------TATGCTATGGACTAC-TGGGGTCAAGGAAACTTAGTCACCGTCTCCTA
SFA 6B4.2     -------------AAGATGATGGTTACGACTACTCTGTTG---ATGGACTCC-TGGGGTCTGAGGAGCAC
SFA 6B5.1     ----------------GATGATGATTACCCATAC---TCTGCTATGGACTCC-TGGGGTCAAGGATT
SFA 7B1.2     ----------------GACTATGGT---ACCTAC---TATGTTATGGATAAC-TGGGGTCAAGGAAC
SFA 7B7.1     ----------------GACTATGGT---ACCTAC---TATGTTATGGATAAC-TGGGGTAAAGGAC
SFA 9B3.1     ----------GGTTATGATTACGTC---TTCTAT---TCT---ATGGACTAC-TGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCCA
SFA 9B6.1     -------------TATGATTACGAC---------GATGCT---ATGGACTAT-TGGGGTCAAGGAACCTCAGTCACCGTCTCCTCGCC
SFA 9B8.1     ----------GGCTTTGGTCCC---------TACTATTCT---ATGGACTGC-TGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAG
 BALB/k 158
SFA 7B2.1     ----------------------GGTGACTACTTT-----------GACTAC-TGGGGTCTAAGGCAC
SFA 7B3.1     ----------------GACTATGGT---ACCTAC---TATGTTATGGACGAC-TGGGGTCAAGGAGCCTCAGGCACCGTCTCCTCAGCC
SFA 7B5.1     -------GATAGACGCTCGGGTTTCGGTGCTTTG-----------GACTAC-TGGGGTCAAGGAA
SFA 7B8.1     ----------------TGGGATGGT------TACCTCTATGTTATGGACTAC-TGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGC
SFA 7B10.2    ----------------TGGTACCCTGAGAGG-----------TTGAACTAC-TGGGGTCAAGGCAC
SFA 7B11.1    ----------------TGGGACTATGCT--------------ATGGACAAC-TGGGGTCAAGGAGACACAGACACCGTCTCTGTTTG
SFA 7B15.1    -------------TCTGGGTCCTATGCG--------------AAGGACTAC-TGGGGTACAGGWM
SFA 7B19.1    ----------------TGGTACCCTGAGAGG-----------TTGAACTAC-TGGGGTCAAGGCACTACTCTCACAGACTCCAAAGC
 CBA/Ca 158
JCB 2B5.1.1   ----------------------GGTGACTACCTT-----------GACTAC-TGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCCA
JCB 2B29.1    ----GTCTACTATGATTACGACAGG---TTCTTCGATGTC-------------TGGGGCGCAGGGACCACGGTCACCGTCTCCTCAGCCA
JCB 3B6.1     ----------------------GGTGAATTTCTT-----------GACTAC-TGGGGTCAAGGCACNGCTCTCACAGTCTCCTCAGC
JCB 3B8.2     ----------------------GGTGAATTTCTT-----------GACTAC-TGGGGTCAAGGCACCACTCTCACAGTCTCCTCAGCC
JCB 4B2.1     ----------------------GGTAACTTCTTT-----------GACTAC-TGGGGTCAAGGCACCACTCTCACAGTCTCCTCAGCC
JCB 4B3.1     ----------------------AGCGACTTCTTT-----------GACTAC-TGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCC
JCB 4B4.3     ----------------GGT------GACTAC---CAT---------GACTAC-TGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCCA
JCB 4B40.7    ----------------GCT------GACTAT---CAT---------GACTAC-TGGGGCAAGGCGCCGCTCTCACAGTCTCCTCAGC
LD6 A7.6      ----------------------TCGTATTTCTTT-----------GACTAC-TGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCCA
LD6 A12.2     ----------------------TCGTACTACTTT-----------GACTAC-TGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCCA
LD6 D9.1      ----------------------TCGTATTTTTTT-----------GACTAC-TGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCC
  Frames      ------------------------CDR3-----------------------<-FR4-start---JH--------------------
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Nucleotide sequences of BALB/k and CBA/Ca influenza HA specific immunoglobulin light chain V domains: Signal and FR1

```
  Frames      -----signal---------------------------------<-FR1-start-----------------------------------------------FR1-end->
 BALB/k 198
SFA 2B1.1     --------NTGTTGCTGCTGCTATCGGTATCTGGTACCTGTGGAGACATTTTGATGACCCAGTCTCCATCCTCCCTGACTGTGTCAGCAGGAGAGAAGGTCACTATGAGCTGC
SFA 2B3.1     TTTCTTTGTATTTCGTGTTTCTCTGGTTGTCTGGCATTGACGGAGACATTGGGATGACCCAGTCTCACAAAATCATGTCCACATCAGTTGGAGACAGGGTCAGCATCACCTGC
SFA 2B7.2     -----NTNATGTTGCTGCTGCTATCGGTATCTGGTATCTGTGGAGACATTTTGATGACCCAGTCTCCATCCTCCCTGACTGTGTCAGCAGGAGAGAAGGTCACTATGACCTGC
SFA 2B8.1     -----NAGGYTGYWGGTGCTGCTGTGCTTACAGGTGCCAGATGTGACATCCAGATGACTCAGTCTCCAGCCTCTCTATCTGCATCTGTGGGAGAAACTGTCACCATCACATGT
SFA 3B2.1     NAGGTCCTGGGGTTGCTGCTGCTGTGCTTACAGGTGCCAGATGTGACATCCAGATGACTCAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAAACTGTCACCATCACATGT
SFA 3B3.1     ----------------NCTGCTGTGCTTACAGGTGCCAGATGTGACATCCAGATGACTCAGTCACCAGCCTCCCTATCTGCATCTGTGGGAGAAACTGTCACCATCACATGT
SFA 3B7.1     --------NNATCCATACTGCTCTGGTTATATGGTGCTGATGGGAACATTGTAATGACCCAATCTCCCAAATCCATGTCCATGTCAGTAGGAGAGAGGGTCACCTTGAACTGC
SFA 3B10.1    TAGGTCCTGGGTTGMTGCTGCTGTGGCTTACAGGTGCCAGATGTGACATCCAGATGACTCAGTCTCCAGCCTCCCTGTCTGCATCTGTGGGAGAAACTGTCACCATCACATGT
SFA 3B12.1    -----NNATATCCAATACTGCTCTGGTTATATGGTGCTGATGGAAACATTGTAATGACCCAATCTCCCAAATCCATGTCCATGTCAGTAGGAGAGAGGGTCACCTTGAGCTGC
SFA 3B14.1    --NNTGTAGCTGTTGTGCTGCTGTGCTANGTAGGTGCCAGATGTGACATCCAGATGACTCAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAAACTGTCACCATCACATGT
SFA 6B1.1     ----------------NCGCGCTGGGTTCCAGGTTCCACTGGTGACATTGTGCTGACACAGTCTCCTGCTTCCTTAGCTGTATCTCTGGGGCAGAGGGCCACCATCTCATAC
SFA 6B4.2     ----------------------GTTGTCTGGTGTGTTGACGGAGACAGTGTGATGACCCAGTCTCACAAATTCATGTCCACATCAATAGGAGACAGGGTCAGCATCACCTGC
SFA 6B5.1     ----------------------------NTGGCCGTTGACGGAGACATTGTGATGACCCAGTCTCACAAATTCATGTCCACATCAGTAGGAGACAGGGTCAGCATCACCTGC
SFA 7B1.2     -------------------------------NNTGTTGACGGAGACATTGTGATGACCCAGTCTCACAAATTCATGTCCACATCAATAGGAGACAGGGTCAGCATCACCTGC
SFA 7B7.1     ---------------------------------NNGGACGGAGACATTGTGATGACCCAGTCTCACAAATTCATGTCCACATCAATAGGAGACAGGGTCAGCATCACCTGC
SFA 9B3.1     ------------------------------------------GACATTGTGATGACCCAGTCTCACAAATTCATGTCCACATCAGTAGGAGACAGGGTCAGCATCACCTGC
SFA 9B6.1     --------------------GTCTCAGTCATAAWGTCCAGAGGACAAATTGTTCTCACCCAGTCTCCAGTAATCATGTCTGCATCTCCTGGGGAGAAGGTCACCTTGACCTGC
SFA 9B8.1     AGGATCCTGGGTTGCTGCTGCTGTGGCTTACAGGTGCCAGATGTGACATCCAGATGACTCAGTGTCCAGCCTCCCTATCTGCATCAGTGGGAGAAACTGTCACCATCACATGT
 BALB/k 158
SFA 7B2.1     --------------------------------NNWWCCAGCAGTGATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGC
SFA 7B3.1     ------------------------------------------GACAGTGTGATGACCCAGTCTCACAAATTCATGTCCACATCAGTAGGAGACAGGGTCAGCATCACCTGC
SFA 7B5.1     ------------------------------------------------------------------------------------------NNCAGAGGCCACCATCTCCTG
SFA 7B8.1     ----------------NTGGCCCGTGTGGCAGTTGACGGACGACACTGTGNGATGACCCAGTCTCACAAATTCATRYCCACAYCAATAGGAGACAGGGTCAGCATCACCTGC
SFA 7B10.2    ---------------------------------------CAGTGTGACATSSAGATGACACAGTCTCCATCCTCACTGTCTGCATCTCTGGGAGGCAAAGTCACCATCACTTGC
SFA 7B11.1    ------------------------------------------------------------------------------------NNGGGGCAGAGSSCCMMYAYYYCATGC
SFA 7B15.1    TCCTGCTATGGGTGCTGCTGCTCTGGGTTCCAGGTTCCACAGGTAACATTGTGTTGACCCAATCTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGAGGGCCACCATATCCTGC
SFA 7B19.1    ------------------------------------------NACAGCCAGATGACACAGTCTCCATCCTCACTGTCTGCATCTCTGGGAGGCAAAGTCACCATCACTTGC
 CBA/Ca 158
JCB 2B5.1.1   ----------------------------CCTGCTTCCASAAGTRATGTTGTRATGACCCAAACTCCAGTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGC
JCB 2B29.1    ----------------------------NNTGGTGTTGACGGAGACATTGTGATGACCCAGTCTCACAAATTCATGTCCACATCACTAGGAGACAGGGTCAGCATCACCTGC
JCB 3B6.1     ---------------------------ATTCCTRCTTCCAGCAGTDATRTTGTGATRACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATGAAGCCTCCATCTCTTGC
JCB 3B8.2     ------------------------------------------GATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGC
JCB 4B2.1     ----------------------------CCWGCTTCCAGCAGTGATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGC
JCB 4B3.1     --------GTTGGTCTGATGTTCTGGATTCCTGCTTCCTGCAGTGATGTTTTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGC
JCB 4B4.3     ---------------------------NNTCCTGCTTCCAGCAGTGATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATTTTTTGC
JCB 4B40.7    ---------------------------TTTCCTGCTTCCAGCAGTGGTGTTGTGATGACCCATACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGC
LD6 A7.6      -----NGGTTGGTGCTGATGTTCTGGATTCCTGCTTCCAGCAGTGATGTTGTGATGACCCAGACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGC
LD6 A12.2     ----------------NTGTTCTGNATTCCTGCTTCCAGCAGTGATGTTGTNATNACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGC
LD6 D9.1      --------------------GGCTGTATTCCTGCTTCCAGCAGTGATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATYTCTTGC
  Frames      -----signal---------------------------------<-FR1 start-----------------------------------------------FR1-end->
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Nucleotide sequences of BALB/k and CBA/Ca influenza HA specific immunoglobulin light chain V domains: CDR1 and FR2

