Human $CD4^+$ T Cell Recognition of Influenza A Haemagglutinin

by

Colin Malcolm Gelder M.B. B.S.

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Department of Immunology,

St Mary's Hospital Medical School,

Imperial College of Science, Technology and Medicine,

University of London.

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Abstract

$CD4^+$ T cell recognition of haemagglutinin from influenza A/Beijing/32/92 (H3N2)(HA Beij), has been examined in 3 groups of unrelated adults differing in MHC haplotype. Group 1, 12 donors naturally infected with influenza during November and December 1993, a time when A/Beijing/32/92 like strains circulated London. Group 2, 6 subjects with no history of influenza infection or vaccination during the preceding 4 years, but who made polyclonal T cell responses to HA. Group 3, 12 donors who were vaccinated with a trivalent influenza subunit vaccine containing HA Beij. Short term T cell lines were selected during 3 weeks *in vitro* with full length HA Beij. The specificity of $CD4^+$ T cells for HA epitopes was examined using a panel of 118 peptides spanning the entire sequence of HA Beij.

$CD4^+$ T cell HA recognition by all 3 groups of donors was dominated by responses to highly conserved regions of HA which have not been subject to frequent drift mutation, and resulted in cross reactive recognition of H1, H2 and H3 influenza A viruses. The HA2 subunit, previously widely regarded as non-immunogenic for T cells, induced strong responses in every donor. Following natural infection two conserved regions of HA (residues 303-323 and 407-442) were recognised by every donor in groups one and two. Despite the frequent recognition of these regions the powerful influence of MHC class II over epitope selection was clearly evident. In particular following natural infection one pair of unrelated donors expressing HLA-DR1, 11 and -DQ2, 5 alleles, but differing in age, nationality, and previous exposure to influenza A, recognised identical HA peptides; furthermore 6 unrelated individuals expressing HLA-DR7, 15 alleles recognised identical HA peptides following influenza subunit vaccination.

The relationship between the HA peptide recognition and relative MHC-HA peptide binding affinity has been explored and found to be complex.

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Abbreviations

Units of measure

s	second
min	minute
h	hour
d	day
wk	week
mo	month
yr	year
nM	nanomolar
mM	millimolar
M	molar
ml	millilitre
l	litre
ng	nanogram
µg	microgram
mg	milligram
g	gram
G	gravitational units
cpm	counts per minute

General abbreviations

APC	antigen presenting cells
Chap.	Chapter
CTL	cytotoxic T lymphocyte
DNA	Deoxyribonucleic Acid
Fig.	Figure
IFN	Interferon
MHC	Major Histocompatibility Complex
NK	Natural Killer
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cells
RNA	Ribonucleic acid
TCR	T cell antigen receptor
[3]TdR	[3]methyl thymidine

Special abbreviations

HA	haemagglutinin

Aichi	A/Aichi/68 (H3N2)
Beij	A/Beijing/32/92 (H3N2)
gm	geometric mean
HA Aichi	HA A/Aichi/68 (H3N2)
HA Beij	HA A/Beijing/32/92 (H3N2)
MTSE	*Mycobacterium tuberculosis* soluble extract.

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1.5 AIMS

1.1 Overview

Influenza A remains a major cause of human morbidity and mortality especially in the elderly (Ashley *et al.,* 1991). Antibodies, $CD8^+$ T cells, and $CD4^+$ T cells, all play important roles in host defence against influenza infection (reviewed by Mitchell *et al.,* 1985). This thesis describes a detailed investigation of human $CD4^+$ T cell recognition of influenza A haemagglutinin (HA) in different MHC haplotypes. HA is the viral surface coat glycoprotein responsible for virus-host-cell fusion (reviewed by Webster *et al.,* 1992), and the primary target of host neutralising antibodies (reviewed by Potter and Oxford 1979, Wiley *et al.,* 1981). Though human $CD4^+$ T cells are capable of recognising a range of influenza antigens (Lamb *et al.,* 1982), their response to HA is of particular importance because it is the primary component of the widely used HANA influenza subunit vaccines. $CD4^+$ T cells have a central role in the control of influenza infection, for example regulating the production of neutralising antibodies, immunoglobulin class switching, and affinity maturation (Burns *et al.,* 1975 , Anders *et al.,* 1979). In addition they amplify the $CD8^+$ T cell cytotoxic response (Biddison *et al.,* 1981), mainly directed towards internal viral proteins (reviewed by McMichael, 1994), that are generally highly conserved in structure amongst human influenza A viruses (reviewed by Smith and Palese, 1989). $CD4^+$ T cells may also participate in viral clearance more directly by the secretion of interferon γ (reviewed by Askonas, 1988). Surprisingly, until now, there had never been a systematic examination of $CD4^+$ T cell recognition of HA in the MHC-polymorphic human population, indeed the anti-influenza responses of only a single donor had been mapped in any detail (Lamb *et al.,* 1982b).

The remainder of this chapter is arranged into 4 sections: The first reviews antigen recognition by $CD4^+$ T cells, with a particular focus on MHC-peptide interactions;

the second outlines the biology of influenza A; the third reviews the specific immune response to influenza; and the final section lays out the aims of this thesis.

1.2 Antigen recognition by $CD4^+$ T cells

T cells recognise antigen in association with other cells

T cells recognise antigen in association with other cells. This discovery followed the observation by Mosier (1966) that depletion of macrophages by adherence resulted in poor B and T cells responses to antigen. It was later shown that T cell responses could be completely abolished by thorough T cell purification (Seeger and Oppenheim, 1970), that responses could be reconstructed by the addition of macrophages, but not macrophage conditioned medium, and that the degree of reconstruction was proportional to the degree of macrophage replacement (Waldron *et al.,* 1973).

Nature of antigen recognised by $CD4^+$ T cells

T cells antigen recognition is independent of tertiary protein structure (reviewed by Unanue, 1984). Detailed studies of guinea pig T cell recognition of insulin, and the murine recognition of cytochrome C demonstrated that T cell antigen responses are directed towards small fragments of a protein antigen, and that the response to the whole antigen could be reproduced with peptides (Rosenthal, *et al.,* 1977, Barcinski and Rosenthal 1977, Thomas *et al.,* 1981, Corradin and Chiller, 1979, Ultee *et al.,* 1980, Matis *et al.,* 1983).

T cell responses are MHC restricted

Evidence that T cell antigen recognition is MHC restricted came from 3 independent experimental systems: Firstly it was found that, T cells could only

provide help for antibody production if they shared MHC determinants with the B cells (Kindred and Shreffler, 1972, Katz *et al.,* 1973). Secondly, primed $CD4^+$ T cells responded well to PPD presented by autologous antigen presenting cells (APC) but not by MHC incompatible APC (Rosenthal and Shevach, 1973a, Shevach and Rosenthal, 1973b). Similar observations were made in the CTL response to haptens (Shearer, 1974), viruses (Zinkernagel and Doherty, 1974), or minor MHC antigens (Gordon *et al.,* 1975, Bevan *et al.,* 1976). Thirdly, anti-MHC antibodies blocked the interaction between APC and T cells (Shevach, *et al.,* 1974, Frelinger, *et al.,* 1975).

Antigen processing is required for T cell activation

Ziegler and Unanue (1981,1982) working with *Listeria monocytogenes* made 2 critical discoveries: Firstly, that if APC were pre-pulsed with antigen and then fixed after an interval, they retained their ability to present antigen to $CD4^+$ T cells.. Secondly, they discovered that treatment of APC with lysomotropic agents (e.g. ammonium chloride or chloroquine) completely abrogated their processing and presenting capacity. From these results they concluded that after an initial period all the signals necessary for T cell activation are displayed on the APC cell surface and that antigen processing involved proteolytic cleavage of foreign antigens. These results were confirmed in several other experimental systems (reviewed by Allen, 1987). It was then discovered that APC fixed before antigen exposure, though unable to present intact antigen, were able to present a tryptic digest of ovalbumin, indicating that T cells respond to actively processed peptide fragments of antigen (Shimonkevitz *et al.,* 1984). Similar results were obtained in other systems, though the amount of processing required varied considerably between antigens (reviewed by Allen, 1987).

T cell antigen receptor structure

The majority of T cells express an antigen receptor comprised of a 40-60 kD α chain and a 40-50 kD β chain, both of which have variable and constant regions (reviewed by Matis, 1990). The variable region of the α chain contains 102 residues, and is comprised of V (variable) J (junctional) and C (constant) segments encoded by separate genes. The 119 amino acid β chain also contains V, J, and C segments, plus an additional D (diversity) segment. The genes encoding these segments undergo somatic rearrangement in the thymus in a very similar fashion to immunoglobulin genes, generating a highly diverse repertoire of T cell receptors (reviewed by Hedrick and Eidelman, 1993)

The great majority of sequence variation amongst individual TCRs is located in 3 hypervariable domains within the variable region. Two of these domains, named CDR1 and CDR2, are encoded for by V segment genes, and the third, CDR3, is encoded in the α chain by V and J genes and in the β chain by V, J and D genes. The CDR3 domain is more diverse than the other hypervariable domains because of template independent addition, or loss of nucleotides at V-(D)-J junctions (Davis and Bjorkman,1988). The importance of the hypervariable domains was confirmed by studies which demonstrated that artificial mutations within them abolished T cell antigen recognition (Patten, *et al.,* 1993, White, *et al.,* 1993, Nalefski, *et al.,* 1992). By comparison with the structure of immunoglobulin it was proposed that all 3 CDR loops would interact with the MHC-peptide complex, but that the CDR3, loop would be the most important contact with the antigenic peptide (Chothia *et al.,* 1988, Davis *et al.*, 1988). Recent X-ray crystallography studies on the TCR β chain by Bentley and co-workers (1995) have supported this model of the αβ heterodimer's structure, and suggested that the antigen binding site of TCR consists of 4 regions that adopt loop structures, and form the

antigen-MHC interaction site. The CDR1 and CDR2 loops, contact the α-helices of the MHC molecule while, the hypervariable CDR3 loop interacts mainly with the peptide. The fourth MHC loop also appears to be involved in interaction with peptide (Bentley *et al.*, 1995).

The framework regions of the TCR variable region are similar to the corresponding regions of immunoglobulin (Novotny *et al.*, 1986, Bentley *et al.*,1995).

The C-regions of the α and β chains contain between 138 and 179 amino acids, and each consist of 4 functional domains encoded by separate exons. The amino distal domain of the C region probably forms a structure very similar to the Ig constant region domain. The second domain contains a cysteine residue which forms a linkage between the 2 chains The third domain is the transmembrane domain consisting of 20-24 amino acids. An unusual feature of this segment is the presence of charged amino acids which are critical for CD3 polypeptide interaction (see below). The carboxy terminal domains of both C regions contain only 5 to 12 residues, and do not participate in intracellular signalling (see below).

T cell activation and signal transduction

Antigen binding to the TCR initiates a series of intracellular signalling events which culminate in T cell activation. Signalling is initiated by the non-covalently associated CD3 complex and ζ chain, rather than the TCR itself. The former complex comprises a 25-28 kD γ chain, a 20kD δ chain and a 20 kD ε chain. 90% of TCR-CD3 complexes are associated with ζ chains homodimers, and 10% are associated with a heterodimer of one 16 kD ζ chain and one 22 kD η chain (Weiss, 1991). TCR and CD3 proteins are mutually dependant, cell lines lacking

any component of either complex do not express the other (Weiss and Littman, 1994).

Genes encoding the highly homologous γ,δ, ε chains are all located on human chromosome 11. The γ, δ, and ε chains consists of an N-terminal extracellular Ig like domain, a short connecting peptide, a transmembrane segment, and a cytoplasmic tail. Their transmembrane segments contain a negatively charged residue which is absolutely required for association with the TCR. Their cytoplasmic domains contain between 44 and 81 residues, and each includes a sequence of 17 amino acids, termed the antigen recognition motif (ARM), in which the motif tyr-X-X-leu is repeated twice (Samelson *et al.*, 1992, Weiss, 1993). The ζ and η chains which are encoded by alternatively spliced transcripts of a gene located on human chromosome 1, and differ only in their long cytoplasmic tails (113 and 155 amino acids respectively). The cytoplasmic tail of the ζ chain contains 3 ARMs. Their extracellular domains are short containing only 9 amino acids, and like the CD3 peptides their transmembrane domains include a negatively charged amino acid.

The interaction between protein tyrosine kinases (PTK) and the ARAMs of CD3 or the ζ chain results in tyrosine-phosphorylation of several intracellular proteins (reviewed in Weiss and Littman, 1994). There are 2 classes of PTKs: the Src family, and the Syc family (Chan *et al.*, 1992). Lck is the most important member of the Src family (Straus *et al.*, 1992, Molina *et al.*, 1992), and its function is modulated by dephosphorylation of a regulatory sequence by CD45 (Weiss *et al.*, 1994). Zap-70 is the most important member of the Syc family. This molecule does not associate with the TCR in its resting state, but it is rapidly recruited to the CD3-ζ chain complex following ARAM phosphorylation by Src PKCs (Chan *et al.*, 1992). Zap-70 activation leads to phosphorylation and stimulation of the γ1

isoform of phospholipase C, which results in increased phospholipid hydrolysis and the production of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Imboden *et al.*, 1985). These induce a rapid and sustained rise in intracellular free Ca^{2+} , and the activation of protein kinase C. These events trigger the activation of nuclear transcription factors and the promotion of nuclear transcription of several genes including IL-2 (reviewed in Weiss *et al.*, 1994).

Role of co-stimulatory molecules in CD4$^+$ T cell activation

T cell activation is greatly potentiated by co-stimulation of CD4 or CD8 molecules. At the same time that TCR binds to the MHC-peptide complex, CD4 binds to the β2 segment of MHC class II molecule, which leads to the formation of a stable ternary complex (Cammarota *et al.*, 1992). The cytoplasmic tail of CD4$^+$ associates with Lck (Glaichenhaus *et al.*, 1991), and amplifies signal transduction by as much as 100-fold (Janeway and Bottomly, 1994).

Numerous other membrane proteins also promote cell adhesion, and influence antigen recognition (reviewed in Collins *et al.*, 1994). Some, such as CD2 and LFA-1, are capable of transmitting intracellular signals which either enhance TCR-initiated signalling process or stimulate other, TCR-CD3 independent, pathways (Wacholtz *et al.*, 1989, Bierer *et al.*, 1993). The most important co-stimulatory pathway for IL-2 production and clonal expansion, is believed to be the interaction of CD28 with B7 proteins (B7-1 and B7-2) on the surface of antigen presenting cells (reviewed by Allison, 1994).

1.3 MHC

Introduction

In 1909 Tyzzer demonstrated that experimental tumour susceptibility in mice was not inherited in a simple mendelian fashion. In 1914 Little proposed his "Genetic Theory Of Tumour Transplantation" which postulated that tumour susceptibility was a polygenetic trait. Little and Tyzzer then experimentally verified this theory by performing large outbreeding experiments. These indicated that 14-16 genes controlled tumour susceptibility, though their function remained obscure (reviewed by Klein 1975).

In 1933 Haldane suggested that tumour immunity was directed against alloantigens rather than tumour specific antigens, and predicted the existence of antigenic differences between individuals which were similar to blood groups (reviewed by Klein 1975).

During the 1930s Gorer confirmed the presence of these alloantigens, and demonstrated that tumour susceptibility segregated to a minimum of 4 groups of genes. Mice that rejected tumours developed allo-antibodies which shared specificity with his blood group defining sera. These findings showed that tumour susceptibility and alloantigen genes were identical, and demonstrated the immunological basis of experimental tumour rejection (reviewed by Klein, 1975). These observations were extended to non-malignant transplant rejection by a number of workers most notably by Medawar (reviewed by Klein, 1975).

In 1948 Snell named the genes responsible for transplant rejection the "Histocompatibility Antigens", and started to breed mice strains differing only in these genes (H genes). He eventually produced several dozen congenic lines. These were very similar to their originating strains - one notable exception being the A.CA line which carried the fused tail gene. As the localisation of this gene was known its close linkage to the histocompatibility genes allowed their

localisation to linkage group IX. Snell also observed that tumour resistance genes varied in strength (reviewed by Klein, 1975).

In 1956 Counce demonstrated that the H-2 gene was by far the strongest histocompatibility gene, and it was subsequently named the major histocompatibility complex. Snell continuing his genetic studies and Gorer using refined serological techniques then demonstrated that the enormous complexity of the H-2 system. This was confirmed by the demonstration of crossover events within the H-2 locus. Analysis of these recombinants revealed that the H-2 locus comprised several regions designated D, C, V, E, A, K (reviewed by Klein, 1975).

Studies on the human MHC started later than the murine studies detailed above and followed the advent of allogeneic blood transfusion and organ donation. It was noted that patients who rejected kidneys or had transfusion reactions often developed circulating antibodies (Dausset, 1959). In the presence of complement these sera would lyse lymphocytes from some but not all third parties. Panels of antisera were set up and the antigens recognised by these sera became known as the Human Leukocyte Antigens (HLAs). Family studies were used to map these genes. The first 3 genes defined by purely serological approaches were designated HLA-A, HLA-B, HLA-C. The second region of MHC genes to be mapped was identified initially by their capacity to stimulate allogenic cells in the mixed lymphocyte reaction (MLR). This region was named the D region and genes within it were named were named HLA-DR, -DP, and -DQ. The diversity of human MHC genes was subsequently found to be far greater than in the mouse (reviewed by Hansen *et al.*, 1993).

Immune response genes

In a series of elegant experiment McDevitt and colleagues demonstrated that the murine response to branched multichain synthetic polypeptides was a

quantitative genetic trait (McDevitt and Sela, 1965). By performing a series of backcross experiments they firmly linked Ir genes into the MHC (McDevitt and Tynan, 1968, McDevitt and Chintz, 1969). This important discovery lead to 3 theories of T cell immune recognition. The first proposed that the immune response genes coded for T cell antigen receptors. The second postulated that separate receptors for MHC gene products and antigen existed on antigen presenting cells. The third proposed that T cells recognise "altered self" which comprised a combination of MHC and antigen.

The first theory was disproved by a series of bone marrow chimera experiments examining the response to antigens under Ir gene control. In this system responder status was not dependant on the genotype of the T cells, but on the genotype of the background in which they had developed and been exposed to antigen (Longo and Schwartz, 1980, Singer, *et al.* 1981).

Strong evidence in support of a single receptor model of T cell antigen was provided by an experiment in which 2 T cell hybridomas with different antigen and MHC specificites were fused: If the 2 receptor model of antigen recognition was correct new specificites might be expected, however the new hybridoma showed only the antigen-MHC patterns of its parent cell lines (Kappler *et al.*, 1981). Schwartz and co-workers (Heber-Katz *et al.*, 1983) examining the murine response to cytochrome C provided additional evidence for the single receptor model of antigen recognition. They were able to identify T cells specific for pigeon cytochrome C that also responded to moth cytochrome C presented by cells with the Ea^kEb^k class II background. These cells also responded to moth cytochrome C presented on an Ea^kEb^b background, but failed to respond to pigeon cytocrome presented by cells with this background. The best explanation for these results was that the Ea^kEb^b molecule could interact with appropriately only

moth but not pigeon cytocrome, and that Ea^kEb^k could interact with both forms of cytocrome (Racioppi *et al.,* 1991).

Rock and Benacerraf (1983) then demonstrated that 2 antigens recognised by 2 different T cell clones on the same MHC background could interfere with each others presentation.

Evidence that MHC interacted directly with peptide then came from Babbitt and co-workers (Babbitt *et al.,* 1985) who used synthetic peptide antigen and immunoaffinity purified class II MHC molecules to show direct allele-specific binding of the isolated components (Babbitt *et al.,* 1985). These findings were confirmed by Buus and co-workers who additionally demonstrated that the antigen-class II complexes were remarkable stable (Buus *et al.,* 1986).

Immunodominance

A protein antigen contains a large number of potential T cell epitopes. Usually however T cell responses are focused upon only a few "immunodominant" epitopes (Solinger *et al.*, 1979 , Hurwitz *et al.*, 1984). Several mechanisms contribute to this phenomenon, including antigen processing (Adorni *et al.,* 1988, Brett *et al.,* 1988), the ability of processed peptide to bind MHC class II molecules (Buus *et al.,* 1986), competition amongst peptides for MHC binding (Babbitt *et al.,* 1986, Buus *et al.,* 1987), the presence an appropriate repertoire of TCR (Schaeffer *et al.,* 1989), and the presence of a T cell repertoire capable of recognising the MHC-peptide complex (Vidovic and Matzinger, 1988). Failure of MHC molecules to bind peptides is an important reason for the failure of T cell recognition of an antigen, and a clear relationship between immunodominance and MHC class II binding affinity has been reported (Liu *et al.,* 1993, Nikcevich *et al.,* 1994).

Genetics of MHC

The human MHC is large and highly polymorphic, extending over 3500Kb of DNA on the short arm of chromosome 6. Within each HLA locus many genes exist in alternative forms (alleles). Because of this complex polymorphism most individuals are heterozygous at the great majority of their HLA loci. Because recombination frequency within the MHC is relatively small, distinguishable alleles at different HLA-linked loci usually segregate together as a haplotype. The location of many loci within the human MHC complex was established using large family studies, before the application of molecular genetic techniques and pulsed field electrophoresis defined the physical localisation of loci within the HLA complex (Trowsdale *et al.*, 1991). The human MHC contains 3 important regions, the MHC class I region which contains HLA-A, -B, -C; the MHC class II region which includes the HLA-DR (and -DRW), -DQ, -DP, and -DM, as well as TAP and LMP; and the class III region which encodes various complement proteins, as well as TNF and HSP70. The MHC also contains many genes (and pseudogenes) whose function remains unclear (reviewed by Hansen *et al.*, 1993). HLA-DR, -DRW, -DQ and -DP present antigen to $CD4^+$ T cells. The relative surface expression of these proteins varies between cell types. HLA-DQ and DP have variable α and β chains, while only the HLA-DRβ chain is variable. Thus while an individual normally expresses only 2 forms of HLA-DR, they have the potential to express 4 functional forms of both HLA-DQ and -DP.

The 1994 listing of HLA alleles included 50 HLA-A, 99 HLA-B, 34 HLA-Cw, 105 HLA-DR, 14 DRW, 15 DQA, 26 DQB, 8 DPA, and 48 DPB alleles (Bodmer *et al.*, 1994). Table 1.1 shows the frequency of MHC class I and II alleles in Oxfordshire.

Structure of MHC class II

Most detailed structural information is available on HLA-DR, it comprises non-covalently associated α and β polypeptide chains. Both contain peptide binding, immunoglobulin like, transmembrane, and cytoplasmic regions. Each chain contributes 4 stands to a β pleated sheet, which forms the floor of the antigen binding cleft, and one α helix to a side wall (reviewed in Abbas *et al.*,1994). The MHC class II binding pocket is open at both ends (Stern *et al.*, 1994), allowing a central segment of the antigenic peptide (usually about 9 amino acids in length) to be held within the peptide binding cleft, while its N- and C- termini protrude outside (Stern *et al.*, 1994). The ability of MHC class II to form stable complexes with a variety of peptides is conferred by a combination of specific and non-specific peptide-MHC interactions. Non-specific hydrogen bonds and Van der Waals interactions form between the backbone of the antigenic peptide and conserved residues of the MHC molecule (Brown *et al.*, 1993). Specific peptide-MHC interactions occur between pockets in the MHC-peptide binding cleft, and amino acid side chains of antigenic peptides (Garrett *et al.*, 1989, Brown *et al.*, 1993). The residues forming these pockets display a high degree of polymorphism between different MHC class II alleles, leading to differences in peptide binding specificity.

The most detailed investigations into antigenic peptide-MHC interactions have been carried out using HLA-DR1 and a peptide representing residues 306-318 of influenza A Texas/77 HA: X-ray crystallography, showed that DRB1*0101 has 5 pockets which interact with bound antigenic peptide (Stern *et al.*, 1994). The largest and most important peptide binding pocket is near the edge of the peptide binding groove. Although the majority of residues forming this pocket are conserved, there is a Gly/Val dimorphism at position 86 of β chain, which

correlates with the observed binding preferences of several HLA-DR molecules (Busch *et al.*, 1991). The remaining 4 pockets are located throughout the peptide binding cleft and are smaller and more tolerant of peptide side-chain variability (O' Sullivan *et al.*, 1991). Flanking MHC regions may also have some influence over peptide binding affinity (Malcherek *et al.*, 1994), and T cell antigen recognition (Vignali and Strominger, 1994).

In the HA 306-318 DRB1*0101 system a number of antigenic-peptide side chains that do not affect antigen binding to MHC but dramatically alter T cell responses have been identified (Rothbard *et al.*, 1991, Alexander *et al.*, 1993). X-ray crystallography of this interaction showed that these side chains point upwards and out of the peptide binding groove, and are likely to be directly accessible to the TCR (Stern *et al.*, 1994). Polymorphic MHC residues located near the highest point of the β-chain helical region also appear to make contact with TCR and may influence antigen recognition (Kreiger *et al.*, 1991). Antigen recognition may also be influenced by allelic polymorphisms in internal MHC residues, and the relative flexibility the backbone of a peptide (Stern *et al.*, 1994).