```
  Frames      -------CDR1-----------------------------------------<-FR2-start------------------------FR2-end->
 BALB/k 198
SFA 2B1.1     AAGTCCAGTCAGAGTCTTTTAGCTAGTGGCACCCAAAATAACTACTTGGCCTGGCACCAGCAGAAACCAGGACGATCTCCTAAAATGCTGATAATT
SFA 2B3.1     AAGGCCAGTCAGGATGTG-----------------AGTAATGATGTAGCCTGGTATCAACAGAAACCAGGACAATCTCCTAAACTACTGATTTAC
SFA 2B7.2     AAGTCCAGTCAGAGTCTTTTAGCTGGTGGCACCCAAAATAACTACTTGGCCTGGCACCAGCAGAAACCCGGACGATCTCCTAAAATGCTGATAATT
SFA 2B8.1     CGAACAAGTGGGAATATT-----------------CACAATTATTTAGTATGGTATCAACAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTAT
SFA 3B2.1     CGAGCAAGTGGGAATATT-----------------CACAATTATTTAGCATGGTATCAGCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTAT
SFA 3B3.1     CGAGCAAGTGGGAATATT-----------------CACAATTTTTTAGCATGGTATCAGCAAATACAGGGAAAATCTCCTCAGCTCCTGGTCTAT
SFA 3B7.1     AAGGCCAGTGAGAATGTG-----------------GATACTTATGTATTTTGGTATCAACAGAAACCAGAGCAGTCTCCTAAACTACTGATATAC
SFA 3B10.1    CGAGCAAGTGGGAATATT-----------------CAGAATTATTTAGTATGGTATCAGCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTAT
SFA 3B12.1    AAGGCCAGTGAAAATGTG-----------------GATTCTTCTGTATTCTGGTATCAACAGAAACCAGGGCAGTCTCCTAAATTGTTGATATAC
SFA 3B14.1    CGAGCAAGTGGGAATATT-----------------CACAATTATTTAGTATGGTATCAGCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTAT
SFA 6B1.1     AGGGCCAGCAAAAGTGTCAGTACATCTGGC------TATAGTTATATGCACTGGAACCAACAGAAACCAGGACAGCCACCCAGACTCCTCATCTAT
SFA 6B4.2     ACGGCCAGTCAGGATGTG-----------------AGTACTGCTGTAGCCTGGTATCGACAGAAACCAGGGCAATCTCCTAAACTACTGATTTAC
SFA 6B5.1     AAGGCCAGTCAGGATGTG-----------------AGTTCTGCTGTAGCCTGGTATCAACAAAAACCAGGGCAATCTCCTAAACTACTGATTTAC
SFA 7B1.2     AAGGCCAGTCAGGATGTG-----------------AGTGCTGCTGTAGCCTGGTATCAACAGAAACCAGGACAATCTCCTAAACTACTGATTTAC
SFA 7B7.1     AAGGCCAGTCAGGATGTG-----------------AGTGCTGCTGTAGCCTGGTATCAACAGAAACCAGGACAATCTCCTAAACTACTGATTTAC
SFA 9B3.1     AAGGCCAGTCAGGATGTG-----------------AGTACTGCTGTAGCCTGGTATCAACAGAAACCAGGACAATCTCCTAAACTACTGATTTAC
SFA 9B6.1     AGTGCCAGCTCAAGTGTA--------------AATTCCATCTACTTGTACTGGTACCAGCAGAAGCCAGGATCCTCCCCCAAAATCTGGATTTAT
SFA 9B8.1     CGAGCAAGTGGGAATATT-----------------CACAATTATTTAACATGGTATCAGCAGAAACAGGGAAAATCTCCTCACCTCCTGGTCTAT
 BALB/k 158
SFA 7B2.1     AGATCTAGTCAGAGCCTTGTACACAGTAATGGA---AACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTAC
SFA 7B3.1     AAGGCCAGTCACGATGTG-----------------AGTACTGCTGTAGCCTGGTATCAACAGAAACCAGGACAATCTCCTAAACTACTGATTTAC
SFA 7B5.1     CAGAGCCAGCAAAGTGTTGATGATTATGGC------ATTAGTTTTATGAACTGGTTCCAACAGAAACCAGGACAGCCACCCAAACTCCTCATCTAT
SFA 7B8.1     AAGGCCAGTCAGGATGTG-----------------AGTGCTGCTGTAGCCTGGTATCAACAGAAACCAGGACAATCTCCTAAACTACTGATTTAC
SFA 7B10.2    AAGGCAAGCCAAGACATT-----------------AACAAGTATATAGCTTGGTACCAACACAAGCCTGGAAAAGGTCCTAGGCTGCTCATACAT
SFA 7B11.1    AGGGCCAGCAAAAGTGTCAGTACATCTGGC------TATAGTTATATGCACTGGTACCAACAGAAACCAGGACAGCCACCCAAACTCCTCATCTAT
SFA 7B15.1    AGAGCCAGTGAAAGTGTTGGTAGTTATGGC------AATAGTTTTATGTACTGGTACCAGCAGAGACCAGGACAGCCACCCAAACTCCTCATCTAT
SFA 7B19.1    AAGGCAAGCCAAGACATT-----------------AACAAGTATATAGCTTGGTACCAACACAAGCCTGGAAAAGGTCCTAGGCTGCTCATACAT
 CBA/Ca 158
JCB 2B5.1.1   AGATCTAGTCAGAGCCTTGTACACAGTAATGGA---AACACCTATTTACACTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAGTTCTTGATCTAC
JCB 2B29.1    AAGGCCAGTCAGGATGTG-----------------AATACTGCTGTAGCCTGGTATCAACAGAAACCAGGACAGTCTCCTAAACTACTGATTTAC
JCB 3B6.1     AGATCTAGTCAAAGCCTTGTACACAGTAATGGA---AACACCTTTTTACATTGGTACCTGCAGAAGCCAGGCCAGTCACCAAAGTTCCTGATCTAC
JCB 3B8.2     AGATCTAGTCAGAGCCTTGTGCACAGTAATGGA---AACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAGTTCCTGATCTAC
JCB 4B2.1     AGATCTAGTCAGACCCTTGTACACAGTAATGGA---AACACCTATTTACATTGGTACCTGCAGCAGCCAGGCCAGTCTCCAAAGCTCCTGATCTAC
JCB 4B3.1     AGATCTAGTCAGAGCATTGTAGATAGTAATGGA---GACACCTATTTAGAATGGTACCTGCAGAAACCAGGCCAGTCTCCAAAGCTCCTGATCTAC
JCB 4B4.3     AGATCTAGTCAGAGCCTTGTACACAGTAATGGA---AACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTAC
JCB 4B40.7    AGATCTAGTCAGAGCCTTGTACACAGTAATGGA---AACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTAC
LD6 A7.6      AGATCTAGTCAGACCCTTATACACACTCATGGA---GACACCTATTTACATTGGTACCTGCAGAGGCCAGGCCAGTCTCCAAAGCTCCTGATCTAC
LD6 A12.2     AGATCTAGTCAGAGCCTTATACACACTAATGGA---AACACCTATTTACATTGGTACCTGCAGAGGCCAGGCCAGTCTCCAAAGCTCCTGATCTAC
LD6 D9.1      AGATCTAGTCAGAGCCTTATACGCACTGATGGA---AACACCTTTTTACATTGGTACCTGCAGAGGCCAGGCCAGTCTCCAAAGCTCCTGATCTAC
  Frames      -------CDR1-----------------------------------------<-FR2-start------------------------FR2-end->
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Nucleotide sequences of BALB/k and CBA/Ca influenza HA specific immunoglobulin light chain V domains: CDR2 and FR3

```
  Frames      ---------CDR2--------<-FR3-start----------------------------------------------------------------------------FR3-end->
 BALB/k 198
SFA 2B1.1     TGGGCTTCCACTAGGGTTTCTGGAGTCCCTGATCGCTTCATAGGCAGTGGATCTGGGACGGTTTTCACTTTGACCATCAACAGTGTTCAGGCTGAAGATCTGGCTGTTTATTACTGT
SFA 2B3.1     TCGGCATCCTACCGATATACTGGAGTCCCTGATCGCTTCACTGGCAGTGGATCTGGGACGGCTTTCACTTTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCATCTTATTACTGT
SFA 2B7.2     TGGGCATCCACTAGGGTATCTGGAGTCCCTGATCGCTTCATAGGCAGTGGATCTGGGACGGATTTCACTCTGACCATCAACAGTGTGCAGGCTGAAGATCTGGCTGTTTATTACTGT
SFA 2B8.1     AATGCAAAAACCTTAGCAGATGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACACAATATTCTCTCAGGATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGT
SFA 3B2.1     GATGCAGAGACCTTAGCAGAAGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACACAATATTCTCTCAAGATCAACAGCCTGCAGCCTGAGGATTTTGGAAGTTATTACTGT
SFA 3B3.1     GATGCAGAAACCTTATCAGATGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACACAGTTTTCTCTCAAAATCGACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGT
SFA 3B7.1     GGGGCATCCAACCGGTACACTGGGGTCCCCGATCGCTTCACAGGCAGTGGATCTGCAACAGATTTCACTCTGACCATCAGCAGTTTGCAGACTGAAGACCTTGCAGATTATCACTGT
SFA 3B10.1    AATGCAAAAACCTTAGTAGATGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACACAATATTCTCTCAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGT
SFA 3B12.1    GGGGCATCCAACCGGTACACTGGGGTCCCCGATCGCTTCACAGGCAGTGGATCTGCAACAGATTTCACTCTGACCATCAGCAGTGTGCAGGCTGAAGACCTTGCAGATTATCACTGT
SFA 3B14.1    AATGCAAAAACCTTAGCAGATGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGTACACAATATTCTCTCAGGATCAACAACCTGCAGCCTGAAGATTTTGGGAGTTATTACTGT
SFA 6B1.1     CTTGTATCCAACCTAGAATCTGGGGTCCCTGCCAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACCCTCAACATCCATCCTGTGGAGGAGGAGGATGCTGCAACCTATTACTGT
SFA 6B4.2     TCGGCATCCTACCGGTTCACTGGAGTCCCTGATCGCTTCACTGGCAGTGGATCTGGGACGGATTTCACTTTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCACTTTATTACTGT
SFA 6B5.1     TGGGCATCCACCCGGCTCACTGGAGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTATACTCTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCACTTTATTACTGT
SFA 7B1.2     TCGGCATCCTACCGGAACACTGGAGTCCCTGATCGCTTCACTGGCAGTGGATCTGGGACGGATTTCACTTTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCAGTTTATTACTGT
SFA 7B7.1     TCGGCATCCTACCGGAACACTGGAGTCCCTGATCGCTTCACTGGCAGTGGATCTGGGACGGATTTCACTTTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCAGTTTATTACTGT
SFA 9B3.1     TCGGCATCCTACCGGTACACTGGAGTCCCTGATCGCTTCACTGGCAGTGGATCTGGGACGGATTTCACTTTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCAGTTTATTACTGT
SFA 9B6.1     AGCACATCCAACCTGGCTTCTGGAGTCCCTGCTCGCTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGCATGGAGGCTGAAGATGCTGCCTCTTATTTCTGC
SFA 9B8.1     AATGCAGAAACCTTAGCAGATGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACACAATATTCTCTCAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGT
 BALB/k 158
SFA 7B2.1     AAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGC
SFA 7B3.1     TCGGCATCCTACCGGTACACTGGAGTCCCTGATCGCTTCACTGGCAGTGGATCTGGGACGGATTTCACTTTCACCATCAGCACTGTGCAGGCTGAAGACCTGGCAGTTTATTACTGT
SFA 7B5.1     GCTGCATCCAACCAAGGATCCGGGGTCCCTGCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTTCAGCCTCAACATCCATCCTATGGAGGAGGATGATACTGCAATGTATTTCTGT
SFA 7B8.1     TCGGCATCCTACCGGAACACTGGAGTCCCTGATCGCTTCACTGGCAGTGGATCTGGGACGGATTTCACTTTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCAGTTTATTACTGT
SFA 7B10.2    TACACATCTACATTACAGCCAGGCATCCCATCAAGGTTCAGTGGACGTGGGTCTGGGAGAGATTATTCCTTCAGCATCAGCAACCTGGACCCTGAAGATATTGCAACTTATTATTGT
SFA 7B11.1    CTTGCATCCAACCTAGAATCTGGGGTCCCTGCCAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACCCTCAACATCCATCCTGTGGAGGAGGAGGATGCTGCAACCTATTTCTGT
SFA 7B15.1    CTTGCATCCAACCTAGAATCTGGGGTCCCTGCCAGGTTCAGTGGCAGTGGGTCTAGGACAGACTTCACCCTCACCATTGATCCTGTGGAGGCTGATGATGCTGCAACCTATTACTGT
SFA 7B19.1    TACACATCTACATTACAGCCAGGCATCCCATCAAGGTTCAGTGGAAGTGGGTCTGGGAGAGATTATTCCTTCAGCATCACCAACCTGGACCCTGAAGATATTGCAACTTATTATTGT
 CBA/Ca 158
JCB 2B5.1.1   AAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTCGAGGCTGAGGATCTGGGAGTTTATTTCTGC
JCB 2B29.1    TCGGCATCCTACCTGAACACTGGAGTCCCTGGTCGCTTCACTGGCAGTGGATCTGGGACGGATTTCACTTTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCAGTTTATTACTGT
JCB 3B6.1     AACGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGACTTTATTTCTGC
JCB 3B8.2     GAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGC
JCB 4B2.1     AAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGATTTTATTTCTGC
JCB 4B3.1     AAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTACTGC
JCB 4B4.3     GAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGAGGAGGCTGAGGATCTGGGAATTTATTTCTGC
JCB 4B40.7    AAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCACTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAATTTATTTCTGC
LD6 A7.6      GAGGTTTCCAAACGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCGGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGC
LD6 A12.2     AAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGC
LD6 D9.1      GAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACATTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGC
  Frames      ---------CDR2--------<-FR3-start----------------------------------------------------------------------------FR3-end->
```