The structures of HLA-DRW, -DQ, and -DP are likely to be similar to HLA-DR.

Structure of MHC class I

Human MHC class I molecules are also comprised of the 44kd α chain, and the 12kd β_2 microglobulin chain (β_2m). The non-polymorphic β_2M chain which is encoded by a gene on chromosome 15, is not directly attached to the cell membrane, and non-covalently interacts with immunoglobulin like segment of the α chain (see below)(Ploegh *et al.,* 1981). The α chain contains 4 segments; an N-terminal peptide binding-domain containing 2 highly polymorphic α helices, each of about 90 amino acid residues; a structurally conserved extracellular

immunoglobulin like region also containing a 90 residue alpha helix; a transmembrane region of 25 residues; and a cytoplasmic tail containing about 30 residues. The peptide antigen binding cleft, which measures 30A by 10A, is located in the membrane distal segment of the molecule. The floor of this cleft is made up of an 8 stranded β pleated sheet, and the walls are 2 α helices (Bjorkman *et al.*, 1987). Both α segments of the membrane distal segment of the MHC class I heavy chain contribute equally to the peptide binding region, which has closed ends. This restricts the length of peptides which can be bound to 9-11 residues, though some variation in length can occur because of folding of peptide within the groove (Guo *et al.*, 1992).

The great majority of polymorphic residues in MHC class I are located in either the α helical side walls or the β strands that form the floor of the peptide binding cleft, and are orientated in such a way that their amino acid side chains point into the peptide binding cleft (Parham *et al.*, 1988). Like MHC class II the peptide binding pocket contains pockets and these contain the most polymorphic MHC residues (Saper *et al.*, 1991). Like MHC class II two forms of interaction occur between antigenic peptides and the MHC class I molecule. Firstly, non-specific interactions occur between highly conserved MHC class I residues in the binding cleft and both the amino- and carboxyl-termini of the antigenic peptide and its backbone. Secondly, allotype-specific binding occurs between pockets in the peptide binding cleft and the side chains of the antigenic peptide (Madden *et al.*, 1992, Falk *et al.*, 1991, Jardetzky *et al.*, 1991).

1.4 Assembly Pathways

Class II MHC

MHC Class II molecules present exogenous proteins to $CD4^+$ T cells. They are synthesised on the cytosolic surface of the ER, and are transported into the lumen during synthesis, where they form a complex with calnexin and the invariant chain (Cresswell *et al.*, 1990). The latter is a non-polymorphic, non-MHC encoded protein which is believed to prevent endogenous peptide binding to MHC class II (Roche and Cresswell, 1990). Residues 83-107 form invariant chain's MHC class II binding site - the class II-associated invariant chain peptide (CLIP) region (Riberdy *et al.*, 1992).

The invariant chain directs the class II- invariant chain complex into the low pH vesicles (Peters *et al.,* 1991), where they remain for approximately 5h. During this time the invariant chain is cleaved, generating a truncated peptide which remains bound to the MHC class II molecule (Davidson *et al.,* 1991). The importance of the invariant chain is shown by Ii-negative fibroblasts (Layet *et al.*, 1991, Anderson *et al.*, 1992) , and B lymphocytes from Ii-deficient mice (Bikoff, 1992, Viville *et al.*, 1993), which demonstrate decreased MHC class II assembly, inefficient cell surface transfer, and reduced stability of the few dimers which successfully form.

MHC class II associated antigen can be derived from either extracellular proteins or endocytosed cellular proteins (Germain and Rinker, 1993). Processing occurs in acidic proteolytic endosomal or lysosomal vesicles, which fuse with the MHC class II containing vesicles (Davidson *et al.*, 1991, Germain *et al.*, 1991). A protease promotes the removal of the invariant chain fragment and allows access to the MHC class II binding site (Blum *et al.*, 1988). The MHC encoded HLA-DM gene product is required for efficient invariant chain release and MHC class II

loading (Fling *et al.*,1994, Morris *et al.*, 1994). Empty MHC class II molecules are relatively stable in the absence of peptide binding (Stern *et al.*, 1992).

Class I MHC molecules

Class I MHC molecules presents endogenously produced proteins, which can be either host derived or produced by intracellular micro-organisms to $CD8^+$ T cells. MHC class I molecules are synthesised on the cytoplasmic face of the endoplasmic reticulum and translocated into the lumen during synthesis. Newly synthesised α chains bind to free β_2 microglobulin and calnexin (Degen and Williams, 1991). The latter retains the class I molecule in a partially folded state, folding of the molecule and its stability is completed by binding to peptide antigen.

The major pathway for degradation of cytoplasmic proteins involves the LMP complex of cellular proteasomes (Goldberg and Rock, 1992) This complex, which is encoded by genes in the MHC, produces short peptides which are transported into the lumen of the endoplasmic reticulum (ER) by a complex of 2 ATP dependent, transport associated antigen processing molecules (TAP-1 and TAP-2) (Shepherd *et al.*, 1993). Antigenic peptides are held in the ER until loaded into empty MHC class I molecules. The class I MHC-peptide complex is then transported to the cell membrane. Binding of peptide fragments to MHC class I molecule is essential for their stability, and cell lines deficient in TAP genes have very low levels of class I MHC surface expression (Townsend *et al.*, 1989).

$CD4^+$ T cell effector functions

$CD4^+$ T cells are important regulators of the immune response because of their capacity to secrete regulatory cytokines (e.g. IL-2, IL-4, IL-5, IL-10) and IFN-γ.

Murine $CD4^+$ T cell can be divided into 2 functional classes based on their cytokine secretion profiles (Mosmann *et al.,* 1986). Th1 cells produce IL-2, IFN-γ and lymphotoxin (TNF-β), which promote macrophage activation, antibody dependant cytotoxicity and delayed type hypersensitivity reactions, and are typically elicited by intracellular pathogens. In contrast Th2 cells secrete IL-4, IL-5, IL-10, and IL-13, and provide help for B cell differentiation and effector functions, and are typically stimulated by helminths and allergens (reviewed by Mosmann *et al.,* 1989, Zurawski *et al.,* 1994). As their respective cytokines function antagonistically, Th1 and Th2 T cells might mutually regulate each other (reviewed by Seder *et al.,* 1994). A third subset of $CD4^+$ T cells, which have an intermediate pattern of cytokine secretion has also been reported, the Th0 subset (Firestein *et al.,* 1989), and Th1 and Th2 cells probably differentiate from these cells (reviewed by Swain *et al.,* 1991).

It is much less clear whether defined populations of Th1 and Th2 $CD4^+$ T cells occur in man. Most human $CD4^+$ T cell populations produce IL-2, IL-4, and IFN-γ, though in varying quantities (Umetsu *et al.,* 1988). Human $CD4^+$ T cells are therefore usually described as "Th1 like" or "Th2 like".

1.2 Biology Of Influenza A

I: Virology

Introduction

Influenza is responsible for severe epidemics/zoonotics in many species (reviewed by Webster *et al.,* 1992). Human influenza virus was first isolated in 1933 (Smith *et al.,* 1933). It is a negatively stranded RNA virus and a member of the *Orthomyxoviridae*. Influenza viruses are divided into types, A, B and C, on the basis of their nucleoprotein (NP) and matrix protein (M1) genes. Most epidemics and all pandemics so far have been due to influenza type A.

Structure, genes, and proteins of influenza A

Influenza A virus particles are 80-120nm in diameter, and have a pleomorphic spherical or filamentous appearance. They consist of a host derived lipid envelope from which 2 types of spikes protrude; rod shaped HA, and mushroom shaped neuraminidase (reviewed by Webster *et al.,* 1992). These are anchored into matrix protein 1. Inside matrix protein are the ribonuclear protein (RNP) structures and the polymerase proteins (Murti *et al.,* 1992). The virus genome consists of 8 single stranded RNA segments encoding 10 proteins (reviewed by Lamb 1989.) Their genomic organisation and function is summarised in Table 1. HA is discussed in more detail below.

Structure and function of haemagglutinin

Haemagglutinin

~~HA~~ (HA) is a 224.6 kD glycoprotein, named because of its ability to agglutinate erythrocytes by attachment to host sialic acid glycoprotein receptors (Hirst, 1942).

HA was shown by chemical cross linking and X-ray crystallography studies to be a trimer of non-covalently linked monomers (Wiley *et al.,* 1977, Wilson *et al.,* 1981), which are synthesised as a single polypeptide chain in the rough

endoplasmic reticulum of the host cell, and subsequently cleaved by extracellular host enzymes into 2 disulphide linked polypeptide chains (Klenk *et al.,* 1975). The HA1 subunit of human H3 influenza A viruses usually contains 328 residues and the HA2 subunit usually contains 221 residues. Cleavage does not affect antigenicity or receptor binding (Lazarowitz *et al.,* 1971, 1973a, b), but is essential for infectivity (Lazarowitz and Choppin 1975, Klenk *et al.,* 1975), pathogenicity (Bosch *et al.*, 1979, Rott 1979), and spread (Rott *et al.*, 1980).

The HA monomer is 13.4 nm long, and has a globular membrane distal region made up entirely of HA1 on top of an elongated stem comprised of HA2 and the remainder of HA1. HA is glycosylated, though the number and location of the glycosylation sites vary. The carboxy terminus of HA2 anchors the monomer in the viral membrane with its final 15 C-terminal residues being internal (reviewed by Wharton *et al.*, 1989).

The host-cell receptor binding site of HA is located at its membrane distal tip (Rogers *et al.*, 1983). The base of the binding site is formed by residues 98^{Tyr} and 153^{Trp}, the rear is formed by residues 190^{Glu} and 194^{Leu} the left side by residues 224-228 (reviewed by Wharton, 1989). The 10 N-terminal residues of HA2 are required for virus-host fusion. this area is highly conserved in structure and has considerable structural homology with the fusion proteins of paramyxovirus and Sendai virus (reviewed by Wiley and Skehel, 1977).

Life Cycle of influenza A

The life cycle of influenza A commences with viral attachment to host cell surface receptors, and endocytosis. A fall in endosomal pH triggers a conformational change in HA, which leads to membrane fusion (reviewed by Wharton *et al.,* 1989, Wharton *et al.*, 1995). Influenza nucleocapsids migrate to the host nucleus, and their polymerase complexes initiate mRNA transcription. Translation of host mRNA is blocked (Katze *et al.*, 1986b). Influenza RNA polymerases requires priming with host derived mRNA 5' fragments (Bouloy *et al.*, 1978, 1979). Viral

mRNA is polyadenylated (Hay *et al.*, 1977a) and subject to post transcriptional methylation and splicing. In the early stages of infection mRNA transcripts are translated to produce primarily NP and NS1. Later the principal products are M1, HA, and NA. HA and NA are post translationally processed and transported to the cell surface where they integrate into the cell membrane (*reviewed* by Krug *et al.*, 1989).

Viral replication requires the production of full length copies of "positively stranded" complimentary RNA (cRNA) which are used as templates for the production of daughter negatively stranded genomic RNAs (vRNAs). Initially the production of all 3 types of RNA is coupled, later only vRNAs are produced. An increase in the concentration of NP and NS1 in the nucleus is thought to be responsible for the switch (reviewed by Krug *et al.*, 1989).

An increase in the concentration of M1 in the nucleus is associated with the migration of nucleocapsids into the cytoplasm for assembly into progeny viral particles. A core of nucleocapsids becomes encased in M1 protein and buds outwards through the cell membrane, enclosing itself as it does so. Finally HA is cleaved extracellularly (reviewed by Krug *et al.*, 1989).

Antigenic variation amongst influenza A viruses

RNA virus polymerases have an error rate of approximately 1 in 10^4 bases per replication cycle compared to an error rate of 1 in 10^9 bases for DNA polymerases (Holland *et al.*, 1982). Coupled with a fast replication cycle this allows rapid mutation.

Variation in surface coat glycoproteins allows serological classification of influenza viruses (WHO memorandum 1980). To date 14 subtypes of HA, and 9 types of NA have been described. Between serological types surface coat proteins vary by up to 75%, within a serological type variation is between 1-15%. In contrast the internal proteins are relative stable, showing about 8% variation between subtypes (Winter and Field 1981, Huddlestone and Brownlee 1982).

Changes in surface coat proteins take 2 forms, a gradual change due to small numbers of residue substitutions (antigenic drift), which is interspersed by periodic dramatic changes in structure (antigenic shifts). The latter usually cause severe pandemics.

Three subtypes of influenza A viruses have been identified in man: The H1N1 subtype is believed to have been responsible for the 1918/19 pandemic (Spanish influenza), the H2N2 subtype which emerged in 1957 (Asian influenza), and H3N2 subtype which emerged in 1968 (Hong Kong Influenza). There is archeoserological evidence that H1, H2, and H3 influenza A viruses have circulated in a cyclical manner for at least 100 years (Mansurel and Marine 1973, Mansurel and Heijtink 1983), though the association of HA and NA genes may have varied. Indeed there is some archeoserological evidence that the 1889-90 pandemic may have been caused by a H2N8 influenza A virus (Mulder and Mansurel, 1958).There are also sporadic reports of human influenza due to viral subtypes normally associated with disease in other species (Webster *et al.*, 1981, Lvov *et al.*, 1983).

Nature of Antigenic Shifts

There is no evidence that a long term human reservoir of influenza A exists (reviewed by Murphy and Webster, 1990). The question of how human influenza subtypes reappear on a cyclical basis therefore arises. One explanation is that antigenic shift is due to the re-assortment of human and avian influenza A viruses which act as a natural reservoir for influenza genes (reviewed by Murphy and Webster, 1990). Examples of all known HA and NA genes are found in avian influenza species (reviewed by Webster *et al.*, 1992). Detailed phylogenetic studies of these viruses has indicated that they are probably the original source of influenza viruses in all other host species (reviewed by Webster *et al.*, 1992). Furthermore the H3N2 virus responsible for the "Hong Kong" pandemic contains H3 HA, NA, and PB1 genes from an avian donor and its N2 NA and 5 other genes from the

preceding H2N2 human strain (Fang *et al.*, 1981, Kawaoka *et al.*, 1989), the H2N2 influenza A virus responsible for the "Asian" influenza pandemic obtained its HA, NA, and PB1 genes from an avian host and the remaining genes from the preceding H1N1 strains (Gething *et al.*, 1980, Kawoka *et al.*, 1989, Schnurrenberger *et al.*, 1970). Such genetic re-assortment probably occurs in an intermediate host, which is most likely to be the pig (Lin *et al.*, 1994). Reassorted influenza viruses have recently been isolated from pigs (Castrucci *et al.*, 1993, Shu *et al.*, 1994), and there is evidence that they can infect humans (Claas *et al.*, 1994).

Antigenic shift could also occur if an avian influenza virus became directly infectious for man. This is mechanism has been proposed as the cause of the "Spanish" influenza pandemic (reviewed by Webster *et al.*, 1992).

A third possible mechanism of antigenic shift, is the emergence of influenza from long term "storage". The H1N1 virus which reappeared in 1977 had exactly the same genes as the virus which disappeared in 1957. Though it is possible the virus persisted in a frozen lake, it is now widely believed that the emergence of this virus was the result of a laboratory accident in the USSR (Dr B. Mahy speaking at Ninth International Conference on Negative Strand Viruses 1995).

Antigenic Drift

Human H3N2 influenza A viruses, have a residue substitution of about 1.1%/yr (Both *et al.*, 1983, Wilson and Cox 1990). Convalescent sera studies and gene sequence analysis (reviewed by Wharton *et al.,* 1989), indicate that amino acid substitutions primarily occur in the HA1 subunit. Between 1968 and 1988 73 substitutions occurred in HA1 compared to 12 in HA2 (Wharton *et al.*, 1989). HA1 substitutions occur in 5 main clusters in the distal domain, named antigenic sites A-E (Wiley *et al.*, 1981), and appear to prevent the binding of neutralising antibody. The receptor binding pocket itself is usually spared, probably because mutations in this region impair its function.

The location of several important neutralising antibody sites has been established: Site A is centred around a protruding loop containing residues 140-146; site B is at the extreme membrane distal tip of HA and is centred on a loop containing residues 155-60 and residues 188 and 189 from the α-helix which forms the edge of the receptor-binding pocket; site C is situated at the base of the globular domain in the anti-parallel strands of HA1 on the top of the molecule; site D is situated near the trimeric interface of the globular domains of HA1 on top of the molecule; and site E is near the bottom of the globular distal domain between sites C and A (Wiley *et al.,* 1981, reviewed by Wharton *et al.*, 1989). The importance of these sites was confirmed by the finding that monoclonal antibody selected mutant viruses have amino acid substitutions in these regions (Laver *et al.*, 1979, Laver *et al.,* 1981, Daniels *et al.*, 1983, Newton *et al.*, 1983), and confirmed by crystallographic studies localising antibody binding to these sites (Knossow *et al.*, 1984).

The antigenic sites of H1 influenza A viruses are believed to be in similar positions to those of H3 influenza A (reviewed by Wharton *et al.,* 1989).

II Human Influenza Type A

History of Human Influenza

Influenza epidemics have probably occurred for 2,000 yrs (reviewed by Stuart-Harris *et al.*, 1985). The first pandemic about which there is good serological evidence occurred in 1889-90, and followed a 43 yr period with little influenza (reviewed by Stuart-Harris *et al.*, 1975). Influenza epidemics occurred regularly with variations in severity until 1915. The most severe pandemic ever recorded occurred in 1918. Its exact origins are unknown, but it is known to have occurred amongst members of the American Expeditionary Forces landing in Europe during the summer of 1918. It rapidly spread and had an attack rate of 30-40% in children and 20-30% in adults. A second wave of influenza followed a few

months later, and a third occurred in the spring of 1919. Both were very severe, and associated with a large number of cases of pneumonia, with a case fatality rate approaching 50%. At least 1/3 of deaths occurred in young adults. This pandemic was responsible at least 20 million deaths, more than the fighting in The First World War (reviewed by Stuart- Harris *et al.*, 1985), indeed 80% of the fatalities in the American army during the First World War were due to influenza, and the German commander in Chief blamed the failure of the Marne offensive and the loss of the war on this pandemic (reviewed by Murphy and Webster, 1990).

As reviewed by Stuart-Harris and colleagues (1985), between 1920 and 1940 there was a decline in influenza mortality to pre 1918-9 levels, but not to pre 1890 levels. Severe epidemics occurred in 1940, 1943, and 1951. In 1957 a pandemic due to H2N2 influenza started in China. This was again notable for a very high attack rate, reaching 50% in children and 20% in adults. Deaths were mainly confined to the over 55 age group. Further waves of the pandemic occurred in January 1958 and 1959 which were more severe than the original outbreak (reviewed by Stuart-Harris *et al.,* 1985). Epidemics of H2N2 influenza occurred regularly until 1968.

In 1968 a pandemic caused by the H3N2 subtype originated in Hong Kong. Again several waves occurred. The highest attack rates were in children. In adults the highest mortality was in the 45-64 age group, with sparing of the elderly. This is thought to be due to the circulation of H3 influenza viruses prior to the emergence of the H1N1 strains (reviewed by Stuart-Harris *et al.,* 1985). Since 1969 there have been regular epidemics of influenza due to H3N2 influenza viruses.

In 1977-8, a pandemic, mostly confined to the under 25 age group, due to H1N1 influenza occurred. The re-emergence of H1N1 influenza appears to have been due to a laboratory accident (see above). It is particularly interesting that at

present both H3 and H1 influenza A viruses are coexisting rather than the usual occurrence of one strain replacing the other (reviewed by Smith and Palese 1989).

Pathology of influenza

Influenza's incubation period is 1-5 d. Droplet spread is the basis of infection. The virus replicates, and is recoverable, throughout the respiratory tract. After experimental infection virus replication peaks at 48 h, and declines slowly thereafter, with little shedding after detectable after 6-8 d, except in children who shed virus for up to 13 d (reviewed by Murphy and Webster 1990).

Pathological changes in uncomplicated cases occur throughout the respiratory tract, though most significant pathology arises in the lower respiratory tract. Bronchoscopy of uncomplicated influenza shows acute diffuse inflammation of the larynx, trachea, and bronchi. There is generalised mucosal inflammation and oedema (reviewed by Murphy and Webster 1990). Microscopically the columnar and ciliated epithelial cells become vacuolated, oedematous and loose cilia before desquamating. Desquamation starts 1 d after infection and exposes the basement membrane (reviewed by Murphy and Webster 1990). Submucosal oedema and hyperaemia occurs with infiltration of neutrophils and mononuclear cells (Martin *et al.,* 1957). Viral antigen is present predominantly in the epithelial and mononuclear cells (Mulder *et al.,* 1979).

After 3-5 d the epithelium begins to show signs of repair, but complete resolution with recovery of tracheobronchial clearance probably takes 1 mo (Camner *et al.,* 1973, reviewed by Murphy and Webster 1990), though functional pulmonary abnormalities persist beyond this time (Halt *et al.,* 1976, Little *et al.,* 1976).

Epidemiology

Pre-school and school age children are the major vectors of influenza transmission (reviewed by Murphy and Webster 1990). Influenza is reported to affect 5-15% of the population during a normal annual outbreak (reviewed by Palache 1992).

Higher attack rates occur in closed communities such as nursing homes where attack rates can reach 60% with a case fatality rate of 30% (Ruben 1986).

Clinical features of influenza in man

Epidemics usually cause similar symptoms (reviewed by Stuart-Harris *et al.*, 1985): An abrupt onset of headache, shiver, and dry cough occurs 48h after infection, and is followed by sudden rise in temperature to 38-40°C and an intensification of headache, weakness, and myalgia in limb and/or back muscles. Sleep is disturbed, the nose becomes obstructed (though profuse nasal discharge is unusual), cough becomes frequent and irritating, and is accompanied by some degree of substernal soreness. The throat is dry but rarely sore. Symptoms last between 2d-5d. Fever often occurs only for 24h, but is often followed by a secondary rise on d2 or d3.

The initial dry cough is replaced by one productive of scanty mucoid or mucopurulent sputum. The pulse rate is variable, and minor ECG changes have been described. Blood pressure is usually slightly raised.

Recovery in uncomplicated cases is usually rapid once fever has disappeared, but tiredness, depression, and the cough may continue for several days. Most subjects return to work within 7d to 10d.

In infants influenza A is associated with fever in excess of 39°C, moderate illness, coryza, cough and irritability. Otitis media, pneumonitis, and hoarseness or croup occur in 25% (Wright *et al.*, 1977). Complications other than otitis media in older children are unusual. Neonates receive some protection form the placental transfer of antibody but can still suffer from fever, croup, bronchitis or pneumonia (Puck *et al.*, 1980).

Women in the last trimester of pregnancy appear to be at particular risk from the pulmonary complications of influenza (Petersdorf *et al.*, 1959). No definite effects on the foetus have been demonstrated.

Complications of Influenza

Serious complication of influenza are rare in healthy adults, but more frequent and dangerous in the elderly and chronically ill (Glezen, 1982, Barker and Mullooly 1982). In the USA 20,000 deaths are attributed to influenza and its complications annually, the majority in the elderly (Liu and Kendal, 1985, Williams *et al.,* 1988).

By far the most common complication of influenza is tracheo-bronchitis.

Pneumonia associated with influenza is usually due to bacterial secondary infection though primary viral pneumonia can occur. Bacterial secondary infection (usually due to *Streptococcus pneumoniae*) normally arises in those with pre-existing lung or valvular cardiac disease. Infections can either be synchronous with influenza or occur up to 7d later. ~~for these infections~~. Severe secondary infections can also occur in otherwise healthy individuals, the organism responsible for these is usually *Staphylococcus aureus* (Giles and Shuttleworth, 1957, Louria *et al.*, 1959, Schwarzmann *et al.*, 1971).

Influenza is associated with sudden cardiac death though there are few reports of the isolation of influenza virus from cardiac tissue.

Neurological complications include acute encephalitis, and a Guillain-Barré like illness (Flewett and Holt 1958). Rarer complications include coma with focal neurological signs (Dubowitz *et al.*, 1958). The 1918/19 pandemic was associated with encephalitis lethargica and post encephalitic Parkinson's disease (Ravenholt and Foege 1982).

Reye's syndrome has been associated with influenza, particularly type B, but is now believed to be caused by the use of aspirin in febrile children, rather than by influenza per se. Following restrictions on the use of this drug it has almost disappeared in the UK (Glasgow and Moore, 1993).

Influenza vaccination

Introduction

The first attempts at experimental anti-influenza vaccination were made in the 1930s using formaldehyde inactivated virus purified from mouse lung. It was only after the successful propagation of influenza viruses in hen's eggs that the first large scale human field trial of an influenza vaccine could take place in the 1940s. The vaccine used was an inactivated whole virus preparation and was based on the PR/8 virus. It had a 70% success rate in preventing influenza infection by antigenically distinct H1N1 strains (reviewed by Stuart-Harris *et al.,* 1985). In 1947 a severe epidemic occurred due to an H1N1 strain which was distantly related to the PR/8 vaccine strain. Vaccination during this epidemic was unsuccessful, and this led to the policy of incorporating viral strains closely related to potential epidemic strains into vaccines (reviewed by Stuart-Harris *et al.,* 1985).

Such inactivated whole virus vaccines continued to be used until the late 1960s. Unfortunately, as reviewed by Stuart-Harris and colleagues (1985), though they usually conferred "reasonable protection" from influenza, these vaccines were found to be associated with frequent local and systemic reactions. Advances in ultracentrifugation and chemical treatment allowed the removal of unwanted egg proteins. Despite these improvements whole virus vaccines were associated with significant side effects, particularly in children. A number of splitting agents were then tried including, ether, tri-N-butylphosphate, polysorbate 80, and sodium deoxycholate. During the 1970s and 1980s subunit vaccines were developed; these are split vaccines which are highly purified to remove most internal viral proteins, leaving HA and NA (reviewed by Williams and Wood, 1993). These produced more satisfactory vaccines (split-product vaccines) and are the most frequent form of vaccine in use today. Interestingly the method of HA and NA

purification is critical, for example bromelain cleaved HA is reported to be 1000 times less immunogenic than ether split HA (Tyrrell 1974).

Modern inactivated vaccines are derived from allantoic fluid of fertile hen's eggs seeded with a recombinant influenza virus. Recombinants between a highly egg adapted virus and a wild type virus are used (Kilbourne, 1969). Influenza A viruses can be altered antigenically by growth in hen's eggs (reviewed by Murphy and Webster, 1990).

Reaction to the subunit and split influenza vaccines are rare. Guillain-Barré syndrome is the most serious complication associated with influenza vaccination, though the risk of developing this condition is normally less than 1.4 x the base risk (reviewed by Stuart-Harris *et al.,* 1985).

The second form of influenza vaccines are live attenuated vaccines. These were first tested in humans in the 1940s. Such vaccines are produced by altering wild type viruses by a number of means including cold adaptation or the introduction of temperature sensitive mutations, to produce infective but largely non-pathogenic strains. This is often a difficult to achieve, and generally such strains are not particularly infective. Production and safety testing is hampered by the short time interval between the prediction of a particular years influenza strain, and the clinical requirement for the vaccine. Live influenza vaccines have only been subject of extensive clinical trials in the USSR (see below).

Serological response to inactivated influenza vaccines

Several factors determine the serological response to an inactivated influenza vaccine. The most important factor being the quantity of HA and NA contained in the vaccine (Mostow *et al.,* 1970). A 2 dosage regime also appears to induce a better antibody response than a single inoculation, at least on primary exposure or following an antigenic shift (Holland *et al.* 1958, Pandemic Working Group of

MRC Committee on Influenza, 1977, Nicholson *et al.,* 1979). The route of immunisation is also important: intradermal injection is less reliable than s.c. injection. Nasal immunisation and aerosol vaccine delivery produces a less efficient increase in serum antibody than s.c. administration, and interestingly nasal antibodies levels are similar following administration by both routes (reviewed by Stuart-Harris *et al.,* 1985).

The recipient's exposure history is also very important: Higher antibody levels are generated in subjects who have previously been exposed to the same subtype of virus (Pandemic Working Group of the MRC Committee on Influenza 1977), however the nature of the antibody response differs on repeat exposure. Following primary exposure the antibody response is principally strain specific, however following repeat exposure cross reactive antibodies start to take precedence (Oxford *et al.,* 1979), in addition subjects have a tendency to produce neutralising antibodies which are specific for the strain of the influenza subtype to which they were first exposed rather than to the vaccine strain, the phenomenon of "original antigenic sin" (MRC Committee on Influenza Vaccine 1957, Fazekas de St. Groth and Webster, 1966).

Evidence that influenza vaccination is protective

Influenza vaccine trials are often difficult to compare because of differences in methodology. Some trials use as evidence of protection symptom scoring, some viral culture, and others the production of neutralising antibodies. It is usually not clear in the reports of these trials whether subjects have been previously vaccinated, or recently infected with influenza virus. Protection rates with current vaccines as reflected by rises in neutralising antibody are reported to be between 60-90% (Ben-Ahemeida *et al.,* 1993), and reduction in major respiratory tract morbidity is reported to be 70% (Palache, 1992). Protection is greatest when

vaccine and epidemic strains are closely matched (Potter *et al.,* 1977c, reviewed by Palache 1992). Whether similar protection is afforded by vaccines following antigenic shift is less clear. The protection rate in a study following the H2N2 "Asian Influenza" pandemic was 67% in children and 50% in adults (MRC Committee on Influenza Vaccines, 1958). A second trial carried, out following the "Hong Kong" pandemic, failed to demonstrate any protection by inactivated (H3N2) influenza vaccine in school children, despite rises in neutralising antibody (Tyrell *et al.,* 1970). This trial has however been criticised because of a 2-4 mo delay between vaccination and the arrival of the influenza pandemic, furthermore similar trials in the USA were more successful during this pandemic (reviewed by Stuart-Harris *et al.,* 1977).

Live attenuated vaccines ~~is~~ are reported to be as effective as inactivated vaccines in generating neutralising antibody responses (Edwards *et al.,* 1994), and are associated with a protection rate of about 50% from respiratory illness (reviewed by Karzon 1993).

Little research has been carried out on the effect of repeated annual vaccination. One notable trial on boarding school children reported by Hoskins showed protection following a single influenza vaccination, but failed to show any benefit from repeated annual vaccination (Hoskins *et al.,* 1979). Though this study has been criticised because the vaccine and epidemic strains were not always well matched (reviewed by Nicholson 1993). Furthermore as reviewed by Nicholson the levels of neutralising antibody achieved by vaccination in subjects repeatedly vaccinated is lower than those vaccinated for the first time (Nicholson 1993). At present there is therefore no evidence that repeated influenza vaccination is of any benefit to normal individuals.

1.4 Defence Mechanisms Against Influenza A Viruses

I Non-specific defence mechanisms

Human serum and sputum contain inhibitors of influenza infection, including members of the C-type lectin family: Mannose binding protein (Hartshorn *et al.*, 1993), conglutinin (Hartshorn *et al.*, 1993b), and surfactant protein D (Hartshorn *et al.*, 1994).These bind HA, cause viral clumping, and inhibit infectivity. Mannose binding protein also has opsonising and complement activating activity.

Type I IFN production is triggered in influenza infected cells. This induces the synthesis of a number of proteins including the Mx gene product, a 72,000 m.w. protein with specific anti-orthomyxovirus properties (reviewed by Krug 1989). Type I IFN also increases the lytic potential of NK cells for virus infected cells, and increases MHC class I expression aiding $CD8^+$ T cell cytotoxicity.

Type II IFN produced by both $CD4^+$ and $CD8^+$ T lymphocytes in response to influenza infection also plays an important role (see below). However even though experiments using transgenic mice with disrupted IFN-γ receptors demonstrated a compromise in the early non-specific response to influenza, there was no diminution in virus specific T cell mediated immunity: This indicates the IFN-γ produced by immune $CD8^+$ T cells and $CD4^+$ T cells is not essential for viral clearance (reviewed by Doherty 1993).

NK cells are directly induced by viral infections are an important first line defence mechanism. They lyse a wide range of virus infected cells, by a non MHC restricted non specific mechanism (reviewed by Trincheri 1989).

Complement activation and opsinisation, and subsequent phagocytosis also aids in eliminating viruses. Complement binding to antigen also lowers the threshold for B cell activation by at least 2 orders of magnitude, and may influence the class of Ig produced (reviewed by Fearon 1993). Paradoxically opsonised virus may gain easier access to host cells (see below).

II: Antibody

Neutralising antibodies are the primary defence against influenza infection. In humans antibodies are detectable 4d -7d after infection, and reach a peak at 14d-21d (reviewed by Potter and Oxford, 1979). The half life of anti-influenza antibody is variable, depending largely on whether the individual has been previously exposed to influenza: In a study following the antigenic shift in 1968, it was reported to be 28 d (Schild *et al.*, 1977).

Influenza A infection or immunisation triggers the production of antibody directed against most viral proteins. However most important antibodies are neutralising and specific for HA. Most of these are directed against the globular head of HA1, with less than 3% binding to HA2 (Eckert 1973, Brown *et al.*, 1980). Anti-HA antibodies can be strain specific or cross reactive within a subtype (reviewed by Potter and Oxford, 1979). Strain specific neutralising antibodies are the most protective (Virelizier 1975).These "haemagglutination inhibition" antibodies (HI), bind to highly variable regions of HA1 closely related to its sialic acid receptor (Wiley *et al.*, 1982). HI antibodies protect against further infection by the same or closely related strains of influenza, resulting in an inverse relationship between susceptibility and pre-exposure neutralising IgG antibodies levels (Morris *et al.*, 1966, Potter et *al.*, 1977).

The relative proportion of strain specific and cross reactive antibodies produced following infection differs between young children, presumably exposed to influenza for the first time, and adults who have been repeatedly exposed to an influenza subtype (between antigenic shifts), and therefore may have received repeated boosts to their cross-reactive response. The majority of antibody produced by the former are strain specific, whereas the majority of antibodies produced by adults cross react with almost all variants of an influenza A subtype the phenomenon of "original antigenic sin" (S. Fazekas De St Groth and Webster 1966, Oxford *et al.*, 1981).

Interestingly it has also been observed that mice immunised with one influenza strain and subsequently vaccinated with a second strain of the same subtype, produce antibody specific for the original strain's HA (Virelizier *et al.,* 1974). Though anti-HA neutralising antibodies are effective in preventing extracellular viral spread they are of no use once virus has entered a cell, and the neutralising capacity of an antibody *in vitro* often shows little correlation with its protective capacity *in vivo* (reviewed by Potter and Oxford, 1979).

Various subtypes of immunoglobulin probably play important roles in the defence against influenza, in particular secretorary IgA has the potential to neutralise influenza virus in the respiratory tract. However its serum titres are closely related to those of IgG, and it is difficult to disentangle their respective roles (reviewed by Tyrrell and Smith 1979). Passive infusion experiments in mice have clearly shown protection with IgG , either specific for HA or NA alone (Askonas *et al.,* 1982). Antibody against NA may also play a role in protection against influenza A infection: During the 1968 pandemic shift individuals with a high pre-infection anti-N2 NA antibody level had half the infection rate of those with no pre-infection anti N2 NA antibody (Schild *et al.,* 1977). Anti-NA antibodies do not neutralise virus and probably function by inhibiting viral spread, primarily by inhibiting release of virions from infected cells, though they may also be involved in complement mediated lysis of virus particles (reviewed by Mitchell *et al.,* 1985). Antibodies directed against other viral components have been shown to have a similar effect (Treanor *et al.,* 1990). Overall however non-neutralising antibodies are likely to be of secondary importance (McMichael *et al.,* 1983, Potter and Oxford 1985).

Paradoxically the non-neutralising anti-influenza antibody directed against HA or NA may by binding to host Fc receptors allow infectious influenza virus to gain entry into cells more easily, despite of the presence of neutralising antibody, the phenomenon of "antibody-dependant enhancement" (Tamura *et al.,* 1991, Gotoff *et al.,* 1994). There are 2 reasons why this may be of importance in influenza

infection, firstly the boosting of cross reactive antibody responses by repeat infection with different influenza A strains, secondly "original antigenic sin" (see above). However rather than mediating viral escape this may result in increased antigenicity, as dendritic cells (which have Fc receptors) stimulate strong proliferative and cytotoxic responses from $CD8^+$ T cells (Macatonia *et al.*, 1989).

III: The role of $CD8^+$ T lymphocytes in the defence against influenza A

Murine models of CTL recognition of influenza A

Cytotoxic T lymphocytes (CTL) are the principle mechanism of viral clearance once influenza infection is established. The majority of CTL are MHC class I restricted (reviewed by Zinkernagel and Doherty, 1979). After nasal influenza infection of mice pulmonary virus levels reach a maximum during the first 4d and remain high for a further 2d to 3d before declining to below the limits of detection by 10d. The earliest and most active CTL responses occur in the lung and are detectable 3d-4d after intranasal infection, before the production of a primary IgM antibody, with the peak CTL responses occurring at 5d to 7d, before they decline over 2-3 wks (Yap and Ada 1978, Cambridge *et al.*, 1976, Doherty *et al.*, 1977). Preimmunisation accelerates CTL responses by about 2d, and more vigorous and consistent response are elicited (Doherty *et al.*, 1977, Effros *et al.*, 1978). Priming with live virus results in an increase in CTL precursor frequency of between 10-100 fold (Askonas *et al.*, 1982, Owen *et al.*, 1984). Interestingly in order to achieve priming different viral strains have to be used for secondary challenges, otherwise anti HA antibody will neutralise circulating virus and no CTL response is seen (Effros *et al.*, 1977, Greenspan and Doherty 1982).

There is considerable evidence that CTL play a crucial role in host defence against influenza. Firstly there is a correlation between induction of pulmonary CTL activity and the reduction of influenza virus titres (Yap and Ada 1978). Secondly adoptive transfer of a T cell population selected for CTL both protects naive mice against homologous lethal influenza pneumonia and reduces

recoverable infectious virus by 100 to 10,000 fold (Yap *et al.,* 1978, Yap and Ada 1978). Finally reduced mortality and viral titres have been demonstrated in naive mice transfused with a single cross reactive CTL clone (Lin and Askonas 1981). Depletion of $CD8^+$ T cells in mice with antisera before influenza infection leads to a delay in viral clearance (Lightman *et al.,* 1987). Transgenic β_2m (-/-) H-2^b mice, which possess no $CD8^+$ T cells, clear the non-lethal X-31 influenza virus with almost normal kinetics (Eichelberger *et al.,* 1991), and are able to control a low level challenge with the virulent PR8 virus, but are much more susceptible than normal mice (Bender *et al.,* 1992). This partial immunity may be due to $CD4^+$ T cell mediated mechanisms, for example antibody directed cytolysis, IFN-γ production, or $CD4^+$ T cell mediated cytotoxicity.

It is not clear if immunity modulated by CTL is simply related to their cytotoxic ability and it is likely that their ability to secrete IFN-γ on contact with infected cells is also important (Morris *et al.,* 1982).

Specificity of the murine CTL response to Influenza A

Most studies on the specificity of the murine CTL response to influenza A have been carried out on *in vitro* restimulated lymphocytes derived from mice previously infected or immunised. Two populations of influenza A specific CTL are induced by nasal infection, the first and largest population are able to lyse target cells infected with a wide range of influenza A strains, but not influenza B (Zweerink *et al.,* 1977). The smaller second population is specific for the immunising strain or closely related strains (Cambridge *et al.,* 1976, Braciale 1977). Early studies showed that most of the strain specific murine CTL recognised HA, and that most cross reactive CTL recognised internal viral proteins (reviewed by Yewdell and Hackett, 1989). However these studies were limited by problems obtaining antigens of high purity. This was overcome in an elegant series of studies using cells transfected with DNA constructs containing influenza virus genes and target cells infected by recombinant vaccinia viruses

(Vac) encoding influenza virus genes. These experiments showed that nucleoprotein was a major antigenic target of CTL (Townsend *et al.,* 1984, Yewdell *et al.,* 1985). Interestingly not all NP specific CTL clones were cross-reactive for all influenza A subtypes (Townsend and Skehel, 1982, Townsend and Skehel, 1984).

In general the CTL response is much more powerfully induced by infectious virus than inactivated whole virus or subviral preparations, because the response is primarily to newly synthesised viral proteins (reviewed by Yewdell and Hackett, 1989). These findings are in contrast to those for Class II MHC antigen presentation and reflect differences in the antigen processing pathways for the 2 systems. Indeed the murine CTL response to HA has provided clear evidence for the cytosolic processing of antigen presented by MHC class I (Townsend *et al.,* 1986, Hahn *et al.,* 1991). Despite the superiority of live virus in inducing CTL responses, they can be induced both by non-infectious viral particles (Braciale and Yap, 1978) and by purified proteins, for example NP (Wraith and Askonas, 1985). This may be the result of proteins, which are present extracellularly in high concentrations, being taken up into the cell and then gaining access to the endocytotic pathway, or alternatively of antigen taken up in the form of antigen-antibody complexes obtaining access to the cytosolic compartment.
Other influenza proteins including the polymerases PA, PB1, and PB2, and also NS1 have all been demonstrated to be target antigens for murine CTL responses following infection. Interestingly their relative importance differs between mouse strains, for example some recombinant mice are non-responders to NP (reviewed by Townsend and Bodmer, 1989), and CTL responsiveness co-segregates with class I MHC (reviewed by Yewdell and Hackett, 1989).

Human CTL recognition of influenza A

The human CTL response to influenza shares many features of the murine $CD8^+$ T cell response: Cytotoxic T cells can be detected from 4d after infection (McMichael *et al.*, 1983). The responses are also generally widely influenza type A cross reactive (McMichael and Askonas 1978, Biddison *et al.*, 1979), and this cross reactivity has been demonstrated to occur at the clonal level (Fleisher *et al.*, 1982, Braciale *et al.*, 1981). The majority of human CTL recognise internal proteins and a response to HA appears to be rare (reviewed by McMichael 1994). The human anti-influenza CTL response has been shown to involve a number MHC class I specific epitopes, the response to which are mounted in an individual, by limited numbers of closely related T cell clones (Moss *et al.*, 1991). In an extension of this work it has recently been demonstrated that a panel of 12 unrelated HLA-A0201 adult donors use the identical TCRs Vβ gene segments to respond to a dominant matrix protein epitope, and that these Vβ TCRs also have extensive conservation of their CDR3 regions (Lehner *et al.*, 1995).

CTL are undoubtedly important in man in the recovery from influenza infection. In experimental human influenza CTL activity prior to infection correlated with rapid clearing of administered virus in individuals with no antibody immunity (McMichael et *al.*, 1983).

McMichael and co-workers (1983) reported that CTL responses following influenza infection decline with time, and estimated the half life of the response to be 2-3 yrs.

IV: The role of $CD4^+$ T lymphocytes in the defence against influenza A

$CD4^+$ T cells play a central role in defence against influenza infection: The production of neutralising antibody is $CD4^+$ T cell dependant (see below), $CD4^+$ T cell amplify CTL responses (Biddison *et al.*, 1981, Braakman *et al.*, 1986), as well as participating more directly in viral clearance by the secretion of IFN-γ (reviewed by Askonas 1988). $CD4^+$ T cell dependence of neutralising antibody

production was initially demonstrated in thymectomised mice, which failed to mount a neutralising antibody response to HA (Virelizier *et al.,* 1974), and confirmed in nude mice (nu/nu) who were shown not to mount an antibody response against PR8 virus infection (Burns *et al.,* 1975). Anders and co-workers (1981) subsequently demonstrated T cell dependence of secondary antibody responses, and showed that primed T cells are much more efficient in than naive T cells in this regard (Anders *et al.,* 1981). Scherle and Gerhard (1988), using adoptive transfer, reported that individual Th clones specific for either HA, NP, or matrix protein could restore antibody responses to HA or NA in nu/nu mice.

Murine CD4$^+$ T cell recognition of influenza A

Murine CD4$^+$ T cells probably recognise most if not all influenza proteins, though the balance of recognition of internal and external proteins varies according to the experimental system employed. Hurwitz and co-workers (1985) derived a panel of T cell hybridomas from BALB/c mice following priming with PR/8 virus (H1N1) either by foot pad injection of inactivated virus in Freund's complete adjuvant, or by intraperitoneal injection of live virus. They reported that 4/12 hybridomas responded to matrix protein, 3/12 to NP, 1/12 to NA, and 3/12 to HA. One hybridoma had an undefined specificity (Hurwitz *et al.,* 1985). The HA and NA specific hybridomas were found to be subtype specific, whereas the NP restricted hybridomas were cross reactive amongst influenza A subtypes (Hurwitz *et al.,* 1985).

In contrast a group lead by Thomas, using T cell clones obtained following nasal infection of CBA mice with the less virulent X31 virus (which has H3N2 external proteins and PR/8 internal proteins), found that the CD4$^+$ T cell response to influenza was heavily skewed towards the recognition of HA: Of a total of 42 clones isolated from 11 mice, only 9 clones failed to respond to HA, 5 of which were probably NA restricted and 4 were restricted to internal viral proteins (Mills *et al.,* 1986).

Fine specificity of murine CD4$^+$ T cell response to HA

Studies on the fine specificity of CD4$^+$ T cell responses to influenza virus have been largely confined to HA. Atassi and Kurisaki (1984) used a panel of 12 peptides to examine the response of BALB/c mice primed with X-31. The authors reported that 3/12 peptides restimulated well (23-36, 183-199, HA2 56-68), 3/12 were intermediate (HA201-218, 272-288, 300-15), and one stimulated poorly (175-188). As most of the synthetic peptides had been shown to stimulate good B cell responses, some overlap of B and T cell antigen recognition was evident (Atassi and Kurisaki, 1984).

A second group (Anders *et al.,* 1981) examined the responses of CD4$^+$ T cells obtained from BALB/c mice previously primed by intra-peritoneal injection of one of a panel of live influenza A viruses. *In vitro* restimulation revealed that though the immunising strain was recognised most strongly, the response to HA contained components which were widely cross reactive both within and between influenza A subtypes (Anders *et al.,* 1981). This group later reported similar findings following priming with live virus (Katz *et al.,* 1985). Interestingly they considered the HA2 subunit to be an important target for CD4$^+$ T cells, though responses to this subunit were not localised (Katz *et al.,* 1985). Later studies reported that the cross reactive element of the CD4$^+$ T cell response was I-E^d restricted (Brown *et al.,* 1987), whereas the major strain specific response was I-A^d restricted. (Brown *et al.,* 1988).

A third group leąd by Gerhard examined responses of T cell hybridomas derived from BALB/c mice primed either by IP inoculation of live PR8 (H1N1) virus, or by intradermal injection of inactivated PR8 virus in Freund's complete adjuvant. Using a large panel of antibody selected PR8 mutants they reported 3 types of HA specificity. The immunodominant group of hybridomas were sensitive to

mutations around residue 115 which is a conserved region amongst naturally occurring H1 influenza A viruses (though subject to antigenic drift in H2 and H3 influenza viruses), a second group of hybridomas were sensitive to mutations around residues 135 and 136 which is related to an antibody neutralising site and the third to residues 302-313 of PR8, this region is highly conserved amongst H1 influenza A viruses and is equivalent to a human $CD4^+$ T cell epitope from H3 influenza (Hurwitz *et al.*, 1984, Lamb *et al.,* 1982b). The group went on to identified another 5 epitopes 2 of which were I-E^d restricted (159-170, 174-185) and 3 of which were I-A^d restricted (195-209, 212-224, 269-280). There was no correlation between restriction element and propensity of site to drift mutation, and no determinants were found to the HA2 epitope which they regard as immunologically silent (Haberman *et al.,* 1990, Gerhard *et al.,* 1991). Indeed the authors considered that previously reported responses to HA2 (described above) were the result of impurities (Gerhard *et al.,* 1991).

Thomas and co-workers have performed the most detailed studies of $CD4^+$ T cell recognition of HA following priming with live virus. They originally described 33 clones derived from CBA mice following priming with nasal infection with X-31 (H3N2 external proteins and PR/8 internal proteins)(Mills *et al.,* 1986). Six were thought to be duplicates. Of the remaining 27 clones, 20 responded to the tryptic fragment of HA (residues 28-328) and 7 clones were probably restricted to either the N terminus of HA1 or the HA2 subunit (Mills *et al.,* 1986). The 20 clones responding to the trypsin cleavage fragment of HA were examined using a panel of H3 influenza A viruses. Twelve distinct specificities were recorded, varying from clones which only recognised viral strains very closely related to the immunising strain, to clones that were widely cross reactive amongst the H3 subtype. No type specific clones were described. The authors concluded that the majority of $CD4^+$ T cells induced after nasal priming of CBA mice with live virus were sensitive to drift mutations, and that extensive diversity existed in the $CD4^+$

T cell repertoire (Mills *et al.,* 1986). Using an incomplete panel of peptides based on A/Victoria/3/75 (H3N2) they localised the response of 3 clones to residues 53-63 (Mills *et al.,* 1986, Mills *et al.,* 1988). In a follow up study using a panel of 32 overlapping peptides spanning the sequence of A/Aichi/68 HA (H3N2) the responses of a further 5 of the twelve groups of T cell clones were localised (Burt *et al.,* 1989). Three of these were shown to be I-A^k restricted with specificities to 68-83, 120-139, 269-288 and 2 were I-E^k restricted with specificities 226-45 and 246-265. Interestingly the I-A^k restricted clones recognised regions of HA which were closely related to neutralising antibody sites E, A, and C respectively. The response to site 226-45 probably localised to residues 229-41 because of the pattern of response to their panel of influenza viruses (Burt *et al.,* 1989). This region is highly conserved amongst H3 influenza A viruses and most of it is buried within the tertiary structure of HA (Wilson *et al.,* 1981). The response to region 248 to 260 was thought to localise to residues 249-259 again a highly conserved region. The group then examined the response to HA on the I-A^d background (using BALB/c mice) following nasal priming with live virus (Barnett *et al.,* 1989a). Both T cell clones recognising internal viral proteins and surface glycoproteins were obtained, though the former group were not characterised. Of 67 $CD4^+$ restricted clones established from 9 individual mice 5 clones were widely cross reactive and were restricted to either the N-terminus of HA or to the HA2 subunit. When the clones were analysed by both their reactivity to a panel of natural influenza strains and a panel of mutants derived by monoclonal antibodies a total of 31 distinct specificities was evident, suggesting extensive diversity in the $CD4^+$ T cell response especially as no duplicated responses were seen between mice (Barnett *et al.*, 1989b). $CD4^+$ T cell clones derived from 5/9 mice recognised only antibody neutralising sites B (residues 177-199, and 182-199) and 2 mice only recognised site E (residues 56-76). Within these specificities clones were sensitive to drift mutation. All responses were I-A^d restricted. The authors concluded that $CD4^+$ T cells and B cells recognise closely associated

epitopes (Graham *et al.,* 1989). Further analysis of the remaining clones revealed that their responses localised to sites closely related to antibody neutralising sites, and all were sensitive to natural drift substitution; residues 56-76 (site E), 81-91 (site E), 177-99 (site B), 206-227 (site D). (Barnett *et al.,* 1989b). The group later reported that within the major immunogenic region 177-199 there were 2 overlapping I-A and one I-E specificity (Barnett *et al.,* 1990). This group have also reported a Th clone which recognises a conformational determinant on HA1 (Mills *et al.* 1986b).