Nucleotide sequences of BALB/k and CBA/Ca influenza HA specific immunoglobulin light chain V domains: CDR3 and FR4

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 Frames       -------CDR3--------------------<-FR4-start------JH------------<-C-start-------------------------------------
 BALB/k 198
SFA 2B1.1     CAGCAGTCCTACAGCGCT---CCGGTCACG-TTCGGTGCTGGGACCAAGCTGGAGCTGAAA-CGGGCTGATGCTGCACCACTG
SFA 2B3.1     CAGCAACATTATACTACT------CCGGCG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACT
SFA 2B7.2     CAGCAGTCCTACAGCGCT---CCGGTAACG-TTCGGTGCTGGGACCAAGCTGGAGCTGAAA-CGGGCTGATGCTGCACCAACT
SFA 2B8.1     CAACATTTTTGGAATACT---CCGTACACG-TTCGGAGGGGGGACCAAGCTGGAAATAAAA-CGGGCTGATGCTGCACCAACT
SFA 3B2.1     CAGCATTTTTGGAGTACTCCTCGG---ACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCA
SFA 3B3.1     CAACATTTTTGGAATACTCCTCGG---ACG-TTCGGTGGTGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACTGTATCCATCTTC
SFA 3B7.1     GGACAGACTTATAGCTAT---CCGCTCACG-TTCGGTGCTGGSACCAAGCTGGAGCTGAAA-CGGGCTGATGCTTCG
SFA 3B10.1    CAACATTTCTGGAATACTCCTCGG---ACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACTGTATCCATCTTG
SFA 3B12.1    GGACAGACTTACAGCTAT---CCACTCACG-TTCGGTGCTGGGACCAAGCTGGAGCTGAAA-CGGGCTGATGCTGCACCAACTGWWCCATCCTCC
SFA 3B14.1    CAACATCTTTGGAATACTCCTCGG---ACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCGATG
SFA 6B1.1     CAGCACATTAGGGAGCTT------ACACGT-TCGGAGGGGGGACCAAGCNTGGAAATAAAA-CGGGCTGATGCTGCACCAACTG
SFA 6B4.2     CAGCAACATTATGGTACTCCTCCG---ACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACTGTATCCGACCAT
SFA 6B5.1     CAGCAACATTATAACACTCCTCCG---ACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACTGCATCC
SFA 7B1.2     CAGCAACATTATAGTACTCCTCCG---ACG-TTCGGTGGAGGCACCAAGGTGGAAATCAAA-CGGGCTGATGCTGCACCAACTGTATCCATC
SFA 7B7.1     CAGCAACATTATAGTACTCCTCCG---ACG-TTCGGTGGAGGCACCAAGGTGGAAATCAAA-CGGGCTGATGCTGCACCAACTGTATCC
SFA 9B3.1     CAGCGACATTATAGTACTCCTCCG---ACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACTGTATCCAAGCAT
SFA 9B6.1     CATCAGTGGAGTAGTTCC---CCATTCACG-TTCGGCTCGGGGACAAAGTTGGAAATCAAA-CGGGCTGATGCTGCACCA
SFA 9B8.1     CAACATTTTTGGAATACT---CCGTACACG-TTCGGAGGGGGGACCAAGCTGGAAATAAAA-CGGGCTGATGCTGCACCAACT
 BALB/k 158
SFA 7B2.1     TCTCAAAGTACACATGTT---CCGCTCACG-TTCGGTGCTGGGACCAAGCTGGAGCTGAAA-CGGGCTGATGCTGCACCA
SFA 7B3.1     CAACAACATTATAGTACTCCTCCG---ACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCACTGTAT
SFA 7B5.1     CAGCAAAGTAAGGAGGTT---CCGAGGACG-TTCGGTGGAGGCACCAAGCTGGAGATCAGC-CGGGCT
SFA 7B8.1     CAGCAACATCATAGTACTCCTCCG---ACG-TTCGGTGGAGGCACCAAGGTGGAAATCAAA-CGGGCTGATGCTGCACCAACT
SFA 7B10.2    CTACAGTATGATATTCTT---CGG---ACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACTGTATCC
SFA 7B11.1    CAACACAGTAGGGAGCTG------TACACG-TTCGGAGGGGGGACCAAGCTGGAAATCAAA-CGGGCTGATGCT
SFA 7B15.1    CAGCAAAATAATGAGGAT---CCGTGGACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACT
SFA 7B19.1    CTACAGTATGATATTCTT---CGG---ACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACTGTATCCATCTTC
 CBA/Ca 158
JCB 2B5.1.1   TCTCAAAGTACACATGTT---CCAGTCACG-TTCGGCTCGGGGACAAAGTTGGAAATAAAA-CGGGCTGATGCT
JCB 2B29.1    CACCAACATTATAGTTTT---CCATTCACG-TTCGGCTCGGGGACAAATGTGKAAATAAAA-CGGGCTGATGCTGCACCA
JCB 3B6.1     TCTCAAAGTACACATGTTCCTCGG---ACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACTGTATCC
JCB 3B8.2     TCTCAAAGTACACATGTTCCTCGG---ACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCT
JCB 4B2.1     TCTCAAAGTACACATGTTCCTCCGTGGACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCA
JCB 4B3.1     TTTCAAGGTTCACATGTT---CCGTACACG-TTCGGAGGGGGGACCAAGCTGGAAATAAAA-CGGGCTGATGCT
JCB 4B4.3     TCTCAAACTACACATGTTCCTCCGTGGACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCA
JCB 4B40.7    TCTCAAACTACACATGTTCCTCCATGGACG-TTCGGTGGAGGCACCAAGCTGGAAATCAAA-GGGGCTGATGCT
LD6 A7.6      TCTCAGACTTCACATTTTCCTCCG---ACG-TTCGGTGGCGGCACCAAGCTGGAACTCAAA-CGGGCTGATGCTGCACCAACT
LD6 A12.2     TCTCAAAGTACACATTTTCCTCCG---ACG-TTCGGTGGCGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCACCAACT
LD6 D9.1      TCTCAAAGTACACATTTTCCTCCG---ACG-TTCGGAGGCGGCACCAAGCTGGAAATCAAA-CGGGCTGATGCTGCA
  Frames      -------CDR3--------------------<-FR4-start------JH------------<-C-start-------------------------------------
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Appendix II