Diversity of murine CD4$^+$ T cell recognition of HA

The murine CD4$^+$ T cell response to HA is reported to be diverse: For example Gerhard and co-workers reported a large degree of functional diversity amongst the T cell hybridomas specific for residues 110-120 of PR8, which affected both their fine specificity (Haberman *et al.,* 1990) and their TCR gene usage (Taylor *et al.,* 1990). Ffrench and co-workers produced 15 CD4$^+$ T cell clones from BALB/c or DBA/2 mice immunised with a C-terminal fragment of HA1 (residues 305-328) (Ffrench *et al.,* 1989). Responses were focused onto residues 306-319 but their fine specificity and requirement for N and C terminal residues differed. Thomas and co-workers reported that T cell clones raised from different congenic mice differ in their dominant epitope recognition and that there was no obvious structural homology between the epitopes (Smith *et al.,* 1994a). In contrast individual clones raised from single mice differed both in their fine specificity to individual dominant epitopes as determined by panels of naturally occurring and laboratory mutant influenza A viruses (Smith *et al.,* 1994a). Most surprisingly clones derived from a single mouse with different fine specificities had identical productive rearrangements of their TCR Vα and Vβ genes (Smith *et al.,* 1994a). The group's explanation of these findings was that the clones may have originated from a common precursor cell, but subsequently differed in their expression of

accessory molecules, causing some clones to be more tolerant than others of residue substitutions (Smith *et al.,* 1994a, Smith *et al.,* 1994b).

Human CD4$^+$ T cell recognition of influenza A

Less detailed work has been carried out on the human CD4$^+$ T cell response to influenza A. Human CD4$^+$ T cells probably recognise most viral proteins. Lamb and co-workers (1982a) raised a panel of 11 human CD4$^+$ T cell clones specific for A/Texas/1/77 from an adult donor (DR1,3) who had been recently infected with influenza. The clones were obtained by *in vitro* restimulation with formalin inactivated A/Texas/1/77 virus: 5 clones were specific for NA, 4 were specific for matrix protein, one was specific for HA, and one clone was specific for NP. The HA specific clone was subtype specific, the NA restricted clones recognised H3N2 and H2N2 influenza viruses, the matrix and NP specific clones were widely cross reactive amongst human influenza A viruses (Lamb *et al.,* 1982a , Lamb *et al.*, 1982b).

Similar findings were reported by Sterkers and co-workers (1985), who studied the responses of one long term CD4$^+$ T cell line and 5 CD4$^+$ T cell clones obtained form 3 donors who had been vaccinated 4 wks previously with a trivalent influenza vaccine containing inactivated A/Bangkok (H3N2) A/Brazil (H1N1), and B/Singapore/22/79 (influenza type B) (no further details regarding vaccine strains given in paper). The line had clear T helper activity and the clones (though CD4$^+$) were cytolytic. Three clones were broadly cross reactive amongst influenza A viruses, and probably were restricted to internal proteins. One was specific for NA, and one clone and the line (which was DR1 restricted) were specific for HA. Both the HA specific clone and the line responded to H3 and H4 influenza A viruses but not to H1 or H2 influenza A viruses (Sterkers *et al.* 1985).

A third group lead by Braciale produced CD4$^+$ human A/Japan/57 (H2N2) specific T cell clones with cytotoxic activity from one donor (Kaplan *et al.,* 1984). The clones were DR1,5 restricted and showed 3 specificity patterns: Type specific,

subtype specific, and closely related strain specific. None responded to influenza type B. In a follow up study the influenza type A specific clone was shown to be NP specific, and that the other 2 clones to be HA specific (Brown *et al.,* 1991). Fleischer, and co-workers (1985) reported similar findings: 4 $CD4^+$ T cell clones with CTL activity, raised from a single donor (DW1 Dw6) and selected with A/USSR/90/77(H2N2). A large panel of viruses was employed to demonstrate that one clone was HA specific. This clone was widely cross reactive amongst influenza A viruses (H1 - H9 inclusively were tested). Two clones were NP specific, one of these was type specific, and the other responded only to human H1N1, H2N2, and H3N2 viruses. The final clone was matrix protein specific and had a similar pattern of reactivity to the second NP clone (Fleischer *et al.,* 1985).

Fine specificity of human $CD4^+$ T cell response to influenza A

Studies on the fine specificity of the human $CD4^+$ T cell response to influenza have been very limited. Brett and co-workers (1991), reported a high frequency of response to several highly conserved regions of NP using unselected PBMC and a panel of NP specific peptides 13-24 residues in length. In this study the most frequent response was to residues 206-229 (Brett *et al.,* 1991). Rodda, and co-workers (1993) have reported similar findings in an ambitious preliminary study using unselected peripheral blood mononuclear cells and precursor frequency analysis. In both studies the MHC class II haplotypes of the donors was not reported.

There have been no detailed systematic studies of the human $CD4^+$ T cell response to HA and the fine specificity of only 5 T cell clones and one T cell line obtained from only a single donor, who responded strongly to A/Texas/1/77, has been examined in any detail (Lamb *et al.*, 1982b). The clones were raised by *in vitro* restimulation with HA A/Texas/1/77. Epitope mapping was conducted using an incomplete panel of peptides derived from the closely related X-47 virus. These

peptides were designed to map human antibody responses to HA, and therefore covered most of the surface of the 3D structure of HA, but were incomplete in their coverage of internal regions of HA1 and did not span HA2 (Green *et al.*, 1982). The response of the unselected PBMC was dominated by a strong recognition of influenza B, which was twice the magnitude of the response to influenza A. The PBMC response to influenza A was directed to the C-terminus of HA1, residues 306-318. An HA A/Texas/77 $CD4^+$ T cell line and 4 specific $CD4^+$ T cell clones were then selected with HA A/Texas/77 or whole virus, and grown for 8 wks in culture prior to testing their proliferative responses to whole virus, HA Texas, or the panel of peptides. The line and all the clones did not respond to influenza B virus. The strongest response of both the line and of 3/4 clones was to the C-terminus of HA1, and in particular to residues 306-318. Three other regions of HA1 (residues 1-38, 105-40, and 200-228) induced powerful responses in the T cell line. The response of one clone could not be determined. By comparing the response of the 3 clones with specificity for residues 306-318 the authors were able to demonstrate that the presence of at least 2 different antigenic specificities within this region (Lamb *et al.*, 1982b). A follow up study reported the response of 2 new clones, one was specific for residues 104-39 (Lamb and Green 1983). This clone responded to A/Texas/1/77 (H3N2) and A/Victoria/3/75 (H3N2) but not A/Aichi/2/68 (H3N2), nor to A/Japan/305/57 (H2N2), and A/PR/8/34 (H1N1). There are many residue substitutions between H3 and H1 and H2 influenza A viruses in this region, however the structure of A/Texas/1/77 and A/Victoria/3/75 are identical and only 2 residue substitutions occur between A/Texas/1/77 and A/Aichi/68: 123 ^{N} to ^{T} and 127 ^{N} to ^{T} (non-conservative) implying that the response may have involved one or both of these sites. The second new clone recognised only A/Texas/1/77 but its response could not be localised. It was thought to have either responded to an epitope not covered by the incomplete panel of peptides, the HA2 subunit, or a conformational motif.

Braciale and co-workers were able to localise the response of one of their 2 HA specific T cell clones (Brown *et al.,* 1991), by using an incomplete panel of peptides spanning the N terminal region of HA1 to residues 129-140 and showed that the response was DRW11(5) restricted. This region is the subject of antigenic drift in H2N2 influenza A viruses. Detailed examination of a range of naturally occurring H2N2 viruses followed by subsequent sequencing of the corresponding region of HA1 showed that strains with no residue changes were recognised whereas those with residue substitutions were not, and these findings were confirmed using a panel of synthetic peptides with single base substitutions.

Rodda and co-workers (1993) as part of the preliminary study discussed above on the response to NP also examined the response to HA. The results suggested recognition of several conserved regions of HA including regions in the HA2 subunit. Responses within the HA1 subunit were not localised which in many instances makes the differentiation between conserved and variable recognition impossible. This study was also hampered by the use of short peptides (13 residues) which were not tested for mitogenic activity.
Previous studies of human $CD4^+$ T cell recognition of HA have therefore been very limited, though there was an indication that the response may be primarily directed towards conserved regions of the molecule. However as Thomas has observed (B. Thomas, personal communication) these studies have generally used influenza viruses and/or peptides derived from strains which have not circulated for several yrs, which has introduced bias in favour of such a finding.

Diversity of human $CD4^+$ T cell response to HA

There are preliminary indications that the human $CD4^+$ T cell response to HA might be less diverse than the murine response to this antigen. Faith and co-

workers isolated a panel of 7 clones from a single donor which were specific for HA residues 255-270, the clones differed in their fine specificity within this region. Two of these clones, one being DQB1*0602/DQA1*0102 restricted and the other DRB1*1001 restricted, had very similar Vα and Vβ gene usage, differences only occurring in their junctional regions. Four other clones restricted by DRB1*1001 used closely related Vβ genes though their Jβ and Vα gene usage was diverse (Jones *et al.,* 1994). A remarkable conservation in Vβ3 gene usage has recently been reported in $CD4^+$ T cell lines and clones derived from 2 unrelated donors which were raised against HA region 300-323 (Prevost-Blondel *et al.*, 1995).

1.5 AIMS

The primary aims of this study were:

(1) To investigate the human $CD4^+$ T cell repertoire for HA following recent natural influenza infection in adults. In particular to examine whether $CD4^+$ T cells select HA epitopes from regions of the molecule which are closely related to neutralising antibody sites and subject to drift mutation, or whether they focus on more conserved HA sequences.

(2) To examine $CD4^+$ T cell memory by comparing $CD4^+$ T cell recognition of HA in recently infected donors with that of donors with no recent history of influenza infection.

(3) To investigate the adult $CD4^+$ T cell response to HA following vaccination with a currently available subunit influenza vaccine.

(4) To examine the stability of the $CD4^+$ T cell memory response to HA by re-examining donors one and two yrs after natural infection and vaccination.

(5) To explore the MHC genetics of the human $CD4^+$ T cell recognition of HA in an outbred adult population.

Table 1.1, Prevelance of HLA alleles, in Oxfordshire

HLA-A	% Subjects expressing allele	HLA-B	% Subjects expressing allele	HLA-BW	% Subjects expressing allele	HLA-C	% Subjects expressing allele	HLA-DR	% Subjects expressing allele	HLA-DRW	% Subjects expressing allele	HLA-DQ	% Subjects expressing allele
1	31.57	7	26.06	4	59.32	1	5.08	1	18.86	51	29.66	2	41.95
2	52.75	8	23.52	6	86.65	2	7.84	103	3.18	52	57.63	4	3.6
3	27.33	13	3.18			3	11.23	4	35.81	53	54.45	5	28.18
11	13.98	14	2.75			9	5.08	7	25.42			6	40.68
23	2.12	17	0.21			10	6.99	8	4.03			7	34.96
24	15.47	18	7.2			4	18.22	9	1.91			8	18.01
25	3.18	27	9.11			5	20.34	10	1.69			9	8.26
26	3.39	35	15.04			6	19.07	11	13.56				
28	3.81	37	3.39			7	47.46	12	2.12				
29	6.36	38	1.91			8	8.26	13	18.01				
30	3.18	39	4.24			15	0.42	14	4.45				
31	5.72	40	0.85			16	1.06	15	29.24				
32	5.93	41	0.85			1203	0.42	16	0.85				
33	2.33	44	29.24			7/8v	0.32	17	26.27				
34	0.42	45	1.48			702v	0.21						
66	0.21	47	0.21			14	0.21						
68	3.39	49	2.75			12	0.21						
74	0.21	50	1.91										
		51	7.63										
		52	0.64										
		53	0.85										
		55	2.75										
		56	0.85										
		57	9.32										
		58	0.64										
		60	11.02										
		61	2.54										
		62	11.23										
		63	0.42										
		64	1.27										
		65	6.14										
		70	0.85										

Table shows frequency of expression of individual HLA alleles in an Oxfordshire population. Data was collected from 472 individuals by Dr Ken Welsh and Dr Mike Bunce, Tissue Typing Laboratory, Churchill Hospital, Oxford (personal communication). As most donors express two different HLA alleles at each locus percentages add up to more than 100%. Data was collected before 1994 revision to HLA nomenclature (Bodmer *et al.* , 1994). HLA-DR17 is now classifed as DRB1*030X. The DRW allels have also been renamed thus HLA-DRW51 = DRB*50X, -DRW52 = DRB3*0X, and -DRW54 =DRB4*0X.

Table 1.2, Summary of RNA segments and proteins of influenza A

RNA Segment	Length (nucleo-tides)	Protein	Length of nascent peptide, and Structure	Function(s)	Approximate number of molecules per virion
1	2341	PB2	757 aa. Forms complex with PB1 and PA	Viral RNA Transcription.	30-60
2	2341	PB1	759 aa. Forms complex with PB2 and PA	Viral RNA Transcription.	30-60
3	2233	PA	716 aa. Forms complex with PB1 and PB2	Viral RNA Transcription.	30-60
4	1778	HA	566 aa. Trimer of non-covalently linked monomers. Each monomer made up of 2 disulphide bond linked subunits formed by proteolytic cleavage of precursor molecule	(a) binding to sialic acid containing receptor on susceptible host cell surface (b) fusion of endocytosed particle with endosomal membrane liberating viral cores into the cytoplasm. Requires proteolytic cleavage. (c) major target of host neutralising antibodies	500
5	1565	NP	498 aa.	Interacts with RNA to form ribonucleoprotein particles (RNP)	1000
6	1413	NA	454 aa. Forms homotetramer	Cleavage of alpha-ketosidic link between terminal sialic acid and an adjacent D-galactose or D-galactosamine, allowing virus to escape from sialic acid containing structures (eg mucin), allowing access to target cells. Facilitation of HA cleavage Destruction of HA receptor facilitating viral particle escape, prevention of progeny virus reabsorbtion Prevention of viral self-agglutination by removal of sialic acid from complex carbohydrates	100
7	1027	M1	292 aa.	Probably underlines lipid membrane and adds integrity to the bilayer. May play important role in virus assembly.	3000
7, by alternative RNA splicing	1027	M2	97 aa. Tetramer	Membrane protein. probably acts as proton channel to control Golgi pH during HA synthesis, and to allow acidification of centre of virion during uncoating	20-60
8	868	NS1	230 aa.	Probably involved in the shutoff of host cell protein synthesis or synthesis of vRNA	
8, by alternative RNA splicing	868	NS2	121 aa.	Unknown	

Abbreviations: PB2= polymerase associated protein basic-2, PB1= polymerase associated protein basic-1, PA= polymerase associated protein acidic, HA= haemagglutinin, NP= nucleoprotein, NA= neuraminidase, M1= matrix protein 1, M2= matrix protein 2, NS1= non-structural protein 1, NS-2= non-structural protein 2

Chapter 2, Materials and Methods

2.1 Donor Details

I Selection and characterisation

II MHC typing:

(a) Overview

(b) DNA extraction

(c) Molecular MHC Class I and II tissue typing by SS-PCR

2.2 Trivalent Influenza Vaccine and Vaccination Procedure

I Subunit vaccine

II Vaccination procedure

2.3 Antigens, and T Cell Stimuli

I Haemagglutinins

II Panel of Influenza A viruses

III Synthetic peptides:

(a) Design of HA A/Beijing/32/92 peptide panel

(b) Method of synthesis

IV MTSE

V Polyclonal T cell stimulants

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(b) anti-CD3 antibody

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2.8 T Cell Proliferation Assays

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II Bioassay for IL-2

III ELISA for IL-4 and IFN-γ

2.10 MHC Class II Binding Assays Using HA Peptides

I HLA-DRB1* purification

II Peptide binding studies

2.1 Donor Details

I. Selection and characterisation

The response to influenza A HA of 4 groups of unrelated healthy volunteer adult donors was investigated. Most were medical students or employees of SMHMS, or personal friends of C.M.G. or B.A.A..

1) The first group, of 18 donors, was used in preliminary studies of HA responsiveness during September/October 1993, all were employees of SMHMS.

2) The second group of 12 donors had a history of influenza infection during November/December 1993, a time when A/Beijing/32/92 like influenza strains were known to be circulating in London.

3) The third group were 8 donors who mounted responses to HA A/Aichi/68 in polyclonal assays but had no history of influenza for at least 4y.

4) The fourth group of 12 donors kindly agreed to be vaccinated with a trivalent influenza subunit vaccine.

The donors will be described in detail in the individual experimental chapters.

II. MHC typing

a) Overview

Serological tissue typing, which was used for the preliminary studies on HA responsiveness (reported in Chap. 3), and for a few donors in the remaining studies (mostly class I MHC typing), was kindly performed by Mieke Van Dam in the Tissue Typing Laboratory at St Mary's Hospital, London. Dr Ken Welsh very kindly allowed me to perform MHC class I and II typing of the remaining donors, in his laboratory at the Churchill Hospital Oxford, using sequence specific PCR (PCR-SSP)(Bunce *et al.,* 1995). Other than in Chap 3 where donors were typed serologically, the donors' tissue types are presented in the format recommended in "The Nomenclature For Factors Of The HLA System 1994"(Bodmer *et al.,* 1994).

b) DNA extraction

DNA was extracted from frozen PBMC. 2-5 x10^6 cells were pelleted in a 1.5ml Ependorff tube by centrifugation for 30s at 10,000 G, and resuspended in 1.5ml of red cell lysis buffer (RCLB = 0.114M NH_4Cl, 1.0mM$NaHCO_3$). After 60s incubation the sample was centrifuged as above, and the resulting cell pellet resuspended in 640ml of proteinase K buffer (PKB = 100mg proteinase K in 10mls of 1% w/v SDS, 2mM Na_2EDTA pH 8.0- this buffer was stored frozen in aliquots at -20°C), and incubated for 60 min at 55°C in a rotary incubator. 100µl of 6m NaCl was added, and the sample mixed by inversion and vortexed for 6s. Chloroform extraction was followed by centrifugation at 10,000 G for 4 min. The aqueous phase (containing DNA) was carefully removed and transferred to a fresh Ependorff. DNA was precipitated by the addition of 1ml of 95% ethanol and gentle rocking (rather than vortexing in order to prevent DNA shearing). Ethanol was carefully removed leaving DNA, which was washed twice in 70% ethanol. After removal of residual ethanol in a rotary vacuum dryer, DNA resuspended in 150µl H_2O.

c) Molecular MHC class I and II typing by Sequence Specific-Polymerase Chain Reaction (SS-PCR)

SS-PCR tissue typing was performed using a panel of sequence specific primers and the methods developed by Drs M. Bunce and K. Welsh (Bunce *et al.,* 1995). Individual reactions were performed in 13µl volumes, containing 5µl of diluted primers and 8µl of reaction mixture (see below). Tissue typing was performed using pre-aliquoted primers in strips of "96 well" PCR tubes which were stored at
-70°C prior to use.

Because of the small volumes of individual reactions, master mixtures were used and added to the primers in aliquots. To perform standard Class II MHC HLA-DRB1* and HLA-DQB1*typing a 40x master mix was made up as described below and 8µl added to each of 32 pre-aliquoted primer pairs: Reaction mixture for this standard class II MHC typing contained 10µl of DNA solution, 109µl of H_2O, 200µl of TDMH (Taq buffer-deoxyribonucleotides-magnisium-H_20)(see below), and 1µl (3 units) of Taq DNA polymerase (Cetus or Promega).

TDMH was premixed and stored in 0.65ml aliquots at -70°C. 0.65 ml contained: 169µl 10x PCR buffer (=10x solution =670mM Tris Base pH8.8, 166mM Ammonium Sulphate, 0.1% Tween), 12µl of 10x dNTPs (=100mM mix of all 4 nucleotides), 135µl 25mM $MgCl_2$, 334µl H_2O.

MHC class I and HLA DQA1* typing were performed by the same method though the numbers of reactions performed differed (96 and 8 respectively).

All reactions were carried out in a Cetus 96 well thermocycler with pre-heated lids, an overlay of mineral oil was not employed. Thermocycling parameters were as follows: 96°C 1 min followed by 5 cycles of 96°C 20s, 70°C 45s, 72°C 25s, followed by 21 cycles of 96°C 30s, 65°C 50s, 72°C 30s, followed by 4 cycles of 96°C 30s, 55°C 60s, 72°C 120s, followed by a final cycle of 72°C for 5 min.

Reaction products were size fractionated on a 1% agarose gel containing ethidium bromide and photographed under UV light.

2.2 Trivalent Influenza A Vaccine And Vaccination Procedure

I Subunit vaccine

Influvac a trivalent subunit influenza vaccine contained highly purified HA and NA from A/Beijing/32/92 (H3N2), HA A/Singapore/6/86 (H1N1), HA B/Panama/45/90 (influenza B). The vaccine contained 15µg of each HA. The vaccine was kindly donated by Dr R. Brands and Solvay Duphar, Weesp, The Netherlands.

II Vaccination procedure

Informed consent was obtained from every subject prior to vaccination. Ethical approval was obtained from the ethics committee of St Mary' Hospital. Subjects received 0.5ml of Influvac by I.M. injection into the deltoid muscle according to the manufacturers instructions.

2.3 Antigens, and T Cell Stimuli

I Haemagglutinins

Highly purified egg derived X117 recombinant A Beijing/32/92(H3N2) HA was a generous gift of Dr R. Brands, Solvay Duphar B.V., Weesp, The Netherlands. The preparation contained the entire HA1 (amino acid residues 1-328) and HA2 (residues 329-550) subunits and was contaminated with a trace of nucleoprotein.

Purified bromelain cleaved HA A/Aichi/68 (H3N2) was the generous gift of Dr A. Hay and Dr J. Skehel, N.I.M.R., Mill Hill, London, UK. (Brand and Skehel, 1972) and contained residues 1-504 (Wharton *et al.*, 1989)).

Both haemagglutinins were tested for mitogenicity using a 3 wk human $CD4^+$ T cell line specific for *Mycobacterium tuberculosis* soluble extract (MTSE) (details in Chap. 4).

II Panel of Influenza A viruses

The following freeze dried viruses were a kind gift of Dr P. Chakraverty (PHLS, Colindale, London, UK.): A/Formosa/1/47 (H1N1), A/Taiwan/1/86 (H1N1), A/Fiji/2/88 (H1N1), A/Singapore/1/57 (H2N2), A/Victoria/3/75 (H3N2), A/Bangkok/1/79 (H3N2), A/England/427/88 (H3N2). HA titres varied between 1/160 and 1/2560 HAU/ml.

III Synthetic peptides

a) Design of HA Beijing/32/92 peptide panel

A series of overlapping peptides were designed based on the nucleotide sequence of the HA1 subunit of A/Beijing/32/92, and the HA2 subunit of the closely related A/Hong Kong/90. Unpublished sequence information from both viruses was kindly supplied by Dr N. Cox (CDC, Atlanta, GA, USA). A/Hong Kong was used as the template for HA2 peptide synthesis, on Dr Cox's advice, as no sequence information was available on the HA2 subunit of A/Beijing/32/92.