Sequence homologies of influenza virus haemagglutinin specific secondary antibody genes following natural infection of BALB/k and CBA/Ca mice.

Nucleotide sequence comparison of immunoglobulin heavy chain V regions

Tables are arranged by donor and specificity

A value of 1 represents complete identity

Heavy	SFA 2B1.1	SFA 2B3.1	SFA 2B7.2	SFA 2B8.1
SFA 2B1.1	1	0.7230	0.7230	0.7230
SFA 2B3.1	0.7230	1	0.7123	0.7260
SFA 2B7.2	0.7230	0.7123	1	0.8082
SFA 2B8.1	0.7230	0.7260	0.8082	1
SFA 3B2.1	0.7746	0.6625	0.7169	0.7306
SFA 3B3.1	0.7887	0.6360	0.6849	0.6895
SFA 3B7.1	0.5305	0.4625	0.4977	0.4749
SFA 3B10.1	0.8451	0.6833	0.6575	0.6895
SFA 3B12.1	0.5459	0.4589	0.4879	0.4638
SFA 3B14.1	0.6291	0.4917	0.5068	0.5160
SFA 6B1.1	0.8667	0.7282	0.7487	0.7436
SFA 6B4.2	0.4413	0.3958	0.4155	0.4155
SFA 6B5.1	0.4601	0.4274	0.4475	0.4521
SFA 7B1.2	0.4601	0.4250	0.4338	0.4110
SFA 7B7.1	0.4554	0.4231	0.4247	0.4201
SFA 9B3.1	0.8263	0.6625	0.6804	0.6941
SFA 9B6.1	0.8685	0.6958	0.7032	0.6941
SFA 9B 8.1	0.8732	0.6667	0.6804	0.6804
SFA 7B2.1	0.4902	0.4510	0.5033	0.4706
SFA 7B3.1	0.5352	0.4667	0.4658	0.4338
SFA 7B5.1	0.4633	0.4124	0.3729	0.3729
SFA 7B8.1	0.3662	0.3125	0.3470	0.3516
SFA 7B10.2	0.3803	0.3625	0.3333	0.2877
SFA 7B11.1	0.3052	0.3042	0.3105	0.2922
SFA 7B15.1	0.3427	0.3708	0.3242	0.3516
SFA 7B19.1	0.3474	0.3292	0.3607	0.3653
JCB 2B5.1.1	0.4319	0.4000	0.3836	0.4064
JCB 2B29.1	0.4413	0.4017	0.4475	0.4292
JCB 3B6.1	0.4403	0.4088	0.4591	0.4528
JCB 3B8.2	0.3944	0.3875	0.4384	0.4429
JCB 4B2.1	0.4272	0.4125	0.4338	0.4338
JCB 4B3.1	0.4648	0.4042	0.4886	0.4292
JCB 4B4.3	0.4507	0.4167	0.4612	0.4429
JCB 4B40.7	0.4493	0.3720	0.4155	0.3816
LD6 A7.6	0.5305	0.4583	0.4384	0.4247
LD6 A12.2	0.5258	0.4292	0.4429	0.4384
LD6 D9.1	0.5352	0.4356	0.4429	0.4155

Heavy	SFA 3B2.1	SFA 3B3.1	SFA 3B7.1	SFA 3B10.1	SFA 3B12.1	SFA 3B14.1
SFA 2B1.1	0.7746	0.7887	0.5305	0.8451	0.5459	0.6291
SFA 2B3.1	0.6625	0.6360	0.4625	0.6833	0.4589	0.4917
SFA 2B7.2	0.7169	0.6849	0.4977	0.6575	0.4879	0.5068
SFA 2B8.1	0.7306	0.6895	0.4749	0.6895	0.4638	0.5160
SFA 3B2.1	1	0.8246	0.4519	0.6976	0.4734	0.5876
SFA 3B3.1	0.8246	1	0.4561	0.7500	0.4928	0.6316
SFA 3B7.1	0.4519	0.4561	1	0.4630	0.8889	0.4000
SFA 3B10.1	0.6976	0.7500	0.4630	1	0.5314	0.5945
SFA 3B12.1	0.4734	0.4928	0.8889	0.5314	1	0.4444
SFA 3B14.1	0.5876	0.6316	0.4000	0.5945	0.4444	1
SFA 6B1.1	0.8000	0.8051	0.5231	0.8513	0.5179	0.6615
SFA 6B4.2	0.4574	0.4386	0.3926	0.3972	0.4155	0.3191
SFA 6B5.1	0.4316	0.4123	0.4872	0.4444	0.5072	0.3205
SFA 7B1.2	0.3985	0.3728	0.4368	0.4368	0.4879	0.2912
SFA 7B7.1	0.3932	0.3596	0.4402	0.4316	0.4686	0.2735
SFA 9B3.1	0.6858	0.7368	0.4674	0.7165	0.5507	0.5517
SFA 9B6.1	0.6254	0.7281	0.4593	0.6735	0.5169	0.5464
SFA 9B 8.1	0.6289	0.7149	0.4519	0.6769	0.5314	0.5292
SFA 7B2.1	0.4967	0.5033	0.5163	0.4902	0.4837	0.3464
SFA 7B3.1	0.4399	0.4430	0.4778	0.4694	0.5507	0.4433
SFA 7B5.1	0.4237	0.4181	0.4350	0.4463	0.4294	0.3559
SFA 7B8.1	0.3711	0.3333	0.3481	0.3608	0.3913	0.3230
SFA 7B10.2	0.3711	0.3596	0.4259	0.3505	0.4348	0.3849
SFA 7B11.1	0.3574	0.3377	0.2963	0.3196	0.2995	0.3505
SFA 7B15.1	0.3262	0.3070	0.3519	0.3369	0.4058	0.3652
SFA 7B19.1	0.3540	0.3465	0.3630	0.3469	0.3865	0.3540
JCB 2B5.1.1	0.4023	0.3947	0.4176	0.4138	0.5024	0.3142
JCB 2B29.1	0.4402	0.4298	0.4188	0.4359	0.4493	0.3547
JCB 3B6.1	0.4591	0.4465	0.5975	0.4591	0.5786	0.3522
JCB 3B8.2	0.4291	0.3728	0.5074	0.3901	0.5217	0.3121
JCB 4B2.1	0.4265	0.3991	0.4630	0.4516	0.5459	0.3548
JCB 4B3.1	0.3953	0.4035	0.5620	0.4419	0.6522	0.3721
JCB 4B4.3	0.4147	0.4298	0.5969	0.4302	0.6425	0.3682
JCB 4B40.7	0.3913	0.3913	0.5749	0.4300	0.6329	0.3382
LD6 A7.6	0.4683	0.4737	0.5754	0.5119	0.6087	0.4048
LD6 A12.2	0.4841	0.4868	0.5754	0.5357	0.6039	0.4087
LD6 D9.1	0.4667	0.4533	0.6044	0.5022	0.6280	0.4311

Heavy	SFA 6B1.1	SFA 6B4.2	SFA 6B5.1
SFA 2B1.1	0.8667	0.4413	0.4601
SFA 2B3.1	0.7282	0.3958	0.4274
SFA 2B7.2	0.7487	0.4155	0.4475
SFA 2B8.1	0.7436	0.4155	0.4521
SFA 3B2.1	0.8000	0.4574	0.4316
SFA 3B3.1	0.8051	0.4386	0.4123
SFA 3B7.1	0.5231	0.3926	0.4872
SFA 3B10.1	0.8513	0.3972	0.4444
SFA 3B12.1	0.5179	0.4155	0.5072
SFA 3B14.1	0.6615	0.3191	0.3205
SFA 6B1.1	1	0.4872	0.4051
SFA 6B4.2	0.4872	1	0.5342
SFA 6B5.1	0.4051	0.5342	1
SFA 7B1.2	0.3846	0.5211	0.7521
SFA 7B7.1	0.3692	0.5427	0.7222
SFA 9B3.1	0.8974	0.4330	0.4444
SFA 9B6.1	0.8872	0.4184	0.4316
SFA 9B 8.1	0.8564	0.3936	0.4487
SFA 7B2.1	0.4967	0.6667	0.6732
SFA 7B3.1	0.4718	0.4787	0.5385
SFA 7B5.1	0.4181	0.5198	0.5480
SFA 7B8.1	0.3385	0.3652	0.3547
SFA 7B10.2	0.3538	0.3865	0.4103
SFA 7B11.1	0.2615	0.3050	0.3034
SFA 7B15.1	0.3692	0.3794	0.3547
SFA 7B19.1	0.3385	0.3794	0.4103
JCB 2B5.1.1	0.4051	0.3640	0.3675
JCB 2B29.1	0.4103	0.3889	0.4274
JCB 3B6.1	0.4465	0.4528	0.4403
JCB 3B8.2	0.4154	0.4007	0.3803
JCB 4B2.1	0.4359	0.4122	0.3590
JCB 4B3.1	0.4513	0.3915	0.4060
JCB 4B4.3	0.4513	0.3915	0.4017
JCB 4B40.7	0.4513	0.4348	0.4396
LD6 A7.6	0.5179	0.3929	0.4231
LD6 A12.2	0.5231	0.4008	0.4274
LD6 D9.1	0.5282	0.3956	0.4133