Following advice from Prof. D. Wraith (Dept. Pathology, Bristol University) the peptides were specifically designed to avoid glycine or proline as one of the final two C-terminal residues. Both amino acids in these positions allow the reaction of the emerging peptide with its synthesis resin, resulting in possible residue deletion(s) and a low yields.

Additionally PMC arginine and tryptophan Boc were used rather than the alternatives supplied by Cambridge Research Biochemicals as both are superior compounds - see below. This prevented either from being used as a C-terminal residue, as neither was available pre-coupled to the KB resin. These were the only restrictions on peptide design, in particular no bias in design was introduced and results of previous studies of HA responsiveness and known class II MHC peptide binding motifs were not taken into consideration. Peptides were 16 residues in length, overlapped usually by 11 residues, and spanned the entire sequence of the HA precursor molecule HA0.

The composite primary amino acid sequence used to synthesise the peptides and individual peptide sequences are shown in Fig. 2.2; HA1 encompass residues 1-328, residue 329 is cleaved by host proteases, and HA2 encompasses residues 329-550. Peptides were initially employed in pools of 5 peptides. Individual peptides are numbered according to their N-terminal residue. Peptide pools are numbered in the text by the N-terminal residue of their N-terminal peptide, and the N-terminal residue of their C-terminal peptide. In order to simplify the figures, peptide pools listed on the X-axis of graphs are numbered only by the N-terminal residue of their N-terminal peptide.

b) Method of synthesis

With the exception of the two peptides used in Chapter 3 which were synthesised by Dr J.Hayball using an Applied Biosystems Peptide Synthesizer, I synthesised every peptide used in this study using F-moc chemistry on a Multipeptide Synthesis block BT7400 (Cambridge Research Biochemicals, Wirral, UK) with Pepsyn KB resins according to the methods of Prof. D. Wraith (Fairchild *et al.*, 1993). This solid phase system uses a base labile linker. Its advantage over more conventional resin systems is that it allows thorough washing of completed deprotected peptides while attached to the resin, allowing efficient removal of residual TFA and scavengers. The basic principle of synthesis is that amino acids are added to the resin in a sequential C to N terminal manner. The N-termini of each amino acids is protected by a Fmoc (fluorenylmethylmethoxycarbonyl) group, preventing self-coupling and polymer formation. The Fmoc group is subsequently cleaved by piperidine in dimethylformamide (DMF) to reveal the reactive ester. Because the side

chains of some amino acid are reactive, they are shielded by various protective groups (e.g. t-butyloxycarbonyl (Boc)). These are acid labile and are removed at the end of synthesis by TFA treatment. To ease synthesis all amino acids except arginine were used as preactivated esters of pentaflurophenyl (OPFp) or oxo-benzotriazine (ODhbt). Arginine is a particular problem in peptide synthesis as it is difficult to remove its conventional protecting group (Mtr), and this becomes increasingly difficult if more than one arginine residue is present in a peptide. To circumvent this PMC-protected arginine was used. This is not stable as an ester and has to be preactivated {to preactivate 5 equivalents of PMC arginine, PYBOP (Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium) and HOBT (1-Hydroxybenzotriazole) were dissolved in DMF, and 10 equivalents of diisoethylamine added. The reaction was mixed by inversion for 60s prior to addition to the resin}. Peptide synthesis took place on small filters placed inside wells of a Multipeptide Block positioned in a fume hood. The block was linked to a water vacuum pump and a cold solvent trap, allowing rapid removal of reagents. 100mg resin pre-coupled to the first amino acid was washed with DMF four times, then activated with 1ml of 20% piperidine in DMF (two 0.5ml aliquots for 5 min each), washed six times in DMF and dried twice in ether, the second amino acid ester was then added in HOBT/DMF (25 mg/ml) and incubated for one hour, this was followed by 5 washes in DMF, and a return to the start of the cycle for the addition of the next amino acid. This was repeated until the synthesis was complete, and the Fmoc group had been removed from the N-terminal residue. Full length peptides were deprotected with trifluroacetic acid/phenol/ thioanisole/ ethanedithiol in the ratio of 94/2/2/2, and cleaved from the resin using 0.4 M NaOH for 2h. The resulting solutions were neutralised with HCl to yield peptides in saline solution and pH adjusted to 6-8. Peptides were synthesised in batches of 48. Insoluble peptides had their pH increased to pH10. If they remained insoluble they were freeze dried taken up in 100µl formic acid and then into H_20.

Peptides were quantified using a micro BCA assay kit according to the manufacturers instructions (Pierce). Five peptides were chosen at random and run on reverse phase HPLC (high pressure liquid chromatography) using a C18 (Applied Biosystems) and a linear gradient of 1.25% acetonitrile per min in 0.08% trifluroacetic acid in H_20.

Dominant single peaks were obtained though there was variation in the presence of impurities. Figure 2.1 shows the result of the HPLC analysis of one peptide (peptide 268-283).

HA peptides were tested for mitogenic activity as described for HA- see Chap 4, Fig. 4.6.

IV Mycobacterium Tuberculosis Soluble Extract (MTSE)

MTSE was a gift from the MRC Tuberculosis and Related Diseases Unit at the Hammersmith Hospital, London,. UK. It was reconstituted in PBS, filtered through a 0.2μm Millipore filter and stored at -20°C in a stock solution at 1mg/ml.

V Polyclonal T cell stimulants

a) PMA

Phorbol myristate acetate (PMA) was purchased from Sigma and stored at -20°C.

b) Anti-CD3 antibody

Murine OKT-3 anti-CD3 antibody used for T cell activation (ECAC, Porton Down, Salisbury, UK).

2.4 Cytokines

I Human recombinant Interleukin 2

Human recombinant IL-2 was a kind gift of Biogen, England.

II Lymphocult

"Lymphocult T-Lectin Free" (Biotest Folex, Frankfurt, Germany) was used as a source of IL-2. Following batch screening in proliferation assays over a range of concentrations, it was used at an optimal concentration of 10% v/v. This preparation is obtained from PHA stimulated human lymphocytes and predominantly contains IL-2, but includes other cytokines.

2.5 Antibodies

I Anti-human lymphocyte surface markers

Cytofluoremetric analysis was performed using the following fluorescein (FITC)-conjugated, affinity labelled, murine anti-human monoclonal antibodies:

a) anti-CD3 (OKT3) (Ortho Diagnostic Systems, Raritan, NJ, USA).

b) anti-CD25 (Dako, Glostrup, Denmark).

c) anti-CD2 (Becton-Dickinson, Oxford, UK).

d) FITC-conjugated mouse IgG1 (Becton-Dickinson) as a control for non-specific binding.

The following murine monoclonal antibodies were used for MHC class II restriction studies. The antibodies were isolated from hybridoma culture supernatants and purified using protein A Sepharose columns (Pharmacia), and standard methods (Harlow and Lane 1988):

e) anti-HLA-DR (L243) (Lampson and Levy 1980).

f) anti-HLA-DP (B7/21) (Watson *et al.*, 1983).

g) anti-HLA-DQ (SPV-L3), a gift from Dr H.Spitz (DNAX, Palo Alto).

Murine anti-human-CD3, -CD4, -CD8 used for FACS analysis of the developing T cell lines are described below (section 2.8).

2.6 Lymphocyte Culture

I General techniques

All procedures unless otherwise stated were carried out at room temperature in a class II MATS laminar flow tissue culture hood, using standard sterile techniques. Cells were incubated in a copper-lined, humidified Heraeus incubators (95% humidity) at 37^{o} C, in 5% CO_2 in air.

II Media

The following media were used for cell culture:

(a) Basic medium: RPMI 1640 supplemented with 2g/L of sodium bicarbonate (Gibco, Life Technologies, Paisley, Scotland)

(b) Heparinised medium: Basic medium with additional 10 units/ml preservative-free sodium heparin (CP Pharmaceuticals Ltd, Wrexham, UK).

(c) Complete medium: Basic medium supplemented with 2mM L-glutamine (Gibco) and 100 IU/ml penicillin/streptomycin (Gibco), and 5% screened, heat-inactivated human AB^{+} serum (Sigma Ltd Poole Dorset.). AB^{+} serum from a single batch was used for all experiments (batch number 43H0211). This was screened in standard proliferation assays to exclude the presence of inhibitors or mitogens.

(d) FCS medium: Basic medium supplemented with 2mM L-glutamine (Gibco) and 100 IU/ml penicillin/streptomycin (Gibco), and 10% heat-inactivated foetal calf serum (Gibco).

All sera were heat inactivated at 56^{o} C for 30 min in a water bath, and stored at -20^{o}C in aliquots.

III Peripheral blood mononuclear cell separation

PBMC (peripheral blood mononuclear cells) were isolated from 120 ml of fresh heparinised peripheral venous blood, using centrifugation on a discontinuous Ficoll-Paque gradient (Pharmacia Biotech Ltd., Milton Keynes, UK): Fresh whole blood was mixed with an equal volume of heparinised medium and 20ml layered on top of an equal volume of Ficoll-Paque in polypropylene-glycol tube (Falcon 2070 Blue Max, Becton-Dickinson). Centrifugation was conducted at 750G for 20 min at room temperature.

PBMC were removed from the plasma/density gradient interface, transferred to a fresh polypropylene-glycol tube to which at least an equal volume of heparinised medium was added. Samples were centrifuged at 500G for 15 min. Cell pellets were resuspended in 1ml of heparinised medium to which 49ml of heparinised medium was added and the sample mixed by inversion and centrifuged at 300G rpm for 15 min. The PBMC were resuspended in a total of 20ml of complete medium. The number of viable cells was determined using white cell counting fluid (2% glacial acetic acid with gentian violet). Yields varied from donor to donor but were normally about 2.0 $x10^6$/ml (range 0.8-3.0 $x10^6$ cells/ml venous blood).

IV Cryopreservation of cells

PBMCs and T cell lines not used for cell culture were frozen in dimethyl sulfoxide (DMSO)(Sigma Pharmaceuticals, Poole, UK) in FCS. A 15% solution of DMSO in FCS(85%) was added dropwise, with gentle agitation to the cells (at a concentration of 20-40 $x10^6$/ml) in ice cold complete medium. One ml aliquots of this cell suspension was rapidly dispensed into cryovials (Nunc, Gibco), placed in a polystyrene box, frozen overnight at -70^oC, and stored at -180^oC, in the vapour phase of liquid nitrogen.
Cells were thawed rapidly in a 37^oC waterbath, and transferred to a 20ml universal container (Sterilin). Five ml of complete medium were added dropwise with continuous mixing and then a further 5ml added rapidly. The cells were washed, centrifuged at 300G for 10 min, and resuspended in 10ml of complete medium, mixed by inversion, centrifuged at 300G for 10 min. The cell pellet was resuspended in 5 ml complete medium and counted using Trypan Blue (Sigma) exclusion as an index of viability. Viable cell recovery varied between 50-90%.

V Preparation of antigen presenting cells

Autologous frozen PBMC were routinely used as APCs. Immediately before use they were thawed (as above), and irradiated (3000 rads) in a γ-ray cell irradiator (Gamma Cell 1000 elite, Nordion International, Inc., Kanata, Ontario).

VI T cell culture (see Chap 4 for development)

Freshly isolated PBMC were cultured with 0.1μg/ml HA A/Beijing/32/92 (HA A/Beijing/32/92) at a density of 5 x10^6 cells/ 2ml well in complete medium. At 7d the cells were recovered and washed twice using a minimum of 30 ml of plain medium. T cells were restimulated at a density of 1 x 10^6 cells/ 2ml well in complete medium using an equal number of freshly thawed autologous irradiated (3000 rads) PBMC as APC. The APC were prepulsed with 0.1 μg/ml HA Beijing/32/92 (2nd *in vitro* stimulation) or 0.01μg/ml (3rd and 4th *in vitro* stimulations) for 1h immediately prior to use. Lymphocult was added as a source of IL-2 at 24h and 72h. Additionally 1ml of medium from each well was exchanged at 72h with fresh complete medium. T cell lines were maintained in culture for 3 to 4 wks, with a 7d cycle of antigen restimulation as described above.

2.7 T cell proliferation assays

I Polyclonal responses

PBMC (5x10^5/well) were cultured in complete medium {RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin (Gibco, Life Technologies, Paisley, Scotland) and 5% screened, inactivated human AB^+ serum (Sigma, Poole, Dorset, England)}with HA A/Aichi/68 and HA Beijing/32/92 (0.1, 0.5, 1.0, 5.0, 10μg/ml), in triplicate wells of round bottomed 96 well tissue culture plates. Cells were incubated in a copper-lined, humidified Heraeus incubators (95% humidity) at 37° C, in 5% CO_2 in air. On d7, T cell proliferation was measured by pulsing the cultures with tritiated methyl thymidine (1μCi/well, [^{3}H]TdR; Amersham International, Amersham, UK: Specific activity 35Ci/mmol, concentration 1mCi/mmol) and the cultures were harvested (Microcell Harvester, Skatron, Norway) onto nitrocellulose filters (Wallac, Turku, Finland) 8h-16h later. Proliferation was measured by [^{3}H]TdR incorporation and

liquid scintillation spectroscopy (1205 Betaplate, Pharmacia, LKB). A response of 5x the geometric mean (gm) of the background proliferation was taken to be significant.

II Response of T cell lines to HA and HA peptides

HA specific peptide proliferation was examined at 7d, 14d, and 21d. Freshly thawed autologous, irradiated (3000 rads) PBMC (4 $x10^4$ cells/well) were incubated in triplicates with antigen (HA A/Beijing/32/92, 0.01 to 0.1 μg/ml, or HA A/Aichi 1.0 μg/ml), or peptide pools (5 peptides at an individual concentration of 10 μg/ml) for 1h, at 37°C, 5% CO_2 in complete medium and subsequently cultured with $4x10^4$ responder T cells/well in round bottom 96 well plates in a total volume of 200μl. At 48h the cells were pulsed with [^{3}H]TdR 1μCi/well (Amersham International PLC, Amersham, UK) and harvested 16h later. Proliferation, was measured as above. T cell reactivity to individual peptides (10 μg/ml) was assayed at 21d or 28d as described above.

Fig. 4.4 (page 100) shows the results obtained from Donor A $CD4^+$ T cell line at 7d, 14d, 21d using these techniques, and the results of epitope mapping using single peptides at 21d. The results from this donor are typical of those from the majority of donors subsequently examined. There is a progressive fall in the background responsiveness over 3 wk in culture, with the emergence of clear response HA peptide response patterns. Wherever possible responses were examined at 21d.

III MHC restriction studies

Autologous irradiated (3000 rads) PBMC (10^4 cells/well) were incubated for 30 mins with 10 mg/ml of anti MHC class II antibody before the addition of antigen (1 and 5 μg/ml of individual peptides). APCs were then incubated for 1 h at 37°C in round bottomed 96 well plates in complete medium. Finally T cells (10^4 cells/200 μl well) were added, and the cells cultured, pulsed and harvested as described above.

IV Response to panel of influenza A viruses

Between 1-10 HAU of freeze dried influenza A virus were preincubated with autologous

irradiated PBMC (10^4 cells/well) for 1 h, and then cultured with T cells as described above.

2.7 Cytokine Measurements In T Cell Culture Supernatants

I Cell culture supernatants

T cell lines were stimulated with PMA (1ng/ml) and anti-CD3 (see below) or APC and HA (0.01, 0.1 and 1.0 μg/ml) or HA peptides (10μg/ml), at $1x10^6$ cells/2 ml complete medium. The anti-CD3 was precoated onto the tissue culture plate by incubation of a 10μg/ml solution in PBS for 1h at 37°C. Supernatants were harvested at 24h, 48h, and 72h, and stored at -70°C.

II Bioassay for IL-2

IL-2 production by T cells was estimated by the ability of supernatants collected from the cells in culture to induce the proliferation of an IL-2 dependent murine cell line CTLL-2. Because blocking antibodies to T cell IL-2 receptors were not used rather than measuring total IL-2 production, the assay measured the amount of IL-2 remaining in the tissue culture medium after the cultured T cells had taken up a proportion for their own use. The CTLL-2 cell line was cultured in complete medium containing 1 U/ml recombinant IL-2 (Biogen, England). Cells in the log phase of growth were washed three times to remove any residual IL-2, resuspended in IL-2 free FCS medium, and rested overnight. They were plated in 50μl aliquots ($5x10^4$ cells/well) into 96-well flat bottom microtitre plates (Nunc), containing either 50μl samples of doubling dilutions of recombinant IL-2 as a standard, or sequential dilutions of culture supernatants. The cells were incubated for 24h and [^{3}H] thymidine (1μCi/well) added for the final 4h. Proliferation was measured by [^{3}H] thymidine incorporation and liquid scintillation spectroscopy. The concentration of IL-2 in the supernatants was determined by comparing the level of proliferation induced by the supernatants to the proliferation induced by rIL-2 standards.

III ELISA for IL-4 and IFN-γ

Assays were kindly performed using ELISA techniques by Dr W.Holter (Vienna, Austria).

2.10 MHC Class II Binding Assays Using HA Peptides

I HLA-DRB1 purification*

HLA-DRB1* protein was prepared from the Epstein Barr virus transformed B-cell lines by Dr M. Davenport. A 10g pellet was lysed in phosphate buffered saline containing 2% CHAPS (vol./vol.), leupeptin (2μg/ml), pepstatin (2μg/ml), and 5mM EDTA. Cell lysates were centrifuged at 100,000g for 90 mins at 4 °C, and then loaded onto an L-243 affinity column. The column was washed extensively, and then material eluted with 0.05% diethylamine/ 150mMNaCl/ 0.1% CHAPS, pH 11.5. Eluate was concentrated on a Centricon-10 column (Amicon).

II Peptide binding studies

An invariant chain peptide (Ii) containing the CLIP peptide [Ii-(97-120)] was biotinylated using biotinamidocaproate N-hydroxysuccinamide ester (Sigma). Binding of peptides to DRB1*0701 was measured by their ability to inhibit the binding of the biotinylated Ii peptide (LPKPPKPVSKMRMATPLLMQ), using the method of Davenport *et al.,* (1995). On day 1, 96 well flat bottomed ELISA plates were pre-blocked by incubation overnight at 37°c with 200μl per well of 3% BSA in PBS. Plates were carefully dried by flicking immediately prior to use. A second set of 96 well tissue culture plates were conjugated with anti-HLA-DR antibody (L-243, 1μg/ml in PBS, 50μl/well), by incubation as above. On day 2 peptide dilutions were performed in disposable 96 well falcon plates with a starting concentration of 5 mg/ml HA peptide. pH 5 buffer was used as the diluent (MES 1.95g, CHAPS 0.5g, NaCl 4.1g, 20% Na-Azide /L). Diluted peptides were transferred in

20 µl aliquots to the pre-blocked flat bottomed ELISA plates. The L-243 incubated plates were then blocked as above.

20 µl biotinylated invariant chain solution (0.02µg/ml in pH 5 buffer)

and 20µl MHC class II solution were added to diluted peptide solutions, and the plates were incubated overnight at 37°c.

On day 3 the above reaction was neutralised with 10µl neutralising solution (1M Tris pH 8.0 , 10% BSA, 1% tween 0.02% azide), and peptide-MHC-invariant chain solutions transferred to the BSA blocked L-243 plates, and incubated at 37°c for 1h. Plates were then washed three times in PBS Tween (0.1%) and three times in PBS.

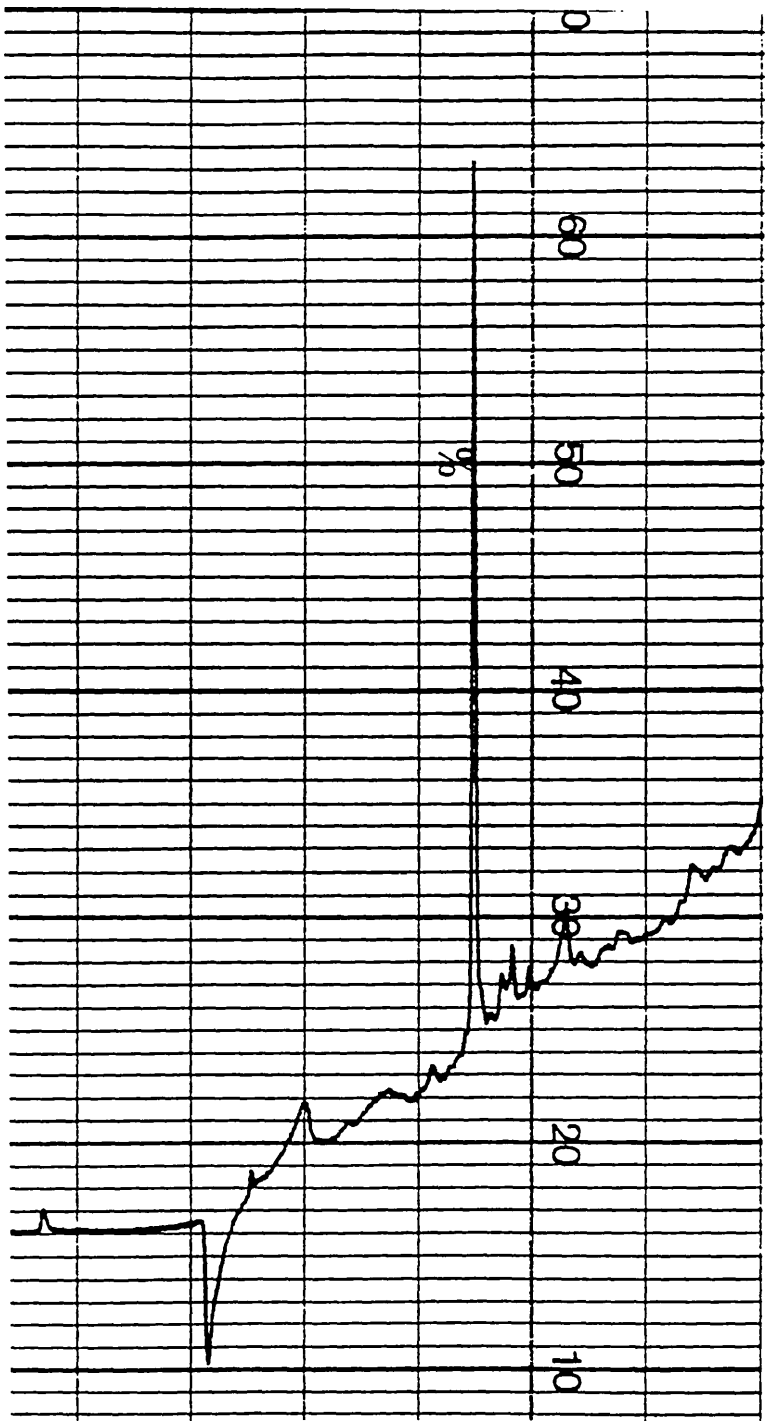
50µl 1/1000 streptavidin-HRP in PBS-Tween 0.1% BSA (buffered with Borate) was then added to each well, and the plates incubated for 15 min 37°c, and then washed as above.

50 µl antiavidin-(Biotinylated) 1/1000 in PBS-tween-BSA was then added and the plates incubated for 30 min at 37°c, and then washed as above. Plates were then incubated with 50 µl streptavidin-HRP for 15 min 37°c and washed as above, which was followed by a final wash in ddH_20.

100µl/well OPD (*o*-phenylenediamine) working solution (40mg/ml in citrate phosphate buffer, 3µl/ml H_2O_2) was then added per well. Plates were incubated at room temperature in a dark box for 15min. Reactions were stopped by the addition of 100µl 12.5% H_2SO_4.