Heavy	SFA 7B1.2	SFA 7B7.1
SFA 2B1.1	0.4601	0.4554
SFA 2B3.1	0.4250	0.4231
SFA 2B7.2	0.4338	0.4247
SFA 2B8.1	0.4110	0.4201
SFA 3B2.1	0.3985	0.3932
SFA 3B3.1	0.3728	0.3596
SFA 3B7.1	0.4368	0.4402
SFA 3B10.1	0.4368	0.4316
SFA 3B12.1	0.4879	0.4686
SFA 3B14.1	0.2912	0.2735
SFA 6B1.1	0.3846	0.3692
SFA 6B4.2	0.5211	0.5427
SFA 6B5.1	0.7521	0.7222
SFA 7B1.2	1	0.9615
SFA 7B7.1	0.9615	1
SFA 9B3.1	0.3640	0.3718
SFA 9B6.1	0.4100	0.4231
SFA 9B 8.1	0.4253	0.4316
SFA 7B2.1	0.6797	0.6601
SFA 7B3.1	0.5402	0.5556
SFA 7B5.1	0.5537	0.5424
SFA 7B8.1	0.3410	0.3333
SFA 7B10.2	0.3755	0.3761
SFA 7B11.1	0.3218	0.2991
SFA 7B15.1	0.3716	0.3675
SFA 7B19.1	0.3985	0.4103
JCB 2B5.1.1	0.3678	0.3675
JCB 2B29.1	0.4231	0.4188
JCB 3B6.1	0.4340	0.4340
JCB 3B8.2	0.3946	0.3932
JCB 4B2.1	0.4023	0.3889
JCB 4B3.1	0.3915	0.3846
JCB 4B4.3	0.4070	0.3974
JCB 4B40.7	0.3816	0.3671
LD6 A7.6	0.4603	0.4487
LD6 A12.2	0.4444	0.4359
LD6 D9.1	0.4311	0.4133

Heavy	SFA 9B3.1	SFA 9B6.1	SFA 9B 8.1
SFA 2B1.1	0.8263	0.8685	0.8732
SFA 2B3.1	0.6625	0.6958	0.6667
SFA 2B7.2	0.6804	0.7032	0.6804
SFA 2B8.1	0.6941	0.6941	0.6804
SFA 3B2.1	0.6858	0.6254	0.6289
SFA 3B3.1	0.7368	0.7281	0.7149
SFA 3B7.1	0.4674	0.4593	0.4519
SFA 3B10.1	0.7165	0.6735	0.6769
SFA 3B12.1	0.5507	0.5169	0.5314
SFA 3B14.1	0.5517	0.5464	0.5292
SFA 6B1.1	0.8974	0.8872	0.8564
SFA 6B4.2	0.4330	0.4184	0.3936
SFA 6B5.1	0.4444	0.4316	0.4487
SFA 7B1.2	0.3640	0.4100	0.4253
SFA 7B7.1	0.3718	0.4231	0.4316
SFA 9B3.1	1	0.7126	0.7011
SFA 9B6.1	0.7126	1	0.8878
SFA 9B 8.1	0.7011	0.8878	1
SFA 7B2.1	0.5033	0.4902	0.4837
SFA 7B3.1	0.4368	0.4762	0.4592
SFA 7B5.1	0.4350	0.4350	0.4294
SFA 7B8.1	0.3525	0.3436	0.3299
SFA 7B10.2	0.3448	0.3952	0.3780
SFA 7B11.1	0.3027	0.3265	0.3196
SFA 7B15.1	0.3295	0.3191	0.3014
SFA 7B19.1	0.3448	0.3265	0.3333
JCB 2B5.1.1	0.3716	0.3678	0.3640
JCB 2B29.1	0.4017	0.3932	0.3932
JCB 3B6.1	0.4528	0.4528	0.4591
JCB 3B8.2	0.3985	0.3901	0.3794
JCB 4B2.1	0.3678	0.3943	0.3835
JCB 4B3.1	0.4302	0.4070	0.4031
JCB 4B4.3	0.4225	0.3992	0.3953
JCB 4B40.7	0.4300	0.4493	0.4444
LD6 A7.6	0.4643	0.4563	0.4841
LD6 A12.2	0.4960	0.4722	0.5000
LD6 D9.1	0.5111	0.4978	0.5111

Heavy	SFA 7B2.1	SFA 7B3.1	SFA 7B5.1	SFA 7B8.1	SFA 7B10.2	SFA 7B11.1	SFA 7B15.1	SFA 7B19.1
SFA 2B1.1	0.4902	0.5352	0.4633	0.3662	0.3803	0.3052	0.3427	0.3474
SFA 2B3.1	0.4510	0.4667	0.4124	0.3125	0.3625	0.3042	0.3708	0.3292
SFA 2B7.2	0.5033	0.4658	0.3729	0.3470	0.3333	0.3105	0.3242	0.3607
SFA 2B8.1	0.4706	0.4338	0.3729	0.3516	0.2877	0.2922	0.3516	0.3653
SFA 3B2.1	0.4967	0.4399	0.4237	0.3711	0.3711	0.3574	0.3262	0.3540
SFA 3B3.1	0.5033	0.4430	0.4181	0.3333	0.3596	0.3377	0.3070	0.3465
SFA 3B7.1	0.5163	0.4778	0.4350	0.3481	0.4259	0.2963	0.3519	0.3630
SFA 3B10.1	0.4902	0.4694	0.4463	0.3608	0.3505	0.3196	0.3369	0.3469
SFA 3B12.1	0.4837	0.5507	0.4294	0.3913	0.4348	0.2995	0.4058	0.3865
SFA 3B14.1	0.3464	0.4433	0.3559	0.3230	0.3849	0.3505	0.3652	0.3540
SFA 6B1.1	0.4967	0.4718	0.4181	0.3385	0.3538	0.2615	0.3692	0.3385
SFA 6B4.2	0.6667	0.4787	0.5198	0.3652	0.3865	0.3050	0.3794	0.3794
SFA 6B5.1	0.6732	0.5385	0.5480	0.3547	0.4103	0.3034	0.3547	0.4103
SFA 7B1.2	0.6797	0.5402	0.5537	0.3410	0.3755	0.3218	0.3716	0.3985
SFA 7B7.1	0.6601	0.5556	0.5424	0.3333	0.3761	0.2991	0.3675	0.4103
SFA 9B3.1	0.5033	0.4368	0.4350	0.3525	0.3448	0.3027	0.3295	0.3448
SFA 9B6.1	0.4902	0.4762	0.4350	0.3436	0.3952	0.3265	0.3191	0.3265
SFA 9B 8.1	0.4837	0.4592	0.4294	0.3299	0.3780	0.3196	0.3014	0.3333
SFA 7B2.1	1	0.6275	0.5490	0.4444	0.4510	0.2484	0.4248	0.4118
SFA 7B3.1	0.6275	1	0.4972	0.3986	0.4227	0.3196	0.3546	0.3878
SFA 7B5.1	0.5490	0.4972	1	0.4068	0.4520	0.2994	0.3446	0.3729
SFA 7B8.1	0.4444	0.3986	0.4068	1	0.4192	0.3230	0.3333	0.3677
SFA 7B10.2	0.4510	0.4227	0.4520	0.4192	1	0.4639	0.3830	0.4399
SFA 7B11.1	0.2484	0.3196	0.2994	0.3230	0.4639	1	0.3688	0.3299
SFA 7B15.1	0.4248	0.3546	0.3446	0.3333	0.3830	0.3688	1	0.5000
SFA 7B19.1	0.4118	0.3878	0.3729	0.3677	0.4399	0.3299	0.5000	1
JCB 2B5.1.1	0.4248	0.3602	0.4068	0.2874	0.2797	0.2797	0.3487	0.3448
JCB 2B29.1	0.4379	0.3761	0.4237	0.3333	0.3675	0.3504	0.3761	0.4145
JCB 3B6.1	0.4771	0.4151	0.4151	0.3333	0.3396	0.2642	0.4025	0.4088
JCB 3B8.2	0.4902	0.3865	0.3898	0.2908	0.3085	0.3298	0.3511	0.3582
JCB 4B2.1	0.4510	0.4194	0.3955	0.2867	0.3333	0.3118	0.3978	0.3835
JCB 4B3.1	0.4575	0.3953	0.4294	0.3062	0.3721	0.2868	0.3682	0.3992
JCB 4B4.3	0.4771	0.4496	0.4350	0.3062	0.3876	0.3101	0.3411	0.3953
JCB 4B40.7	0.4967	0.4493	0.4124	0.3816	0.4444	0.3043	0.3527	0.3623
LD6 A7.6	0.4837	0.4286	0.3955	0.3294	0.4087	0.3254	0.3373	0.3571
LD6 A12.2	0.4967	0.4167	0.4124	0.3413	0.3810	0.3175	0.3373	0.3690
LD6 D9.1	0.4771	0.4222	0.4011	0.3378	0.4089	0.3200	0.3511	0.3511