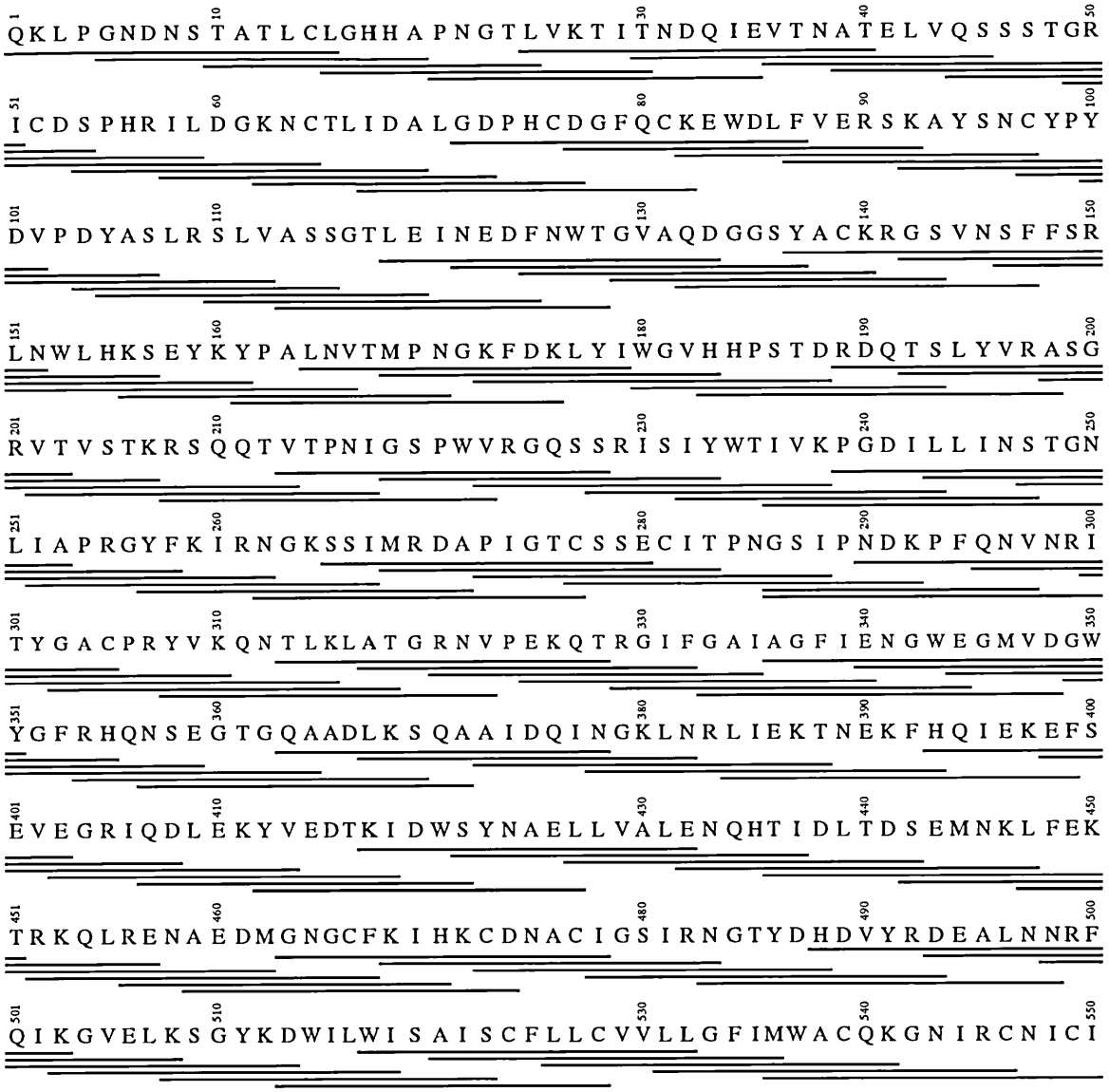
Finally the OD at 492nm was measured using an ELISA plate reader and the IC_{50} of invariant chain binding for each HA peptide calculated using standard methods.

Figure 2.1, HPLC analysis of HA peptide



HPLC was performed on 5 randomly chosen HA peptides using a Hewlett Packard HP1090 with chemstation using a C18 column, and a linear gradient of 1.25% acetonitrile per min in 0.08% trifluroacetic acid in water. Example shown is peptide 268.

Figure 2.2 Sequence of HA A/Beijng/32/92 and HA Beijing/32/92 specific peptides



HA0 sequence used for peptide synthesis of HA Beijing/32/92 specific peptides is shown. HA1 subunit (residues 1-328) is derived from A/Beijing/32/92, HA2 subunit (residues 329-550) is derived from the closely related A/Hong Kong/90 (H3N2), as no sequence information was available regarding the HA2 subunit of A/Beijing/32/92 at the time of peptide synthesis.

Chapter 3, Preliminary Investigation of Polyclonal Proliferative Responses to HA A/Aichi/68 and 2 Human CD4$^+$ T Cell HA Epitopes

3.1 Introduction

During September and October 1993 the polyclonal proliferative responses, of 18 randomly chosen adult donors, to HA A/Aichi/68 (H3N2) were examined. This preliminary study was designed to identify non-responders. A/Aichi/68 had not circulated for 25 yr, during which time considerable drift mutation had occurred. Therefore if responses were found they would probably be directed towards conserved regions of HA.

In addition polyclonal recognition of 2 previously defined epitopes for single T CD4$^+$ cell clones were examined, residues 306-318 (Lamb *et al.*, 1983) and residues 255-70 (A. Faith personal communication).

3.3 Aims

(1) To identify non-responders to HA A/Aichi/68.

(2) To examine human CD4$^+$ T cell recognition of two H3 influenza HA epitopes, which have previously been characterised in single donors, in an outbred population.

3.3 Methods

Subjects

18 members of staff at SMHMS were chosen at random. Donor details are listed in Table 3.1. None had a history of influenza during the preceding 4y. Donors had an equal sex distribution, and their ages ranged from 24yr-42yr. One donor (JL) had been vaccinated with an influenza subunit vaccine 4yr previously.

MHC class II typing

MHC class II typing was performed serologically at St Mary's Hospital, with the exception of donors AY, CJ, JHA, and RL who were tissue typed using SS-PCR at the Churchill Hospital Oxford. In this Chap. donors MHC Class II haplotypes are reported in the format usually used for serological typing.

Polyclonal prolifeerative assays

Proliferative assays were performed as described in Chap.2 using 5% IL-2 (positive control), medium alone (negative control), HA A/Aichi/68 (1, 5, 25 μg/ml), HA 255-70 (1, 5, 25 μg/ml), or HA 306-318 (1, 5, 25 μg/ml).

3.4 Results

PBMC proliferative responses by the donors to HA A/Aichi/68 are shown in Figure 3.1a. 12/18 subjects mounted a response of at least 5x the geometric mean of their background proliferation to HA A/Aichi/68 (donors GH, RL, JHA, PA, KY, MLA, CH, CJ, MK, JHI, JL, AF). 6/12 mounting a response of at least 10,000 cpm (RL, JHA, PA, CJ, JL, AF). Nearly all responses were maximal at an antigen concentration of 5μg/ml, and declined at 25μg/ml. There was no correlation between age (mean age of responder 30.0 yr, mean age of non-responders 30.6 yr), sex, or MHC type and HA recognition.

No response was seen to the 255-70 HA peptide. Donors GH and KY recognised the 306-318 peptide, though the magnitude of donor the former subject's responses was low. Interestingly donor KY's response to whole HA was considerably weaker than her response to this peptide (see discussion).

3.5 Discussion

As a preliminary experiment HA specific proliferative responses by unselected PBMCs obtained from 18 randomly chosen unrelated adult donors were examined. The study was conducted during September and October of 1993, and followed a

period of 4yr with relatively little influenza. 6/ 18 donors were non-responders. 12/18 mounted a significant proliferative response (5x g.m. of control) to HA, even though none had a convincing history of recent influenza. This may reflect either $CD4^+$ T cell memory for HA lasting at least 4 yr, or alternatively that the subjects have had a recent subclinical infection.

No response was seen to peptide 255-70, an epitope originally identified as the target of a single human $CD4^+$ T cell clone, raised from a HLA DR 3,11 donor (A. Faith Personal communication). This region contains only one common residue substitutions among H3 influenza A viruses (residue $260^{M\ to\ I}$ conservative). One other donor in this preliminary study expressed DR3, and by chance none expressed DR 11.Therefore at the time of this study recognition of 255-70 was considered be restricted to the DR 11 haplotype. However later studies showed this not to be the case: 2 DR 11 donors from the study described in Chap. 5 did not recognise this region, whereas 2 DR 3 donors did, and the response of one of these localised to residues 257-273 (Chap.5: Fig 5.1 and Table 5.2).

Peptide 306-318 is the epitope for the original human $CD4^+$ T cell clone described by Lamb (Lamb *et al.,* 1983), and represents a region close to the C-terminus of the HA1 subunit. Other than a single substitution (residue $307^{K\text{to}\ R}$ conservative) the region has not been the subject of antigenic drift. Two donors with different MHC haplotypes mounted a significant response to this peptide. Donor KY interestingly mounted a much stronger response to this peptide than to the whole HA A/Aichi/68 molecule: This may relate to drift mutation occurring in flanking regions, leading to alterations in antigen processing, or possibly recent H1 influenza A virus infection, as there is considerable homology between these influenza subtypes in this region (Fig. 3.2). No association of strength or absence of response to either HA A/Aichi/68 or the 306-318 peptide was seen with any MHC haplotype. As neither residues 255-70 nor 306-318 account for the proliferative $CD4^+$ T cell response to HA A/Aichi/68, other conserved regions of HA are likely to be recog-

nised. This encouraged us to study responses to the entire HA molecule in more detail.

3.6 Conclusions

Polyclonal proliferative T cell responses by 18 unrelated adults to HA derived from A/Aichi/68, an influenza A virus which had not circulated for 24yr, were examined. 12 subjects responded, none had a history of influenza during the previous 4 yr. Six non-responders were identified. No association with age, sex, or class II MHC type and responder status was found.

Recognition of 2 peptides representing previously described human $CD4^+$ T cell HA epitopes were examined. No response was seen to a peptide representing residues 255-70 of HA A/Aichi/68, and only 2 subjects responded to a peptide representing residues 306-318.

These results suggest that human $CD4^+$ T cell memory lasts at least 4 yr, and that the recognition of conserved regions of HA is an important feature of the human $CD4^+$ T cell response.

Table 3.1, Summary of donors

	Donor	Sex	Age	CLASS II MHC TYPE
AF	Q	M	42	DR3,4
AV	H	F	35	DR1,5
AY	O	F	40	DR10,13
CH	I	F	26	DR10,15 DQ1
CJ	K	F	28	DR13,13
GH	A	M	29	DR4,12 DRW52,53 DQ1,2
JHA	C	M	28	DR1,7
JHI	M	F	30	DR7,15
JL	N	M	42	DR1,4
KY	E	F	23	Not done
LJ	P	F	26	DR4,7
MK	L	M	24	DR7,6
MLA	F	M	29	DR4
MLW	G	F	24	DR4,15
PA	D	F	25	Not done
RL	B	M	34	DR1, DQ5
SD	J	F	33	DR1,3
TB	R	M	26	DR1,7

MHC class II typing was performed serologically, except for donors AY, CJ, JHA, RL who were tissue typed using SS-PCR.

Figure 3.1, Response to HA Aichi/68, and peptides 255-70, and 306-18

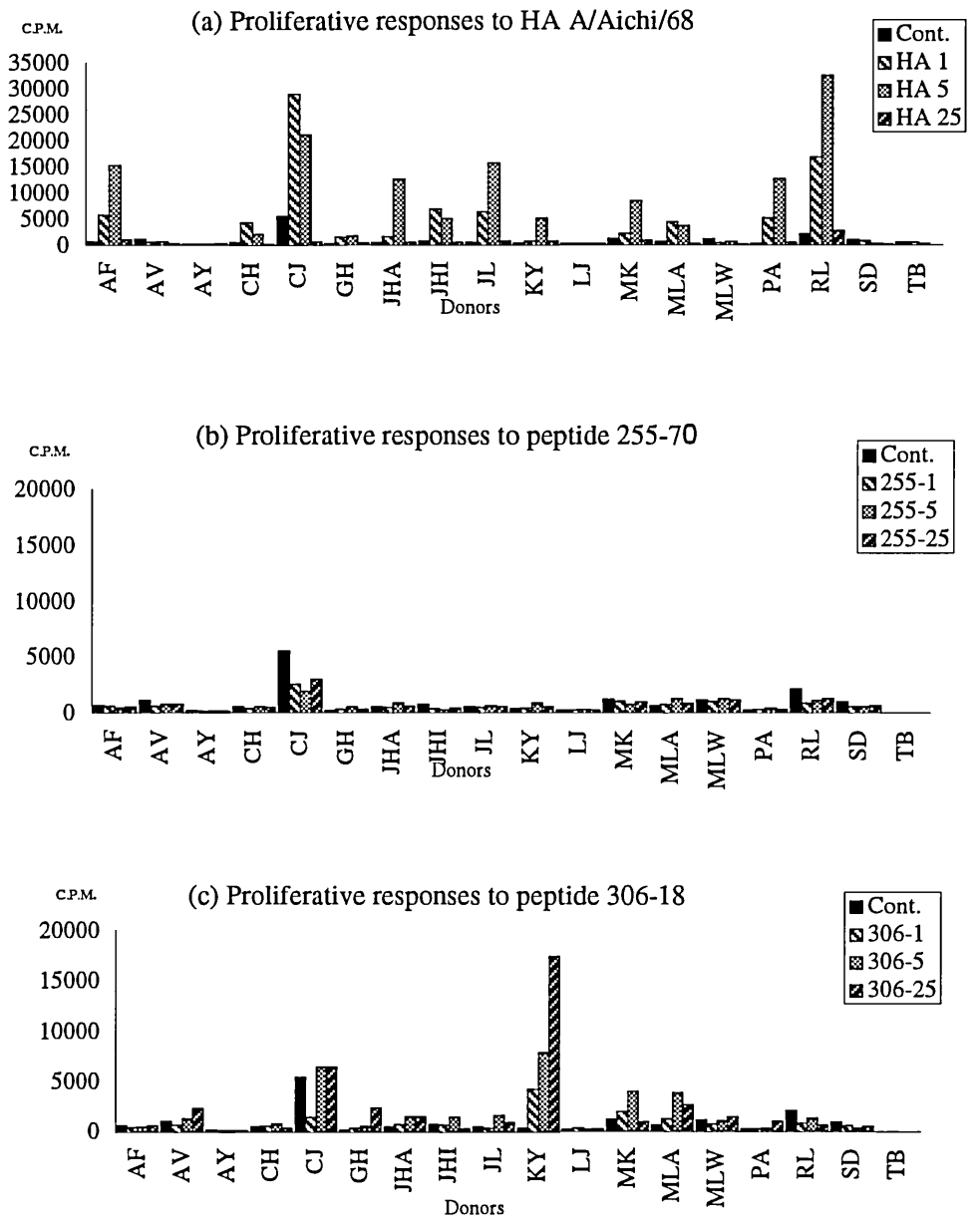


Figure 3.1a shows the g.m. of triplicate well proliferative responses of unselected PBMC derived from 18 unrelated adult donors in a 7d proliferation assay to HA A/Aichi/68 (H3N2.).Figure 3.1b and 3.1c show the response to synthetic peptides representing residues 255-70, and 306-318 of HA A/Aichi/68 respectively. 5 x 10^6 cell/ml were cultured in 200 ml round bottomed wells, with medium, or 1, 5, 25 mg/ml of antigen. Responses of 5x the g.m. of control proliferation were regarded as significant. Cont = control, HA = HA Aichi, 255 = 255-70 peptide, 306 = 306-18 peptide. 1= 1µg/ml, 5 = 5µg/ml, 10 = 10 µg/ml. Y axis represents c.p.m..

Figure 3.2, Comparison of C-terminal amino acid sequences of HA-1 subunits from H1, H2, H3 influenza A viruses

	300	301	302	303	304	305	**306**	**307**	**308**	**309**
A/AICHI/68 (H3N2)	I	T	Y	G	A	C	**P**	**K**	**Y**	**V**
A/BRAZIL/78 (H1N1)	V		I		E					
A/SINGAPORE/57 (H2N2)	L		I		E					
	310	**311**	**312**	**313**	**314**	**315**	**316**	**317**	**318**	319
A/AICHI/68 (H3N2)	**K**	**Q**	**N**	**T**	**L**	**K**	**L**	**A**	**T**	G
A/BRAZIL/78 (H1N1)	**R**	**S**	**T**	**K**		**R**	**M**	**V**		
A/SINGAPORE/57 (H2N2)		**S**	**E**	**K**		**V**				
	320	321	322	323	324	325	326	327	328	329
A/AICHI/68 (H3N2)	M	R	N	V	P	E	I	Q	S	R
A/BRAZIL/78 (H1N1)	L			I		S		E		
A/SINGAPORE/57 (H2N2)	L					Q				

Chapter 4, Development of Cell Culture Methods and Functional Assays

4.1 Introduction

In late November 1993 a severe outbreak of influenza A occurred in London, which followed a period of several years with little severe influenza. Causative strains were closely related to A/Beijing/32/92 (H3N2). Advanced warning of the epidemic came from preceding outbreaks in Australia and the USA. This was an excellent opportunity for a detailed investigation of human $CD4^+$ T cell response to HA following natural infection. In contrast to the previous limited human studies, we planned to use HA from the infecting strain to select HA specific $CD4^+$ T cell lines, and then use a similarly specific panel of HA peptides to investigate HA epitope recognition. This strategy would overcome the two fundamental problems of all previous human studies: The use of HA and /or HA peptides from influenza viruses which had not circulated for many years and their very small sample size (see Chap. 1).

Dr R. Brands, Solvay Duphar, Weesp, The Netherlands generously provided highly purified HA A/Beijing/32/92, and the 1993/4 influenza A subunit vaccine (which contained HA and NA from Beijing/32/92, in combination with HA and NA from an H1 influenza A virus and an influenza B virus). Dr N. Cox, Head of the Influenza Section of the Center For Disease Control, Atlanta, USA very kindly supplied the unpublished amino acid sequence of the HA1 subunit of HA A/Beijing/32/92. The HA2 subunit of this strain had not been sequenced but it was believed to be closely related to that of A/Hong Kong/90.

Details of peptide design, synthesis, and sequence are given in Chap. 2. In brief 16 amino acid peptides overlapping by 11 residues were synthesised. They spanned the entire sequence of HA A/Beij/32/92. Other than for chemical reasons, the

peptides had a regular overlap of 11 residues. Previous studies of HA epitope recognition, and putative MHC class II motifs was not allowed to influence their design.

The responses of $CD4^+$ T cell lines rather than T cell clones was examined, as the use of clones would have severely restricted the number of donors examined, and would have lead to the selection of a small number of dominant epitopes that were not necessarily representative of the overall $CD4^+$ T cell repertoire. This might have introduced bias in favour of conserved epitope recognition.

4.2 Aims

(1) To establish reliable methods for the investigation of human $CD4^+$ T cell recognition of HA A/Beijing/32/92, which would enable us to examine a substantial number of donors.

(2) To select $CD4^+$ T cells using HA from an influenza A virus as closely related to the donors infecting strains, and then use a similarly specific panel of HA peptides spanning the entire molecule, to map epitope recognition.

4.3 Donor Recruitment

Donors with a convincing history of influenza like illness during November and December 1993 were recruited. A convincing history of influenza was taken to be severe malaise, headache, muscle pains, temperature, and a sore throat, resulting in several days in bed. Donor details are given in Chap. 5. They were either friends or colleagues of BAA or CMG, or medical students at St Mary's Hospital Medical School. In order to investigate the MHC genetics of human $CD4^+$ T cell responses to HA, detailed class I and II MHC Typing was performed on every donor using the Phototyping method in the laboratory of Dr K. Welsh, Churchill Hospital, Oxford (Chap. 2).

4.4 Preliminary Experiments

PBMC were obtained from 2 donors with a history of recent influenza. Polyclonal proliferative assays for HA A/Beij/32/92 and HA A/Aichi/68 responsiveness followed using methods previously established in the laboratory. In parallel PBMC were cultured with either 1.0 or 5.0 μg/ml HA A/Beij/32/92 in 2 ml of complete medium, in 24 well flat bottomed tissue culture plates, using the culture conditions described above. Two different plating densities were employed (1 x 10^6 and 5x10^6 cells /2 ml well).

Results of these polyclonal proliferative assays at the 2 different densities were similar, and the results obtained at a plating density of 5μg/ml are shown in Fig. 4.1. The optimal concentration of HA A/Beij/32/92 for the induction of a polyclonal proliferative response to HA A/Beij/32/92 was found to be at or below 0.1 μg/ml. Most interestingly doses of 5μg/ml, and above, abrogated the proliferative response. In contrast HA A/Aichi/68 specific proliferation continued to increase with increasing doses of antigen (see below). Similar results were obtained from 4 other donors (not illustrated).

Developing T cell lines were recovered at 7d, washed twice in basic medium, and replated at a density of 1 x 10^6 cells/2ml well with an equal number of irradiated (3000 rads) autologous PBMC as APC, and 10% v/v Lymphocult-T as a source of IL-2. APC were freshly thawed from liquid nitrogen vapour phase storage immediately prior to use. The T cell lines were further supplemented with 10% Lymphocult on 10d of culture, and harvested and restimulated at 14d and 21d as above. Responsiveness to HA and HA peptides was investigated at 7d, 14d, and 21d. 1 x 10^6 T cells were cultured in triplicate with an equal number of autologous irradiated PBMC as APCs, and HA or peptide pools (0.1μg/ml, 1.0μg/ml, or 10.0 μg/ml each peptide per pool) in 96 well round bottomed tissue culture plates

(200µl volume). At 48h the plates were pulsed with [3]TdR and harvested 16h later. Results are expressed as cpm. of [3]TdR incorporation (see below). Table 4.1 shows the cell recovery at 7d, 14d, and 21d.

At 7d there was no difference in the cell recovery between a plating density of 1 or 5 x10^6 PBMC/2ml well. But it was clear that an initial stimulation with 1µg/ml rather than 5µg/ml HA A/Beij/32/92 resulted in a higher cell recovery, and this concentration of antigen was used to restimulate the developing T cell line. At 14d no viable cells were recovered from donor two's line. The recovery from donor one's line was satisfactory but no further expansion was seen following the third *in vitro* stimulation.

The results of the proliferative assays to HA and peptides were disappointing. At 7d no response of > 5 x gm. of the background (T cells, APCs, and complete medium) was seen from either donor to HA or the HA peptides (donor one's results in Fig.4.2). At 14d and 21d only the line from donor one could be investigated, and responses to individual peptides at 0.1 µg/ml, 1.0 µg/ml and 10 µg/ml were examined. T cells from this line did not respond to the peptides at 0.1µg/ml (which are therefore not illustrated). Responses to 1µg/ml and 10µg/ml are shown in Fig. 4.2. Stronger responses occurred to 10µg/ml of peptide than to 1µg/ml, and there was some increase in proliferation to HA peptides at 21d.

The decrease in polyclonal response to HA A/Beij/32/92, and the falling recovery of cells after *in vitro* stimulation at a concentrations of 5µg/ml and above was unexpected. The concern was that the HA A/Beij/32/92 preparation was toxic. However it had been highly purified and was known to contain very little endotoxin (R.Brands personal communication). An alternative explanation for these findings was clonal exhaustion, though this did not explain the difference in potency of the HA A/Beij/32/92 and HA A/Aichi/68 preparations: This might have related to purity or possibly partial degradation of HA A/Aichi/68 during

bromelain cleavage. Interestingly it was reported during the development of influenza vaccines that bromelain cleavage substantially reduces the immunogenecity of HA, indeed responses to vaccination with bromelain cleaved HA could only be detected if adjuvants were employed (Jennings *et al.*, 1974, Tyrrell 1974). The concurrent addition of IL-2 and antigen might have contributed to clonal exhaustion by boosting the initial stimulus. The culture technique was therefore modified and APCs were prepulsed with antigen for 1h before the addition of T cells. Furthermore the antigen concentration used for the first and second *in vitro* stimulations was decreased to 0.1μg/ml, and the addition of Lymphocult was delayed by 24h. Finally the number of T cells and APCs in the HA and HA peptide proliferative assays was increased to 4 x 10^4 cells/200μl well. In order to conserve antigen a plating density of 5 x10^6 PBMC/2ml well for the first *in vitro* stimulation, and 1 x10^6 cells/2ml well thereafter was chosen.

Cell recovery from fresh $CD4^+$ T cell lines from the two original donors using modified methods are shown in Table 4.2. Proliferative assays to HA A/Beij/32/92, HA A/Aichi/68, and the peptide pools at 7d, 14d, 21d were performed, and the results obtained from the first donor illustrated in Fig. 4.3. Responses to 0.1μg/ml, 1μg/ml, and 10μg/ml of individual peptides within the pools was examined, however as no response was seen to 0.1μg/ml only the results of experiments with 1.0μg/ml and 10μg/ml are illustrated.

As these modifications to the cell culture technique yielded more satisfactory results, the antigen concentration was further decreased during the third *in vitro* stimulation to 0.01μg/ml. Examination of epitope recognition with 10μg/ml of peptide appeared optimal. These findings were confirmed in experiments on PBMC from two other donors. In addition to examining response to the HA peptide pools we found that the short term $CD4^+$ T cell lines could also be used to examine responses to individual peptides, using the techniques described above.

This method was used for all the experiments which followed (see methods for details).

4.5 Studies with anti-HLA antibodies

In order to examine the balance of HLA-DP, -DQ, and -DR restriction of HA peptide responses experiments were performed using a range of concentrations of anti-class II antibodies (0.1, 1.0, 10.0 μg/ml) and a fixed concentration of the dominant peptide pools (10μg/ml) (details of antibodies in Chap.2.). 4x 10^4 autologous APCs were incubated with anti-class II antibody and dominant peptide pools (10μg/ml of each peptide) for 1h prior to the addition of an equal number of T cells. These experiments were generally unsatisfactory: Though partial inhibition of some responses could be demonstrated with these antibodies the results were variable. Keeping the concentration of the anti-class II antibody constant and varying the concentration of antigen yielded more satisfactory results. Finally by incubating the anti-class II antibodies with the APCs for 30 min prior to the addition of antigen consistent results were obtained. Interestingly it was sometimes necessary to decrease the stimulating dose of antigen by 2 log orders in order to demonstrate any inhibition. Pretreating T cells with anti-class II antibody had no effect. An example of typical experiment using these modified techniques is shown in Chap 5 (Fig. 5.4).

4.6 Examination of $CD4^+$ T Cell Content of Developing Lines

In order to examine the $CD4^+$ T cell content of the emerging lines flow cytometric analysis was performed on two lines at 7d, 14d, and 21d using the following fluorescein-conjugated murine monoclonal antibodies: Anti-Leu 4 (CD3), anti-Leu3a (CD4) , anti-Leu2a (CD8) and a mouse IgG1 control (Becton-Dickinson Oxford, UK). Aliquots of T cell lines containing 0.2x10^6 cells were resuspended in

90µl of ice-cold PBS containing 0.1% NAN_3 (BDH) and 0.1% BSA (PAB). Cells were incubated with saturating concentrations of the FITC-conjugated monoclonal antibodies for 30 min at 4°C. To determine the background fluorescence the cells were incubated with FITC-conjugated mouse IgG1. After washing twice the cells were resuspended in 400 µl of cold PBS and kept on ice until analysed. Fluorescence intensity was determined on 10000 cells from each sample by flow cytometry using an EPICS Profile II analyser (Coulter, Luton, UK), and was measured on a linear scale. All viable cells were included in each determination and in every case the fluorescence profile was unimodal. Viable cells were identified by their ability to exclude propidium iodide (5µg/ml, added prior to FACS analysis). Cell populations were analysed by gating on the volume and light scatter characteristics.

The ratio of $CD4^+$ cells to $CD8^+$ cells steadily increased during 3 wk in culture. The $CD4^+$:$CD8^+$ ratio of a further 4 lines at 21d in culture was examined: At 21d >90% of cells were $CD3^+$, 78-88% of cells were $CD4^+$, and 2-10% of cells were $CD8^+$.

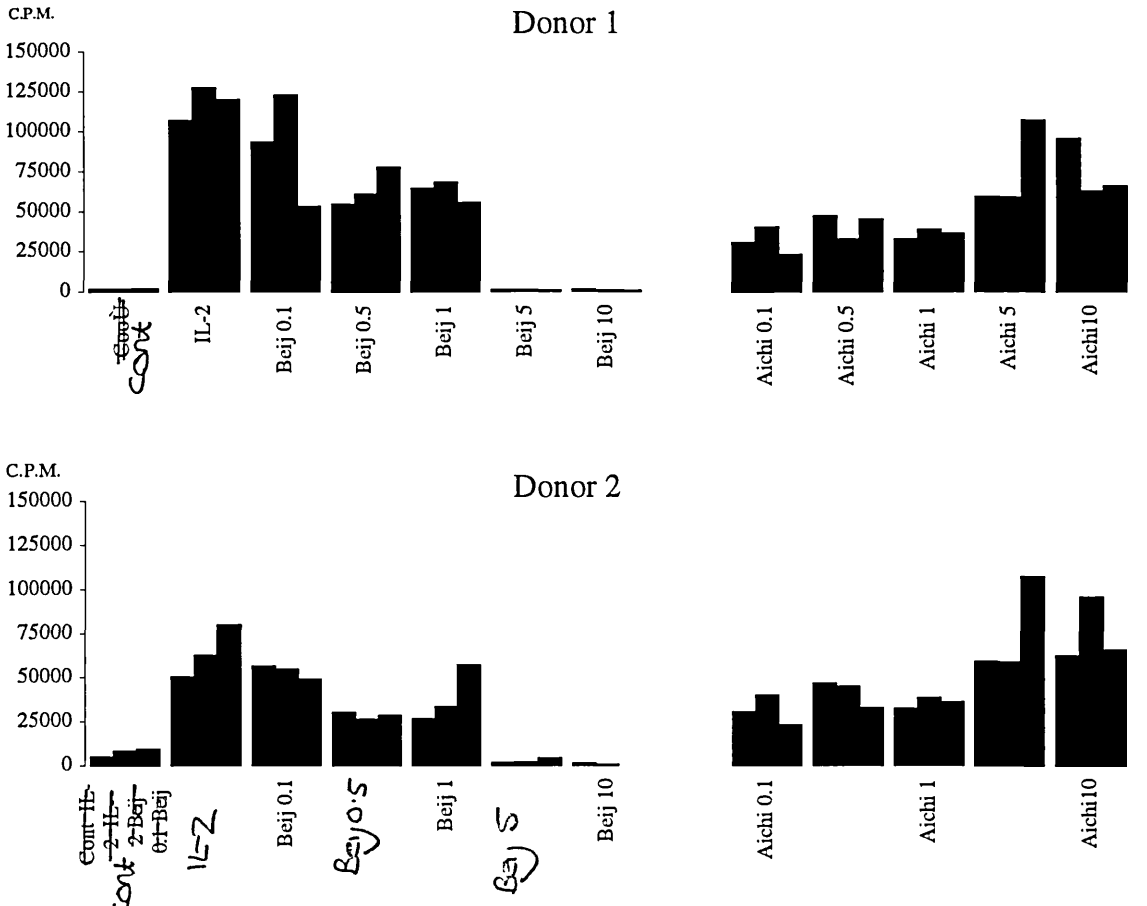
4.7 Examination Of HA Beijing/32/92, HA Aichi/68, And HA Beijing Specific Peptides For Mitogenicity

Finally the HA preparations and peptides were tested for mitogenicity using an MTSE specific 21d T cell line (Fig. 4.5). The line was raised by repeated *in vitro* restimulation of PBMC with MTSE (5µg/ml) prepulsed autologous APC, using the methods described above. No mitogenicity was seen from either HA or any of the peptides.

4.8 Conclusions

Short term $CD4^+$ T cell lines selected with HA A/Beijing/32/92, provided a useful tool for exploring adult T cell recognition of HA. The concentration of antigen used to stimulate the developing T cell lines must be progressively decreased. Optimal $CD4^+$ T cell responses to HA and HA peptides usually also occur at 3 wks.

Figure 4.1, Polyclonal proliferative responses to HA Beijing/32/92 and HA Aichi/68



Polyclonal 7d proliferative assays were established in triplicate wells of round bottomed 96 well tissue culture plates. 5 x 10^6 PBMC from 2 unrelated adult donors with a history of recent influenza were cultured in complete medium 5% CO2 with either HA Beijing/32/92 (Beij), HA Aichi/68 (Aichi), 5% lymphocult-T (IL-2), or medium alone (Cont). Total reaction volume 200µl. Proliferation was measured by [3]TdR incorporation. 0.1, 0.5, 1, 5, 10 refer to concentration of HA Beijing/32/92 (H3N2) or HA Aichi/68 (H3N2) in µg/ml.

Table 4.1, Cell recovery following *in vitro* stimulation with HA A/Beijing/32/92

Donor	*In Vitro* stimulation	Total Number Cells plated x 10^{-6}	Plating density x10^{-6}/ml	HA Beijing concentration	No. cells recovered x10^{-6}	Ratio cells recovered/cells plated
Donor 1	1st	24	1	1μg/ml	24.1	1
		24	1	5μg/ml	4.7	0.2
	1st	30	5	1μg/ml	29.5	1
		30	5	5μg/ml	13	0.4
	2nd	12	1	1μg/ml	25	2.1
	3rd	12	1	0.1μg/ml	12	1
Donor 2	1st	30	5	1μg/ml	16.7	0.6
		30	5	5μg/ml	3.7	0.1
	2nd	12	1	1μg/ml	0	0

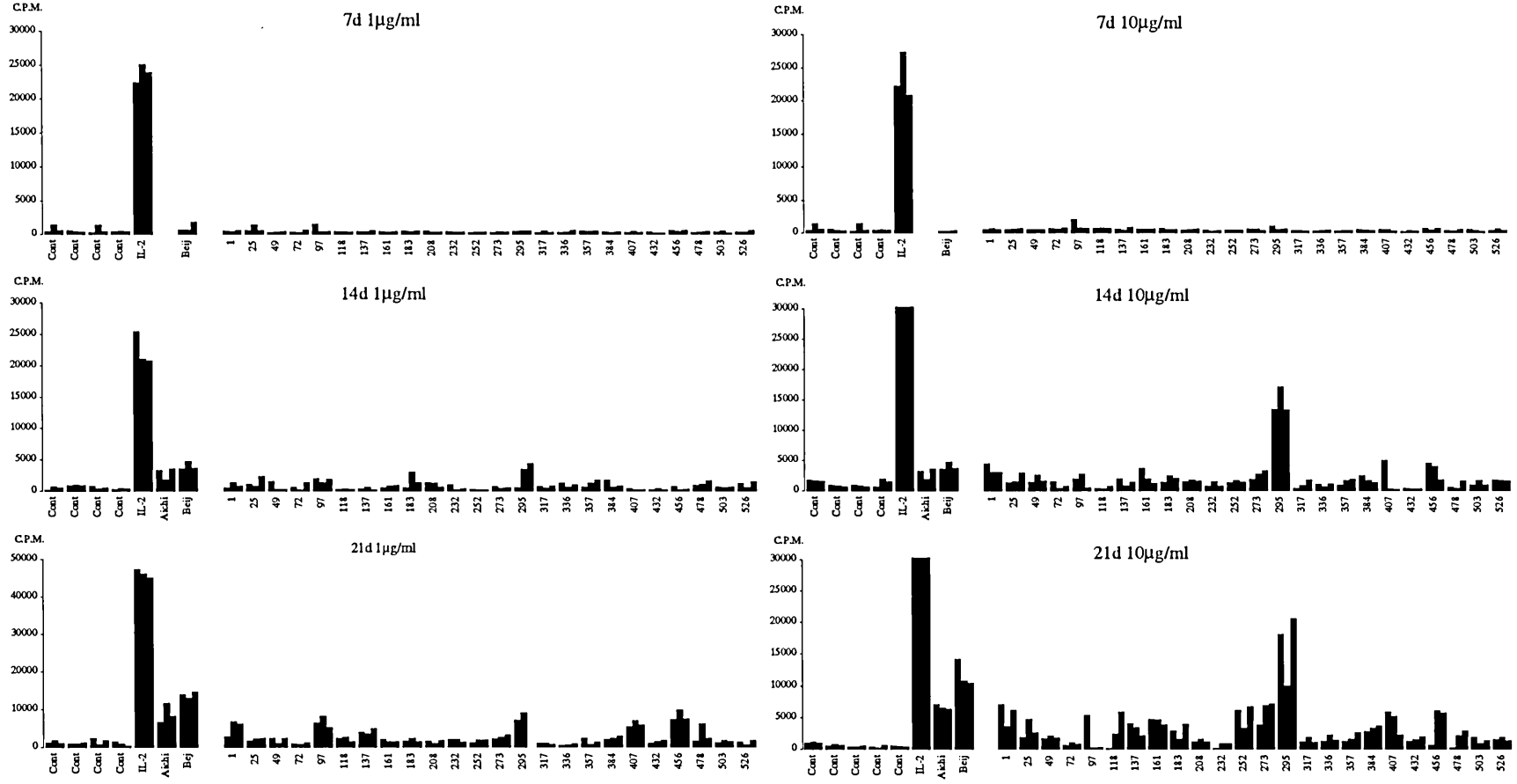
Table shows total number of cells plated at each round of *in vitro* stimulation, and total cell recovery one week later. A ratio of total number of cells recovered to total number of cells plated is calculated for each round of *in vitro* stimulation. Donor 1's PBMC were originally plated in complete medium at 2 different cell densities (1 x 10^6 and 5 x 10^6 cells/2ml well), and with 2 different concentrations of antigen (1μg/ml and 5 μg/ml HA Beijing/32/92). Donor 2's PBMC were plated originally with 2 different concentrations of antigen (1μg/ml and 5 μg/ml HA Beijing/32/92).

Table 4.2, Cell recovery following *in vitro* stimulation with HA A/Beijing/32/92 after modifications to cellular techniques

Donor	*In Vitro* stimulation	Total Number Cells plated x 10^{-6}	Plating density x10^{-6}/ml	HA Beijing concentration	No. cells recovered x10^{-6}	Ratio cells recovered/cells plated
Donor 1 2nd Experiment	1st	40	5	0.1μg/ml	37.8	0.9
	2nd	12	1	0.1μg/ml	19	1.6
	3rd	10	1	0.01μg/ml	32.9	3.3
Donor 2 2nd Experiment	1st	40	5	0.1μg/ml	34	0.6
	2nd	10	1	0.1μg/ml	29	2.9
	3rd	10	1	0.01μg/ml	42	4.2

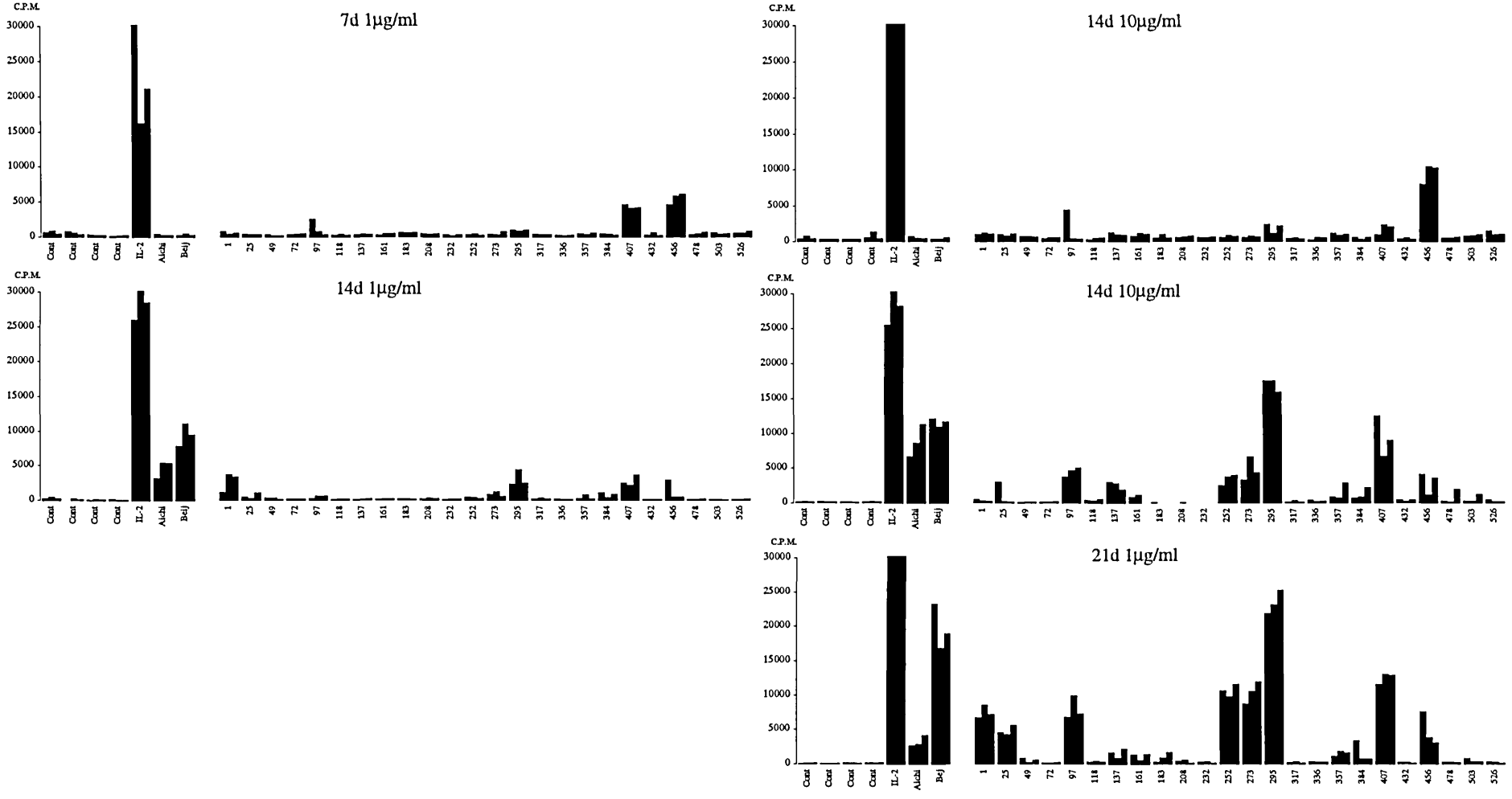
Donor's PBMC were initially plated in complete medium at and 5 x 10^6 cells/2ml well, and 0.1μg/ml HA Beijing/32/92). Other details as Table 4.1

Figure 4.2 Proliferative responses to HA Beijing/32/92, HA Aichi/68, and a panel of HA Beijing/32/92 peptides



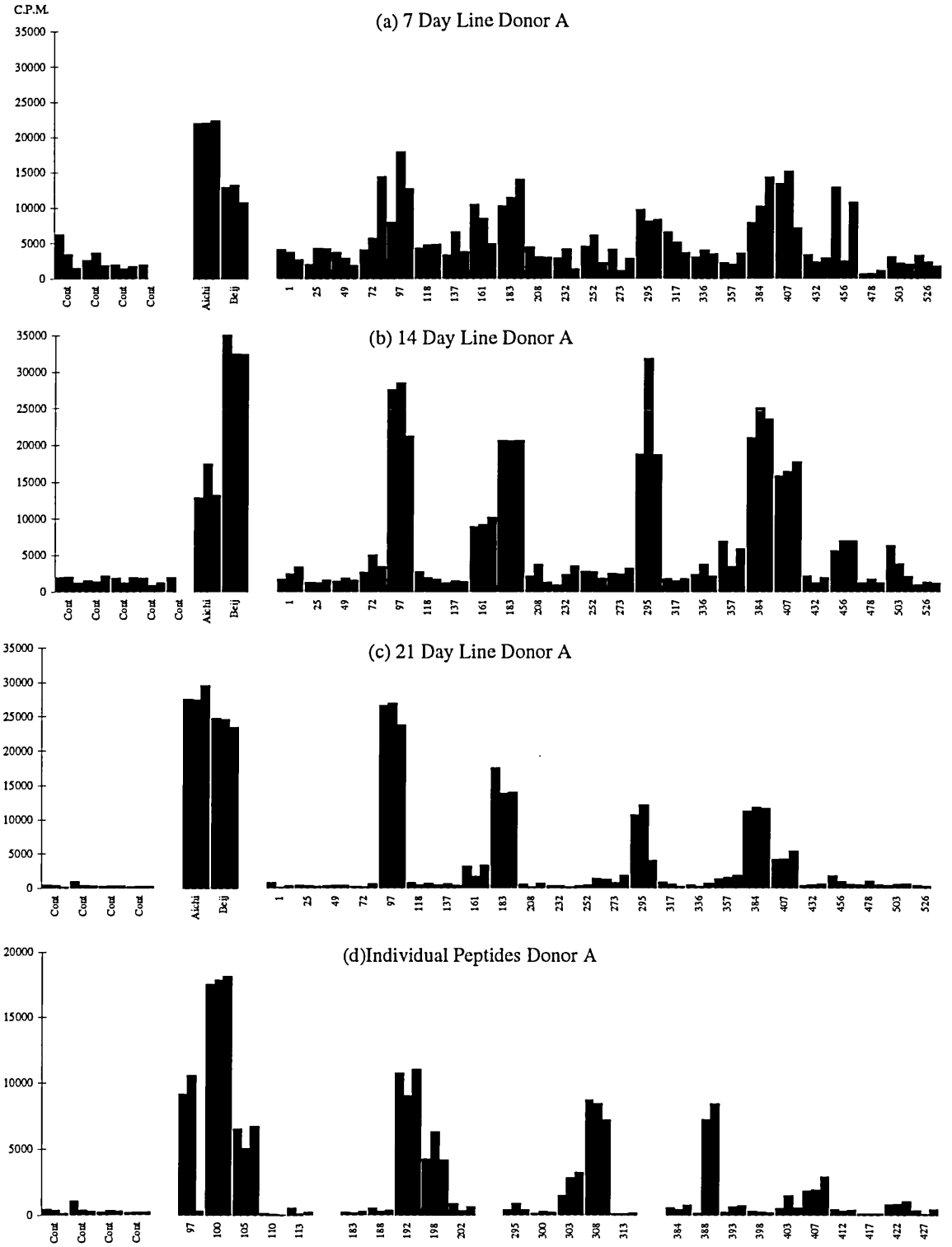
T cell line from donor one was selected with 1μg/ml HA Beijing/32/92 (first in vitro stimulation), 1μg/ml HA Beijing (second in vitro stimulation) , and 0.1 μg/ml HA Beijing (third in vitro stimulation). At d7 (top row), d14 (middle row), and d21 (bottom row), 4 x 10^4 T cells were cultured, in triplicate wells of a 96 well round bottomed tissue culture plate, with an equal number of autologous irradiated PBMC as APC in complete medium, and either 0.1μg/ml HA Beijing/32/92, 1.0μg/ml HA Aichi (Aichi), or peptide pools containing 5 individual 16mer peptides at concentrations of 0.1μg/ml (not illustrated), 1.0μg/ml (left column), or 10 μg/ml (right column) of each individual peptide. Peptide pools numbered by the N-terminal residue of the N-terminal peptide in each pool. Control wells (Cont.) contained T cells, APC, and complete medium. At 48h cells were pulsed with [3]TdR, and harvested 16h later. Y-axis represents c.p.m.of [3]TdR incorporation. Data from individual reactions rather than means of triplicates illustrated.

Figure 4.3 Proliferative responses to HA/Beijing/32/92, HA Aichi/68, and HA Beijing/32/92 peptides following modifications to cellular techniques



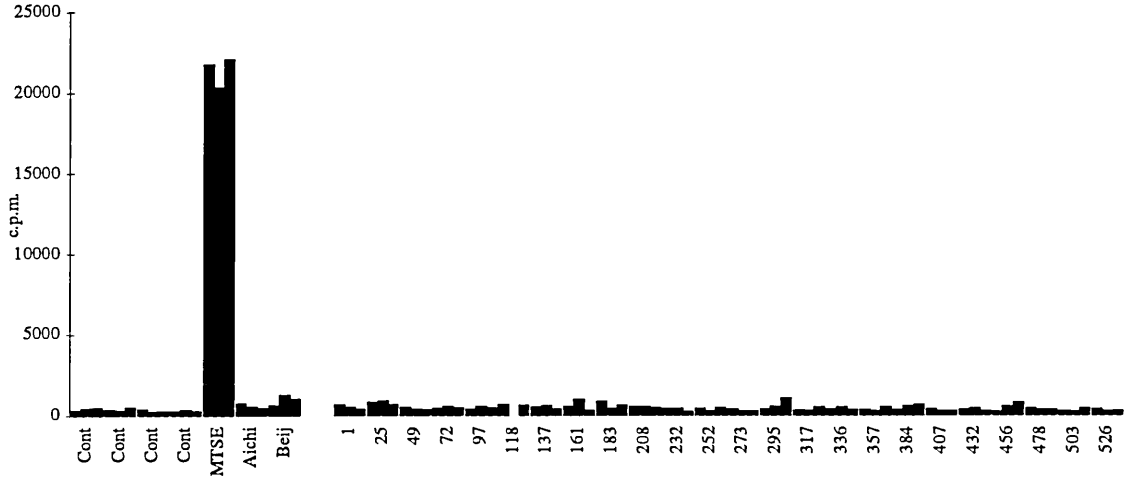
Donor one's T cell line was raised by stimulation of PBMC with 0.1 mg/ml HA Beijing/32/92. Developing line was restimulated at 7d, and 14d with 0.1µg/ml and 0.01µg/ml HA Beijing respectively. At 7d, 14d, and 21d 4 x 10^4 T cells were cultured in triplicate wells of a 96 well round bottomed tissue culture plate, with an equal number of autologous irradiated PBMC as APC in complete medium. Details otherwise as Fig. 4.2.

Figure 4.4, Response of CD4+ T cell line to HA Beijing/32/92, HA A/Aichi/68, and HA Beijing peptides at 7, 14, and 21 days *in vitro*



Figures 4.4a, b, c show response of CD4+ T cell line to HA Beijing/32/92, HA Aichi/68, and HA Beijing/32/92 peptides at 7, 14, and 21 days in vitro respectively. 10 μg/ml individual peptides per pool, details otherwise as Fig. 4.3. Response to individual peptides (10μg/ml) examined at d21.

Figure 4.5 Effect of HA Beijing/32/92, HA Aichi/68, and HA Beijing/32/92 peptides on MTSE specific T cell line



A 3 wk MTSE specific line was raised by weekly in vitro stimulation of PBMC with 5μg/ml MTSE. MTSE= response to MTSE 5 μg/ml, details otherwise as Fig 4.3.