Heavy	JCB 2B5.1.1	JCB 2B29.1	JCB 3B6.1	JCB 3B8.2	JCB 4B2.1	JCB 4B3.1	JCB 4B4.3	JCB 4B40.7	LD6 A7.6	LD6 A12.2	LD6 D9.1
SFA 2B1.1	0.4319	0.4413	0.4403	0.3944	0.4272	0.4648	0.4507	0.4493	0.5305	0.5258	0.5352
SFA 2B3.1	0.4000	0.4017	0.4088	0.3875	0.4125	0.4042	0.4167	0.3720	0.4583	0.4292	0.4356
SFA 2B7.2	0.3836	0.4475	0.4591	0.4384	0.4338	0.4886	0.4612	0.4155	0.4384	0.4429	0.4429
SFA 2B8.1	0.4064	0.4292	0.4528	0.4429	0.4338	0.4292	0.4429	0.3816	0.4247	0.4384	0.4155
SFA 3B2.1	0.4023	0.4402	0.4591	0.4291	0.4265	0.3953	0.4147	0.3913	0.4683	0.4841	0.4667
SFA 3B3.1	0.3947	0.4298	0.4465	0.3728	0.3991	0.4035	0.4298	0.3913	0.4737	0.4868	0.4533
SFA 3B7.1	0.4176	0.4188	0.5975	0.5074	0.4630	0.5620	0.5969	0.5749	0.5754	0.5754	0.6044
SFA 3B10.1	0.4138	0.4359	0.4591	0.3901	0.4516	0.4419	0.4302	0.4300	0.5119	0.5357	0.5022
SFA 3B12.1	0.5024	0.4493	0.5786	0.5217	0.5459	0.6522	0.6425	0.6329	0.6087	0.6039	0.6280
SFA 3B14.1	0.3142	0.3547	0.3522	0.3121	0.3548	0.3721	0.3682	0.3382	0.4048	0.4087	0.4311
SFA 6B1.1	0.4051	0.4103	0.4465	0.4154	0.4359	0.4513	0.4513	0.4513	0.5179	0.5231	0.5282
SFA 6B4.2	0.3640	0.3889	0.4528	0.4007	0.4122	0.3915	0.3915	0.4348	0.3929	0.4008	0.3956
SFA 6B5.1	0.3675	0.4274	0.4403	0.3803	0.3590	0.4060	0.4017	0.4396	0.4231	0.4274	0.4133
SFA 7B1.2	0.3678	0.4231	0.4340	0.3946	0.4023	0.3915	0.4070	0.3816	0.4603	0.4444	0.4311
SFA 7B7.1	0.3675	0.4188	0.4340	0.3932	0.3889	0.3846	0.3974	0.3671	0.4487	0.4359	0.4133
SFA 9B3.1	0.3716	0.4017	0.4528	0.3985	0.3678	0.4302	0.4225	0.4300	0.4643	0.4960	0.5111
SFA 9B6.1	0.3678	0.3932	0.4528	0.3901	0.3943	0.4070	0.3992	0.4493	0.4563	0.4722	0.4978
SFA 9B 8.1	0.3640	0.3932	0.4591	0.3794	0.3835	0.4031	0.3953	0.4444	0.4841	0.5000	0.5111
SFA 7B2.1	0.4248	0.4379	0.4771	0.4902	0.4510	0.4575	0.4771	0.4967	0.4837	0.4967	0.4771
SFA 7B3.1	0.3602	0.3761	0.4151	0.3865	0.4194	0.3953	0.4496	0.4493	0.4286	0.4167	0.4222
SFA 7B5.1	0.4068	0.4237	0.4151	0.3898	0.3955	0.4294	0.4350	0.4124	0.3955	0.4124	0.4011
SFA 7B8.1	0.2874	0.3333	0.3333	0.2908	0.2867	0.3062	0.3062	0.3816	0.3294	0.3413	0.3378
SFA 7B10.2	0.2797	0.3675	0.3396	0.3085	0.3333	0.3721	0.3876	0.4444	0.4087	0.3810	0.4089
SFA 7B11.1	0.2797	0.3504	0.2642	0.3298	0.3118	0.2868	0.3101	0.3043	0.3254	0.3175	0.3200
SFA 7B15.1	0.3487	0.3761	0.4025	0.3511	0.3978	0.3682	0.3411	0.3527	0.3373	0.3373	0.3511
SFA 7B19.1	0.3448	0.4145	0.4088	0.3582	0.3835	0.3992	0.3953	0.3623	0.3571	0.3690	0.3511
JCB 2B5.1.1	1	0.4103	0.7107	0.5977	0.5479	0.4574	0.4612	0.4686	0.4960	0.5079	0.5289
JCB 2B29.1	0.4103	1	0.4780	0.4274	0.4359	0.4402	0.4017	0.4300	0.4231	0.4316	0.4178
JCB 3B6.1	0.7107	0.4780	1	0.9245	0.6981	0.6478	0.6289	0.5535	0.6855	0.7107	0.6855
JCB 3B8.2	0.5977	0.4274	0.9245	1	0.5556	0.5465	0.5426	0.5024	0.5437	0.5556	0.5556
JCB 4B2.1	0.5479	0.4359	0.6981	0.5556	1	0.5349	0.5581	0.4783	0.5238	0.5278	0.5156
JCB 4B3.1	0.4574	0.4402	0.6478	0.5465	0.5349	1	0.8256	0.5700	0.5159	0.5119	0.5422
JCB 4B4.3	0.4612	0.4017	0.6289	0.5426	0.5581	0.8256	1	0.5845	0.5317	0.5278	0.5422
JCB 4B40.7	0.4686	0.4300	0.5535	0.5024	0.4783	0.5700	0.5845	1	0.4928	0.5121	0.5121
LD6 A7.6	0.4960	0.4231	0.6855	0.5437	0.5238	0.5159	0.5317	0.4928	1	0.9048	0.9333
LD6 A12.2	0.5079	0.4316	0.7107	0.5556	0.5278	0.5119	0.5278	0.5121	0.9048	1	0.8800
LD6 D9.1	0.5289	0.4178	0.6855	0.5556	0.5156	0.5422	0.5422	0.5121	0.9333	0.8800	1

Nucleotide sequence comparison of immunoglobulin light chain V regions

Tables are arranged by donor and specificity

A value of 1 represents complete identity

Light	SFA 2B1.1	SFA 2B3.1	SFA 2B7.2	SFA 2B8.1
SFA 2B1.1	1	0.7474	0.9736	0.6175
SFA 2B3.1	0.7474	1	0.7474	0.6351
SFA 2B7.2	0.9736	0.7474	1	0.6386
SFA 2B8.1	0.6175	0.6351	0.6386	1
SFA 3B2.1	0.6246	0.6421	0.6456	0.9509
SFA 3B3.1	0.6070	0.6316	0.6281	0.9404
SFA 3B7.1	0.7298	0.7965	0.7404	0.6105
SFA 3B10.1	0.6316	0.6386	0.6526	0.9684
SFA 3B12.1	0.7368	0.7860	0.7439	0.5930
SFA 3B14.1	0.6140	0.6386	0.6351	0.9754
SFA 6B1.1	0.6094	0.6456	0.6263	0.6211
SFA 6B4.2	0.7368	0.9368	0.7439	0.6105
SFA 6B5.1	0.7474	0.9123	0.7614	0.6351
SFA 7B1.2	0.7509	0.9509	0.7579	0.6211
SFA 7B7.1	0.7509	0.9509	0.7579	0.6211
SFA 9B3.1	0.7544	0.9579	0.7614	0.6246
SFA 9B6.1	0.6667	0.6737	0.6736	0.5965
SFA 9B 8.1	0.6246	0.6386	0.6456	0.9649
SFA 7B2.1	0.6433	0.6456	0.6433	0.6246
SFA 7B3.1	0.7439	0.9474	0.7509	0.6211
SFA 7B5.1	0.5703	0.5863	0.5863	0.5181
SFA 7B8.1	0.7404	0.9404	0.7474	0.6140
SFA 7B10.2	0.6281	0.6526	0.6386	0.7018
SFA 7B11.1	0.6157	0.6392	0.6353	0.6000
SFA 7B15.1	0.6498	0.6632	0.6667	0.6175
SFA 7B19.1	0.6211	0.6421	0.6316	0.7018
JCB 2B5.1.1	0.6400	0.6386	0.6367	0.6140
JCB 2B29.1	0.7404	0.9298	0.7474	0.6211
JCB 3B6.1	0.6300	0.6351	0.6300	0.6105
JCB 3B8.2	0.6367	0.6421	0.6367	0.6140
JCB 4B2.1	0.6300	0.6456	0.6300	0.6175
JCB 4B3.1	0.6600	0.6526	0.6600	0.6456
JCB 4B4.3	0.6367	0.6351	0.6333	0.6105
JCB 4B40.7	0.6367	0.6456	0.6367	0.6175
LD6 A7.6	0.6400	0.6421	0.6400	0.6140
LD6 A12.2	0.6300	0.6421	0.6300	0.6175
LD6 D9.1	0.6367	0.6421	0.6300	0.6070

Light	SFA 3B2.1	SFA 3B3.1	SFA 3B7.1	SFA 3B10.1	SFA 3B12.1	SFA 3B14.1
SFA 2B1.1	0.6246	0.6070	0.7298	0.6316	0.7368	0.6140
SFA 2B3.1	0.6421	0.6316	0.7965	0.6386	0.7860	0.6386
SFA 2B7.2	0.6456	0.6281	0.7404	0.6526	0.7439	0.6351
SFA 2B8.1	0.9509	0.9404	0.6105	0.9684	0.5930	0.9754
SFA 3B2.1	1	0.9474	0.6070	0.9544	0.5930	0.9544
SFA 3B3.1	0.9474	1	0.6000	0.9439	0.5895	0.9439
SFA 3B7.1	0.6070	0.6000	1	0.6105	0.9544	0.6035
SFA 3B10.1	0.9544	0.9439	0.6105	1	0.5965	0.9719
SFA 3B12.1	0.5930	0.5895	0.9544	0.5965	1	0.5895
SFA 3B14.1	0.9544	0.9439	0.6035	0.9719	0.5895	1
SFA 6B1.1	0.6316	0.6140	0.6211	0.6211	0.6281	0.6246
SFA 6B4.2	0.6246	0.6140	0.8000	0.6211	0.7965	0.6175
SFA 6B5.1	0.6421	0.6351	0.8140	0.6456	0.8175	0.6421
SFA 7B1.2	0.6351	0.6211	0.8105	0.6281	0.8105	0.6281
SFA 7B7.1	0.6351	0.6211	0.8105	0.6281	0.8105	0.6281
SFA 9B3.1	0.6386	0.6281	0.8175	0.6316	0.8140	0.6316
SFA 9B6.1	0.6000	0.5825	0.6351	0.6035	0.6456	0.6000
SFA 9B 8.1	0.9579	0.9474	0.6105	0.9614	0.5930	0.9614
SFA 7B2.1	0.6281	0.6140	0.6421	0.6316	0.6281	0.6246
SFA 7B3.1	0.6281	0.6246	0.8140	0.6281	0.8140	0.6316
SFA 7B5.1	0.5181	0.5100	0.5542	0.5060	0.5542	0.5060
SFA 7B8.1	0.6281	0.6140	0.8000	0.6175	0.8000	0.6211
SFA 7B10.2	0.7018	0.6842	0.5965	0.6947	0.5860	0.7018
SFA 7B11.1	0.6000	0.5922	0.6235	0.6000	0.6275	0.6000
SFA 7B15.1	0.6175	0.6175	0.6737	0.6175	0.6737	0.6175
SFA 7B19.1	0.7018	0.6842	0.5895	0.6947	0.5789	0.7018
JCB 2B5.1.1	0.6211	0.6070	0.6316	0.6246	0.6456	0.6175
JCB 2B29.1	0.6281	0.6175	0.8175	0.6246	0.8175	0.6246
JCB 3B6.1	0.6211	0.6140	0.6281	0.6246	0.6351	0.6175
JCB 3B8.2	0.6316	0.6175	0.6386	0.6281	0.6351	0.6211
JCB 4B2.1	0.6281	0.6140	0.6316	0.6316	0.6211	0.6246
JCB 4B3.1	0.6491	0.6351	0.6456	0.6526	0.6351	0.6456
JCB 4B4.3	0.6281	0.6140	0.6386	0.6246	0.6281	0.6175
JCB 4B40.7	0.6281	0.6140	0.6351	0.6316	0.6246	0.6246
LD6 A7.6	0.6316	0.6211	0.6526	0.6281	0.6421	0.6211
LD6 A12.2	0.6281	0.6175	0.6421	0.6316	0.6316	0.6246
LD6 D9.1	0.6246	0.6211	0.6386	0.6211	0.6316	0.6140

Light	SFA 6B1.1	SFA 6B4.2	SFA 6B5.1
SFA 2B1.1	0.6094	0.7368	0.7474
SFA 2B3.1	0.6456	0.9368	0.9123
SFA 2B7.2	0.6263	0.7439	0.7614
SFA 2B8.1	0.6211	0.6105	0.6351
SFA 3B2.1	0.6316	0.6246	0.6421
SFA 3B3.1	0.6140	0.6140	0.6351
SFA 3B7.1	0.6211	0.8000	0.8140
SFA 3B10.1	0.6211	0.6211	0.6456
SFA 3B12.1	0.6281	0.7965	0.8175
SFA 3B14.1	0.6246	0.6175	0.6421
SFA 6B1.1	1	0.6281	0.6246
SFA 6B4.2	0.6281	1	0.9368
SFA 6B5.1	0.6246	0.9368	1
SFA 7B1.2	0.6351	0.9684	0.9404
SFA 7B7.1	0.6351	0.9684	0.9404
SFA 9B3.1	0.6351	0.9684	0.9404
SFA 9B6.1	0.6493	0.6421	0.6421
SFA 9B 8.1	0.6211	0.6140	0.6386
SFA 7B2.1	0.6330	0.6456	0.6351
SFA 7B3.1	0.6351	0.9649	0.9298
SFA 7B5.1	0.7751	0.5783	0.5703
SFA 7B8.1	0.6316	0.9614	0.9263
SFA 7B10.2	0.6351	0.6246	0.6456
SFA 7B11.1	0.9686	0.6235	0.6157
SFA 7B15.1	0.8552	0.6561	0.6526
SFA 7B19.1	0.6281	0.6211	0.6351
JCB 2B5.1.1	0.6330	0.6386	0.6281
JCB 2B29.1	0.6561	0.9404	0.9123
JCB 3B6.1	0.6364	0.6456	0.6351
JCB 3B8.2	0.6296	0.6456	0.6351
JCB 4B2.1	0.6263	0.6456	0.6351
JCB 4B3.1	0.6263	0.6526	0.6421
JCB 4B4.3	0.6263	0.6386	0.6281
JCB 4B40.7	0.6296	0.6491	0.6386
LD6 A7.6	0.6296	0.6456	0.6351
LD6 A12.2	0.6364	0.6456	0.6351
LD6 D9.1	0.6330	0.6491	0.6316

Light	SFA 7B1.2	SFA 7B7.1
SFA 2B1.1	0.7509	0.7509
SFA 2B3.1	0.9509	0.9509
SFA 2B7.2	0.7579	0.7579
SFA 2B8.1	0.6211	0.6211
SFA 3B2.1	0.6351	0.6351
SFA 3B3.1	0.6211	0.6211
SFA 3B7.1	0.8105	0.8105
SFA 3B10.1	0.6281	0.6281
SFA 3B12.1	0.8105	0.8105
SFA 3B14.1	0.6281	0.6281
SFA 6B1.1	0.6351	0.6351
SFA 6B4.2	0.9684	0.9684
SFA 6B5.1	0.9404	0.9404
SFA 7B1.2	1	1
SFA 7B7.1	1	1
SFA 9B3.1	0.9860	0.9860
SFA 9B6.1	0.6526	0.6526
SFA 9B 8.1	0.6246	0.6246
SFA 7B2.1	0.6421	0.6421
SFA 7B3.1	0.9754	0.9754
SFA 7B5.1	0.5823	0.5823
SFA 7B8.1	0.9860	0.9860
SFA 7B10.2	0.6421	0.6421
SFA 7B11.1	0.6275	0.6275
SFA 7B15.1	0.6632	0.6632
SFA 7B19.1	0.6316	0.6316
JCB 2B5.1.1	0.6351	0.6351
JCB 2B29.1	0.9649	0.9649
JCB 3B6.1	0.6351	0.6351
JCB 3B8.2	0.6421	0.6421
JCB 4B2.1	0.6386	0.6386
JCB 4B3.1	0.6491	0.6491
JCB 4B4.3	0.6316	0.6316
JCB 4B40.7	0.6421	0.6421
LD6 A7.6	0.6421	0.6421
LD6 A12.2	0.6421	0.6421
LD6 D9.1	0.6456	0.6456

Light	SFA 9B3.1	SFA 9B6.1	SFA 9B 8.1
SFA 2B1.1	0.7544	0.6667	0.6246
SFA 2B3.1	0.9579	0.6737	0.6386
SFA 2B7.2	0.7614	0.6736	0.6456
SFA 2B8.1	0.6246	0.5965	0.9649
SFA 3B2.1	0.6386	0.6000	0.9579
SFA 3B3.1	0.6281	0.5825	0.9474
SFA 3B7.1	0.8175	0.6351	0.6105
SFA 3B10.1	0.6316	0.6035	0.9614
SFA 3B12.1	0.8140	0.6456	0.5930
SFA 3B14.1	0.6316	0.6000	0.9614
SFA 6B1.1	0.6351	0.6493	0.6211
SFA 6B4.2	0.9684	0.6421	0.6140
SFA 6B5.1	0.9404	0.6421	0.6386
SFA 7B1.2	0.9860	0.6526	0.6246
SFA 7B7.1	0.9860	0.6526	0.6246
SFA 9B3.1	1	0.6526	0.6281
SFA 9B6.1	0.6526	1	0.6000
SFA 9B 8.1	0.6281	0.6000	1
SFA 7B2.1	0.6456	0.6181	0.6246
SFA 7B3.1	0.9825	0.6491	0.6246
SFA 7B5.1	0.5823	0.5542	0.5181
SFA 7B8.1	0.9719	0.6491	0.6175
SFA 7B10.2	0.6421	0.6035	0.6877
SFA 7B11.1	0.6275	0.6627	0.6039
SFA 7B15.1	0.6632	0.6597	0.6140
SFA 7B19.1	0.6316	0.5965	0.6877
JCB 2B5.1.1	0.6386	0.6354	0.6140
JCB 2B29.1	0.9614	0.6667	0.6246
JCB 3B6.1	0.6386	0.6319	0.6105
JCB 3B8.2	0.6456	0.6146	0.6140
JCB 4B2.1	0.6421	0.6146	0.6175
JCB 4B3.1	0.6526	0.6042	0.6421
JCB 4B4.3	0.6351	0.6042	0.6105
JCB 4B40.7	0.6456	0.6076	0.6175
LD6 A7.6	0.6456	0.6076	0.6140
LD6 A12.2	0.6456	0.6250	0.6175
LD6 D9.1	0.6491	0.6111	0.6070

Light	SFA 7B2.1	SFA 7B3.1	SFA 7B5.1	SFA 7B8.1	SFA 7B10.2	SFA 7B11.1	SFA 7B15.1	SFA 7B19.1
SFA 2B1.1	0.6433	0.7439	0.5703	0.7404	0.6281	0.6157	0.6498	0.6211
SFA 2B3.1	0.6456	0.9474	0.5863	0.9404	0.6526	0.6392	0.6632	0.6421
SFA 2B7.2	0.6433	0.7509	0.5863	0.7474	0.6386	0.6353	0.6667	0.6316
SFA 2B8.1	0.6246	0.6211	0.5181	0.6140	0.7018	0.6000	0.6175	0.7018
SFA 3B2.1	0.6281	0.6281	0.5181	0.6281	0.7018	0.6000	0.6175	0.7018
SFA 3B3.1	0.6140	0.6246	0.5100	0.6140	0.6842	0.5922	0.6175	0.6842
SFA 3B7.1	0.6421	0.8140	0.5542	0.8000	0.5965	0.6235	0.6737	0.5895
SFA 3B10.1	0.6316	0.6281	0.5060	0.6175	0.6947	0.6000	0.6175	0.6947
SFA 3B12.1	0.6281	0.8140	0.5542	0.8000	0.5860	0.6275	0.6737	0.5789
SFA 3B14.1	0.6246	0.6316	0.5060	0.6211	0.7018	0.6000	0.6175	0.7018
SFA 6B1.1	0.6330	0.6351	0.7751	0.6316	0.6351	0.9686	0.8552	0.6281
SFA 6B4.2	0.6456	0.9649	0.5783	0.9614	0.6246	0.6235	0.6561	0.6211
SFA 6B5.1	0.6351	0.9298	0.5703	0.9263	0.6456	0.6157	0.6526	0.6351
SFA 7B1.2	0.6421	0.9754	0.5823	0.9860	0.6421	0.6275	0.6632	0.6316
SFA 7B7.1	0.6421	0.9754	0.5823	0.9860	0.6421	0.6275	0.6632	0.6316
SFA 9B3.1	0.6456	0.9825	0.5823	0.9719	0.6421	0.6275	0.6632	0.6316
SFA 9B6.1	0.6181	0.6491	0.5542	0.6491	0.6035	0.6627	0.6597	0.5965
SFA 9B 8.1	0.6246	0.6246	0.5181	0.6175	0.6877	0.6039	0.6140	0.6877
SFA 7B2.1	1	0.6386	0.6024	0.6421	0.6175	0.6627	0.6566	0.6070
SFA 7B3.1	0.6386	1	0.5863	0.9684	0.6456	0.6392	0.6632	0.6421
SFA 7B5.1	0.6024	0.5863	1	0.5823	0.5261	0.8193	0.7952	0.5301
SFA 7B8.1	0.6421	0.9684	0.5823	1	0.6351	0.6275	0.6596	0.6351
SFA 7B10.2	0.6175	0.6456	0.5261	0.6351	1	0.5961	0.6211	0.9895
SFA 7B11.1	0.6627	0.6392	0.8193	0.6275	0.5961	1	0.8784	0.6000
SFA 7B15.1	0.6566	0.6632	0.7952	0.6596	0.6211	0.8784	1	0.6175
SFA 7B19.1	0.6070	0.6421	0.5301	0.6351	0.9895	0.6000	0.6175	1
JCB 2B5.1.1	0.9800	0.6316	0.5904	0.6386	0.6035	0.6549	0.6532	0.5930
JCB 2B29.1	0.6526	0.9544	0.5904	0.9509	0.6561	0.6471	0.6772	0.6456
JCB 3B6.1	0.9733	0.6351	0.6064	0.6386	0.6175	0.6627	0.6667	0.6070
JCB 3B8.2	0.9867	0.6386	0.5984	0.6421	0.6140	0.6588	0.6498	0.6035
JCB 4B2.1	0.9867	0.6351	0.5904	0.6386	0.6140	0.6549	0.6465	0.6035
JCB 4B3.1	0.9600	0.6456	0.5984	0.6456	0.6211	0.6510	0.6532	0.6105
JCB 4B4.3	0.9767	0.6281	0.6064	0.6316	0.6105	0.6549	0.6498	0.6000
JCB 4B40.7	0.9800	0.6386	0.5944	0.6421	0.6140	0.6588	0.6465	0.6035
LD6 A7.6	0.9467	0.6386	0.5663	0.6386	0.6211	0.6510	0.6296	0.6105
LD6 A12.2	0.9833	0.6386	0.5863	0.6421	0.6140	0.6667	0.6498	0.6035
LD6 D9.1	0.9667	0.6421	0.5944	0.6456	0.6140	0.6627	0.6599	0.6035

Light	JCB 2B5.1.1	JCB 2B29.1	JCB 3B6.1	JCB 3B8.2	JCB 4B2.1	JCB 4B3.1	JCB 4B4.3	JCB 4B40.7	LD6 A7.6	LD6 A12.2	LD6 D9.1
SFA 2B1.1	0.6400	0.7404	0.6300	0.6367	0.6300	0.6600	0.6367	0.6367	0.6400	0.6300	0.6367
SFA 2B3.1	0.6386	0.9298	0.6351	0.6421	0.6456	0.6526	0.6351	0.6456	0.6421	0.6421	0.6421
SFA 2B7.2	0.6367	0.7474	0.6300	0.6367	0.6300	0.6600	0.6333	0.6367	0.6400	0.6300	0.6300
SFA 2B8.1	0.6140	0.6211	0.6105	0.6140	0.6175	0.6456	0.6105	0.6175	0.6140	0.6175	0.6070
SFA 3B2.1	0.6211	0.6281	0.6211	0.6316	0.6281	0.6491	0.6281	0.6281	0.6316	0.6281	0.6246
SFA 3B3.1	0.6070	0.6175	0.6140	0.6175	0.6140	0.6351	0.6140	0.6140	0.6211	0.6175	0.6211
SFA 3B7.1	0.6316	0.8175	0.6281	0.6386	0.6316	0.6456	0.6386	0.6351	0.6526	0.6421	0.6386
SFA 3B10.1	0.6246	0.6246	0.6246	0.6281	0.6316	0.6526	0.6246	0.6316	0.6281	0.6316	0.6211
SFA 3B12.1	0.6456	0.8175	0.6351	0.6351	0.6211	0.6351	0.6281	0.6246	0.6421	0.6316	0.6316
SFA 3B14.1	0.6175	0.6246	0.6175	0.6211	0.6246	0.6456	0.6175	0.6246	0.6211	0.6246	0.6140
SFA 6B1.1	0.6330	0.6561	0.6364	0.6296	0.6263	0.6263	0.6263	0.6296	0.6296	0.6364	0.6330
SFA 6B4.2	0.6386	0.9404	0.6456	0.6456	0.6456	0.6526	0.6386	0.6491	0.6456	0.6456	0.6491
SFA 6B5.1	0.6281	0.9123	0.6351	0.6351	0.6351	0.6421	0.6281	0.6386	0.6351	0.6351	0.6316
SFA 7B1.2	0.6351	0.9649	0.6351	0.6421	0.6386	0.6491	0.6316	0.6421	0.6421	0.6421	0.6456
SFA 7B7.1	0.6351	0.9649	0.6351	0.6421	0.6386	0.6491	0.6316	0.6421	0.6421	0.6421	0.6456
SFA 9B3.1	0.6386	0.9614	0.6386	0.6456	0.6421	0.6526	0.6351	0.6456	0.6456	0.6456	0.6491
SFA 9B6.1	0.6354	0.6667	0.6319	0.6146	0.6146	0.6042	0.6042	0.6076	0.6076	0.6250	0.6111
SFA 9B 8.1	0.6140	0.6246	0.6105	0.6140	0.6175	0.6421	0.6105	0.6175	0.6140	0.6175	0.6070
SFA 7B2.1	0.9800	0.6526	0.9733	0.9867	0.9867	0.9600	0.9767	0.9800	0.9467	0.9833	0.9667
SFA 7B3.1	0.6316	0.9544	0.6351	0.6386	0.6351	0.6456	0.6281	0.6386	0.6386	0.6386	0.6421
SFA 7B5.1	0.5904	0.5904	0.6064	0.5984	0.5904	0.5984	0.6064	0.5944	0.5663	0.5863	0.5944
SFA 7B8.1	0.6386	0.9509	0.6386	0.6421	0.6386	0.6456	0.6316	0.6421	0.6386	0.6421	0.6456
SFA 7B10.2	0.6035	0.6561	0.6175	0.6140	0.6140	0.6211	0.6105	0.6140	0.6211	0.6140	0.6140
SFA 7B11.1	0.6549	0.6471	0.6627	0.6588	0.6549	0.6510	0.6549	0.6588	0.6510	0.6667	0.6627
SFA 7B15.1	0.6532	0.6772	0.6667	0.6498	0.6465	0.6532	0.6498	0.6465	0.6296	0.6498	0.6599
SFA 7B19.1	0.5930	0.6456	0.6070	0.6035	0.6035	0.6105	0.6000	0.6035	0.6105	0.6035	0.6035
JCB 2B5.1.1	1	0.6491	0.9633	0.9767	0.9700	0.9433	0.9600	0.9633	0.9300	0.9667	0.9500
JCB 2B29.1	0.6491	1	0.6421	0.6491	0.6456	0.6596	0.6386	0.6491	0.6526	0.6526	0.6561
JCB 3B6.1	0.9633	0.6421	1	0.9733	0.9700	0.9333	0.9600	0.9633	0.9300	0.9633	0.9533
JCB 3B8.2	0.9767	0.6491	0.9733	1	0.9800	0.9467	0.9767	0.9733	0.9467	0.9767	0.9667
JCB 4B2.1	0.9700	0.6456	0.9700	0.9800	1	0.9467	0.9733	0.9767	0.9467	0.9767	0.9600
JCB 4B3.1	0.9433	0.6596	0.9333	0.9467	0.9467	1	0.9367	0.9400	0.9200	0.9433	0.9267
JCB 4B4.3	0.9600	0.6386	0.9600	0.9767	0.9733	0.9367	1	0.9767	0.9433	0.9667	0.9600
JCB 4B40.7	0.9633	0.6491	0.9633	0.9733	0.9767	0.9400	0.9767	1	0.9433	0.9700	0.9533
LD6 A7.6	0.9300	0.6526	0.9300	0.9467	0.9467	0.9200	0.9433	0.9433	1	0.9633	0.9567
LD6 A12.2	0.9667	0.6526	0.9633	0.9767	0.9767	0.9433	0.9667	0.9700	0.9633	1	0.9833
LD6 D9.1	0.9500	0.6561	0.9533	0.9667	0.9600	0.9267	0.9600	0.9533	0.9567	0.9833	1