Chapter 5, Human $CD4^+$ T Cell Repertoire Specific For HA A/Beijing/32/92 Following Recent Natural Infection

5.1 Introduction

Once a reliable method for investigating human $CD4^+$ T cell responses to HA (see Chap. 4) had been established, a detailed study of human $CD4^+$ T cell recognition of HA epitopes following natural infection was commenced. The primary object of this study was to examine the balance of $CD4^+$ T cell recognition between surface regions of HA which are subject to continual antigenic drift (variable regions), and more conserved regions of the whole HA molecule including the HA2 subunit (conserved regions). In contrast to all previous human studies, HA epitope selection would be examined using HA and a panel of HA peptides derived from the infecting influenza virus strain (see Chap. 1).

As a comparison between the $CD4^+$ T cell response to recent natural infection and longer term $CD4^+$ T cell memory, the HA Beijing/32/92 specific responses of 2 groups of unrelated adult donors to HA Beijing/32/92 were investigated. The first group had a history of influenza during November and December 1993 (recent influenza donors). The second group had no history of influenza for at least 4yr, but was known to mount polyclonal proliferative responses to HA.

In order to examine the MHC genetics of the human $CD4^+$ T cell repetoire for HA epitopes, detailed class I and II MHC typing was performed on all donors.

5.2 Aims

(1) To examine human $CD4^+$ T cell recognition of HA A/Beijing/32/92 following natural infection, in an outbred adult population.

(2) To investigate the balance of recognition between conserved regions of HA, and those which have been subject to drift mutation amongst human H3N2 influenza A strains.

(3) To explore the immunogenetics of $CD4^+$ T cell HA epitope selection.

(4) To examine longer term $CD4^+$ T cell memory, by investigating HA recognition by $CD4^+$ T cells of 6 adults with no history of recent infection, but who are known to mount polyclonal responses to HA.

(5) To investigate possible cross reactivity, of the $CD4^+$ T cell response, using a panel of influenza A viruses.

(6) To explore MHC class II restriction of HA epitope recognition.

(7) To examine the balance of IFN-γ and IL-4 production by the HA selected $CD4^+$ T cell lines.

5.3 Subjects

Recent influenza donors: Twelve unrelated normal adult donors (aged 21yr-55yr) with a history of influenza virus infection during November and December of 1993 were studied (recent influenza donors, subjects A-L) 3-6 mo after infection. None had ever been vaccinated against influenza. A positive history of influenza was taken to be fever, headache, sore throat, myalgia, and severe lassitude lasting several days during a period when A/Beijing/32/92 like strains were circulating (see discussion). Many of the subjects had extended periods of lassitude lasting several months and/or persistent cough. 3 of the donors (D, I, L) had been previously included in the study of HA A/Aichi/68 responsiveness during the summer of 1993 described in Chap 3 and were found to be non-responders (donors MLA, AY, TB respectively).

Control donors: A second group of 6 normal adults (aged 28yr-42yr), with no history of influenza like illness during the previous 4yr, but who mounted polyclonal T cell responses to HA, were studied as a comparison (control donors, M-R). Subject details are summarised in Table 5.1. Three of these donors had been regularly exposed to influenza: Donors N and P as practising physicians, and donor O who had worked extensively with influenza A viruses 10yr previously. Donor R was involved in the synthesis of a peptide representing residues 306-318 of HA on a regular basis.

Class I MHC typing was performed by a combination of serological typing and

molecular typing using sequence specific primers (PCR-SSP). MHC class II typing was performed using molecular typing using PCR-SSP, with the exception of one donor (H) who was typed serologically. Tissue types of the donors are presented in the format recommended in "The Nomenclature For Factors Of The HLA System 1994"(Bodmer *et al.*, 1994).

5.3 Results

Subjects

The recently infected donors (with a history of influenza during November/December 1993) had a wide range of MHC class II haplotypes (see Table 5.1). One pair, donors A and B, shared MHC HLA-DRB1* and DQB1*haplotypes (HLA DRB1*0101, 11, DRB3*01/02/03 and DQB1*0301, 0501). A second pair, donors C and D, had identical low resolution MHC class II type (DRB1*04, 15, DRB4*01, DRB5*01, DQB1*0601/2, and 0301), and differed only in HLA DRB1*04 subtype. Three donors (D, I, and L) did not mount significant proliferative responses to HA A/Aichi/68 during the summer of 1993, but recognised both HA Beijing/32/92 and HA A/Aichi/68 following influenza like illness in November/December 1993 (see Chap. 3: Donors MLA, AY, and TB respectively).

The control donors (with no history of influenza during the preceding 4yr) also had a range of class II MHC haplotypes, although 4 shared the DRB1*0101 allele (Table 5.1). Donor O shared low resolution HLA DR and DQ type with donor G, who had a history of recent influenza.

Of the 18 donors in the two groups 7 shared DRB1*0101 DQB1*05 containing haplotype, 5 the low resolution HLA DRB1*04 type (but had several different subtypes), 4 the DRB1*15 haplotype, 3 the DRB1*13 haplotype, 2 the DRB1*07 haplotype, and 2 the DRB1*11 haplotype. In addition the responses of donors with HLA DRB1*0102, -0103, -03, -08, -10, -12 containing haplotypes were examined.

Characteristics of T cell lines

T cell lines derived from the recent influenza donors, and selected with HA Bei-

jing/32/92, expanded at least 4 fold in culture over 3 wk, in contrast those derived from the control donors which, with the exception of donor Q's T cell line, showed no significant expansion (data in Table 5. 1).

Response of $CD4^+$ T cell lines derived from unrelated donors with a history of recent influenza to HA and peptides

The recent influenza donors responded to both HA/Beijing/32/92 and HA/Aichi/68 (Fig 5.1). Marked differences in patterns of peptide recognition were seen between individual T cell lines from donors differing in MHC class II haplotype. Without exception every $CD4^+$ T cell line mounted a marked proliferative response to the HA2 subunit.

When results from individual recent influenza donors during the second and third wk in culture were combined it was found that peptides pools covering almost the entire protein could induce proliferative responses (Fig 5.2). Three regions of HA represented by peptide pools 97-128, 295-328, 407-442, were recognised by 10/12, 12/12, 12/12 donors differing in MHC class II respectively during the second and/or third wk of culture. A further 2 regions 183-217, and 384-418 were seen by 9/12 donors. Four regions of HA represented by peptide pool 208-243, 317-348 and 336-339, 478-513, and 526-550 were less immunogenic (see discussion).

Table 5.2 summarises the results of studies with single peptides, and indicates residues frequently subject to drift mutation amongst a panel of H3N2 influenza A viruses (see Appendix 1). With only the few exceptions described below the strongest proliferative responses localised to identical individual peptides or a pair of overlapping peptides, representing regions of HA that have been structurally conserved over many yr amongst H3 influenza A viruses. It was also notable that the 3 donors (subjects D, I, and L) who were non-responsive to HA A/Aichi/68 prior to natural infection also, preferentially recognised conserved regions of HA. Interestingly pool 407-442 which induced a $CD4^+$ T cell proliferative response in every donor was found to contain at least 2 immunogenic regions (407-422, and

417-437: Table 5.2).

Detailed localisation of HA peptide recognition

The responses of donors **A** + **B** localised to 5 regions of HA as represented by single HA peptides or pairs of peptides. Three of these have not undergone drift mutation amongst human H3N2 influenza A viruses over a 25 yr period (= conserved regions)(residues 97-115, 192-213, and 407-422). Two regions show some variation, the first (303-323) has however only undergone a single highly conservative substitution (307 $^{R\ to\ K}$), and the second (192-213) has undergone 3 conservative residue substitutions (193 $^{S\ to\ N}$, 197 $^{Q\ to\ R}$, 213 $^{I\ to\ V}$).

The response of donors **C** and **D** localised to 3 conserved regions (20-40, 407-422, 417-437), as well as to residues 303-323.

Donor **E**'s HA response was dominated by the recognition of residues 20-40 which are highly conserved.

Donor **F** responded to 9 peptide pools, responses within 7 of these were localised, and with one exception (residues 110-128: which show one conservative 124 $^{G\ to\ D}$ and one non-conservative substitution 126 $^{T\ to\ N}$) were to conserved regions (residues 25-40, 44-59, 97-115, 232-247, 303-323, 407-422, 417-432, 436-451).

Though donor **G** mounted strong responses to residues 20-40, 97-115, 303-323, and 403-422 all of which are conserved (see above), this subject also mounted moderate responses to residues 118-133, 129-144, and 192-207 which encompass parts of antibody sites E, A, and B respectively. Residues 118-133 incorporate antibody site E and includes 3 conservative (124 $^{G\ to\ D}$, 131 $^{T\ to\ A}$, 133 $^{N\ to\ S\ and\ D}$) and 2 non-conservative (122 $^{T\ to\ N}$, 126 $^{T\ to\ N}$) substitutions. Residue 129-144 include the 3 conservative drift mutations residues 131 and 133 and 143 $^{P\ to\ S}$, and 2 non-conservative drift mutations 137 $^{N\ to\ Y}$ (non-conservative), 144 $^{G\ to\ V}$ (non-conservative). Residues 192-207 are located on the edge of antibody site B although only 3 conservative drift mutations are included (residue 193 drifted 193 $^{S\ to\ N}$, and then recently reverted to the A/Aichi/68 sequence, residue 197 $^{Q\ to\ R}$, and 207 $^{R\ to\ K}$).

Donor **H** mounted a proliferative $CD4^+$ T cell response to a large number of peptide pools. The strongest responses localised to 5 HA regions, 2 of which are conserved (residues 303-323, 407-422, 417-432), and 2 regions which have been subject to drift mutation (residues 252-267, which includes a non-conservative substitution ($262^{T\ to\ N}$), and residues 192-207 (see above)).

Donor **I** responded strongly to 5 peptide pools, all these responses localised regions which are generally conserved (residues 97-115, 303-323, 313-328, 378-393, 442-462, 463-478), however this donor did mount a moderate response to residues 113-128 which have undergone one conservative ($124^{G\ to\ D}$) and two non-conservative substitutions (122 and $126^{T\ to\ N}$).

Donor **J** responded to conserved regions (peptides 308-323, 407-422, 417-427).

Donor **K** responded strongly to 3 peptide pools: The response to the first 2 overlapped and were directed to residues 228-247 which includes 2 conservative ($242^{V\ to\ I}$, and $244^{V\ to\ L}$) and one non-conservative ($248^{N\ to\ T}$) substitutions, though the non-conservative drift mutation is on the extreme C-terminus of this region and therefore may not be significant. Donor K's other strong response was to residues 463-483 which have one highly conservative drift mutation ($453^{R\ to\ K}$).

Donor **L** responded most strongly to 3 peptide pools representing the conserved HA2 subunit (which localised to residues 384-399, 403-422, 422-442, 463-483), and mounted a moderate response to one region of HA1 which localised to peptide 232 (see above).

Response of $CD4^+$T cell lines from adult donors with no history of influenza during the preceding 4 years to HA and peptides

The responses of the 6 control donors (M-R) were, in general, to similar regions of HA as those of the recent influenza donors, though not as strong (Figure 5.3). Once again every T cell line responded to the HA2 subunit. Pools 97-128, 295-328, and 407-442 were recognised by 4/6, 5/6, and 6/6 donors respectively.

Influence of MHC class II on T cell recognition of HA peptides

It is striking that the responses of donors A and B (sharing DRB1*0101, 11 DRB3*01/02/03, DQB1*0501,0301) to the peptide pools were very similar; both responded to pools 97-128, 183-217, 295-328, and 407-442, and studies with single peptides demonstrated that the responses were localised to identical peptides or pairs of overlapping peptides within the pools (Table 5.2).

Donors C and D (DRB1*04, 15, DRB4*01, DRB5*01, DQB1*0301, 0601/2) differed in DR4 subtype (0401, and 0408 respectively), but both T cell lines responded strongly to pools 1-25, 25-59, 97-128, 273-305, 295-328, 407-442, and 456-488. An additional response to 252-283 was made by donor D, and again where single peptide studies were undertaken, dominant responses were found to be directed to identical individual peptides (see above and Table 5.2).

Donors G and O who differed in exposure history to influenza, but shared low resolution HLA DR1,4, DRB4, and DQ 3,5 type, made strong responses to identical peptide pools which again were primarily directed towards identical individual peptides or pairs of peptides (see above and Table 5.2), though there were differences in the relative strengths of their responses.

Similarities in patterns of response were also seen between individuals who shared a single class II MHC haplotype. This was particularly evident for HLA-DRB1*0101, as subject G who shared DRB1*0101 and DQB1*0301,05 with subjects A and B and responded to pools 97-128, 183-217, 295-328, 384-418, and 407-442. The responses of subjects M, O, and R who had no history of recent influenza, and who shared the MHC class II alleles DRB1*0101, and DQB1*05, were very similar to A, B, and G, with all 6 lines responding strongly to peptide pools 97-128, 295-328, and 407-442, and where localised these responses were found to be directed to identical individual peptides (Table 5.2).

Subject Q (DRB1*0101,12 DRB3*01/02/03, DQB1*0301, 05) responded to 295 and 407 but not to pool 97 (see discussion).

In contrast marked differences were seen in response patterns of donors with different subtypes of HLA-DRB1*01: neither donor L (DRB1*0102) nor donor N

(DRB1*0103) followed the pattern seen in the DRB1*0101 donors.

Studies with anti-MHC class II antibodies

The results of the MHC restriction studies using antibodies to class II MHC on two T cell lines, are shown as examples in Figure 5.4. The majority of responses that could be inhibited by anti-MHC class II blocking antibodies were primarily blocked by anti-HLA DR antibodies. One important exception was the response to residues 407-422 which appears to be predominantly HLA-DQB1*05 restricted. Interestingly donor D showed some increase in proliferation to peptides 20/25, 97/100, 303/308 following incubation with anti HLA-DP antibodies. The apparent HLA restriction of dominant responses is summarised in Table 5.2.

Influenza Virus Cross Reactivity

Six T cell lines from the recent influenza donors, including all 3 donors (D, I, L) who were non-responsive to HA A/Aichi/68 prior to November/December 1993, were examined for cross reactivity amongst a panel of H1, H2, and H3 influenza A viruses (Figure 5.5). All were cross reactive, although the relative strengths of responses to H1, H2, and H3 viruses varied considerably. T cell line L made a stronger response to H3 viruses than the H1 or H2 viruses. This T cell line was dominated by responses to HA2. Studies with single peptides localised the response to the regions 384-399, 403-422, 427-442, and 463-483 (Table 5.2). In 3 out of 4 of these regions radical changes occur between H3 and H1 and H2 influenza A viruses (residues $388^{\text{T to M}}$, $404^{\text{G to K and R}}$, $412^{\text{Y to K}}$, $417^{\text{F to K}}$, $430^{\text{A to L}}$, and $440^{\text{T to H}}$), which probably account for these observed differences in response.

Cytokine production by HA specific $CD4^+$ T cell lines

The production of IL-4 and INF-γ by HA specific $CD4^+$ T cell lines derived from 6 donors was examined. Responses to both HA A/Beijing/32/92 and dominant HA peptides were found to be primarily Th1-like in nature, with high levels of INF-γ and low or absent levels of IL-4 (data not shown).

5.5 Discussion

This is the first major investigation of human $CD4^+$ T cell recognition of HA following recent natural infection. $CD4^+$ T cell responses of 12 unrelated adults to HA A/Beijing/32/92 (H3N2) were examined 3 mo following natural infection, and compared to the responses of 6 donors with no history of influenza for at least 4 yr. HA was highly immunogenic in all cases. Though the overall pattern of $CD4^+$ T cell HA epitope selection by individual donors, differing in MHC class II haplotype, showed considerable diversity, 2 regions of the molecule (residues 303-323, and 407-442) were recognised by every donor.

Similarity in HA peptide recognition amongst donors sharing MHC class II alleles

MHC class II, and in particular HLA-DR, had the major influence over epitope selection, rather than an individual's exposure history to influenza. It is remarkable that proliferative responses by the short term T cell lines from unrelated donors A and B, who are both DRB1*0101, 11, DRB3*01/02/03, DQB1*0301, 05 were both directed predominantly to peptide pools 97-128, 183-217, 295-328, 384-418 and 407-442, with only some quantitative differences. Analysis using single peptides, revealed that their responses where localised were to identical peptides or pairs of overlapping peptides (Table 5.2). Donors G and O who share low resolution HLA-DR and -DQ type, but differ in exposure history, also recognised identical peptide pools. This similarity in pattern of response was not limited to donors with such close class II MHC matches, as all 7 donors who expressed DRB1*0101 DQB1*05 alleles, irrespective of differences in their exposure history to influenza and other HLA class II alleles, showed similar patterns of T cell recognition, except donor Q who failed to respond to pool 97. All 3 regions are highly conserved amongst H3 influenza A viruses. The regions represented by the second and third of these pools also have homology between H3 and H1 influenza A viruses (see Appendix 1). An explanation for the lack of response to pool 97-128 may be recent infection with an H1 influenza A virus, or alternatively this

donor might lack TCRs capable of mounting a strong response to this region. Unfortunately this donor declined influenza vaccination, which would have helped to resolve this issue.

See Chap. 10 for a detailed comparison between observed MHC associated HA peptide recognition, and published HLA-DR binding motifs.

Differences in HA peptide recognition amongst donors with different subtypes of common serological HLA-DR types

As predicted by peptide binding studies (Busch *et al.,* 1991, Coppin *et al.,* 1993) the subjects who expressed other subtypes of HLA DR1, donors L (DRB1*0102) and N (DRB1*0103), differed in peptide recognition compared to both the DRB1*0101 donors and each other. There are only limited structural differences between these alleles (residues 85 $^{V\ to\ A}$ and 86 $^{G\ to\ V}$ for DRB1*0102, and residues 67 $^{L\ to\ I}$, 70 $^{Q\ to\ D}$, and 71 $^{R\ to\ E}$ for DRB1*0103).

Six donors shared the low resolution HLAB1*04 haplotype: Two of these, donors C (DRB1*0401) and D(DRB1*0408), also shared DRB1*15, and had very similar patterns of peptide responsiveness. The variations between these donors may reflect the slight difference between DRB1*0401 and DRB1*0408 (position 71 $^{K\ to\ R}$). Identifiable patterns of response are also evident for HLA DRB1*07, with donors P, I, and R all responding strongly to pool 456-488.

HA2 induces strong CD4$^+$ T cell responses

Most interestingly HA2 (residues 330-550), which has been regarded as largely non-immunogenic (Becht *et al.,* 1984, Caton and Gerhard 1992), induced strong responses in every donor. Furthermore with the exception of its N and C termini, every peptide pool representing HA2 induced a response in at least 3/12 recent influenza donors. One immunodominant conserved region (residues 407-442) induced responses in 11/12 recent influenza donors during the second or third week in culture, this region is unchanged amongst H3 influenza A viruses and has homology with H1 and H2 viruses. Two additional highly conserved regions

(residues 357-393 and 384-418) were recognised by 8/12 recent influenza donors. There has been only one previous preliminary study of the human $CD4^+$ T cell response to HA2: Unselected PBMC were derived from 14 donors, who were not tissue typed, and limiting dilution analysis showed a T cell responses to at least 3 regions of HA2 (Rodda *et al.,* 1993). One murine study has demonstrated cross reactive $CD4^+$ T cell recognition of HA2 following nasal infection with live virus (though no epitopes were determined) (Katz *et al.,* 1985b). A second murine study demonstrated responses to HA2 following vaccination with HA2 alone, and described two epitopes (residues 425-437, and 499-511)(Jackson *et al.,* 1994).

$CD4^+$ T cell recognition of HA1 is primarily directed towards conserved regions

As regards the HA1 subunit (residues 1-328), 2 regions, residues 97-128 and 295-328 dominated, inducing responses in 10/12 and 12/12 recent influenza donors respectively. Studies with single peptides localised the majority of responses to residues 100-115 and 303-323 which are conserved among H3 influenza A viruses (Table 5.2). The second region has partial sequence homology with H1 and H2 viruses and is closely related to the dominant epitope originally described by Lamb (using a different panel of peptides) (Lamb *et al.,* 1982b, Lamb and Green 1983). One peptide pool which contained a variable region within the H3 influenza A viruses, pool 183-217, induced responses in 8/12 donors. The majority of responses localised within this region were to residues 192-212, which have been relatively conserved during the past decade ($193^{S\ to\ N}$, $197^{Q\ to\ R}$, $201^{R\ to\ K}$, $207^{K\ to\ R}$ - see appendix 1). It is interesting that viral infection induces recognition of several conserved HA epitopes by a large proportion of the population.

Four HA regions were less immunogenic

The first represented by peptide pool 208-243 though generally conserved amongst H3 influenza A viruses contains two important non-conservative residue substitutions. The first $214^{I\ to\ T}$, appears amongst H3 influenza A viruses to be

specific of A/Beijing/32/92, and the closely related isolate A/England/471/93. The second 228 L to Q which occurs in A/Beijing/32/92 and the closely related A/Madrid/252/93 is extremely unusual in mammalian H3 influenza A viruses, but though is a normal egg adaptive mutation induced by passage through eggs, while absent from field isolates (N.Cox personal communication). The second region represented by peptide pools 317-348 and 336-369 contains the cleavage point between HA1 and HA2 (residue 329), as well as the N-terminus of HA2 which appears to be highly conserved among H3 influenza A viruses (though the HA2 subunit of HA Beijing/32/92 has not been sequenced - see Chap. 3). The third and fourth regions, peptide pools 478-513, and 526-550 represent the C-terminal of HA2: Interestingly this region of the HA2 subunit shows considerable variation between H3, and H1 and H2 influenza A viruses (see Appendix 1).

HA recognition by control donors

The HA repertoires of $CD4^+$ T cell lines derived from the control donors M-R were examined as a comparison between 3-6 mo and longer term $CD4^+$ T cell memory. The 2 groups recognised similar peptide pools, though the responses of the control panel were generally weaker. Interestingly the control lines demonstrated a several fold lower expansion *in vitro* compared to those of the recent influenza donors (Table 5.1). The most likely explanation is a lower T cell precursor frequency, implying a decline in circulating $CD4^+$ T cell memory following infection. A similar decline in circulating influenza specific $CD8^+$ T cell memory has been reported (McMichael *et al.,* 1983a) and both findings are in agreement with early epidemiological surveys which showed protection from influenza following natural infection declining after 4yr (Pickles *et al.,* 1947).

Comparison to previous studies

These findings concur with the original human $CD4^+$ T cell studies (which were limited to single donors) where, with the exception of one clone specific for a variable surface region of an H2 influenza A virus (Brown *et al.,* 1991), the re-

sponses were either cross reactive between H3 influenza A viruses (and sometimes H1 and H2 viruses) (Fleischer *et al.,* 1985, Lamb *et al.,* 1982a) or localised to conserved regions of the HA1 subunit (Lamb *et al.,* 1982b, Lamb and Green 1983). But contrast with recent murine studies of live viral infection which have demonstrated strong $CD4^+$ T cell responses to variable epitopes closely related to the antibody neutralising sites (Barnett *et al.,* 1989a, Barnett *et al.,* 1989b, Burt *et al.,* 1989, Graham *et al.,* 1989, Mills *et al.,* 1986a, Thomas *et al.,* 1982) - see Chap.10 for full discussion.

One explanation for these apparent species differences was that the influenza strains which infected my donors were not structurally related to A Beijing/32/92. This seems unlikely as the majority of influenza A virus field isolates obtained in London during November and December 1993 were very similar to A/Beijing/32/92: 4 conservative substitutions ($75^{H\,to\,N}$, $145^{N\,to\,K}$, $201^{R\,to\,K}$, $208^{R\,to\,K}$), 3 intermediate substitutions ($189^{R\,to\,S}$, $214^{T\,to\,I}$, $276^{T\,to\,N}$), and 2 non-conservative substitutions were recorded ($157^{S\,to\,L}$ $219^{S\,to\,F}$ $226^{Q\,to\,L}$)(See Appendix 1: Personal communication J.Ellis, Public Health Laboratory Service, Colindale, England). These changes offer an explanation for the low frequency of response seen to pools 137-171 and 208-243, but do not account for the dominance of $CD4^+$ T cells recognising HA regions conserved within the H3 subtype. Definitive proof that adult $CD4^+$ T cell responses to HA following natural infection are largely directed towards conserved regions of the molecule, would require the infection of volunteers with a known influenza strain. This was not possible in the current study, and an alternative course was followed: Eight volunteers were vaccinated with a subunit vaccine containing A/Beijing/32/92. The results of this study are presented in the following Chap.

Conclusions

The human $CD4^+$ T cell response to HA following natural infection in adults, is dominated by the recognition of highly conserved epitopes located throughout the molecule. The HA2 subunit, previously widely regarded as non-immunogenic,

induced strong responses in every donor. Conserved epitope recognition resulted in functional cross reactivity amongst a panel of influenza A viruses.

MHC class II had a powerful influence over HA epitope selection: This study included one pair of unrelated donors expressing identical HLA DRB1* and DQB1* alleles, and two pairs of donors sharing low resolution MHC class II types, these pairs responded to identical HA peptides, furthermore clearly identifiable patterns of response were seen between donors sharing single MHC class II haplotypes irrespective of the presence of other alleles, and exposure history.

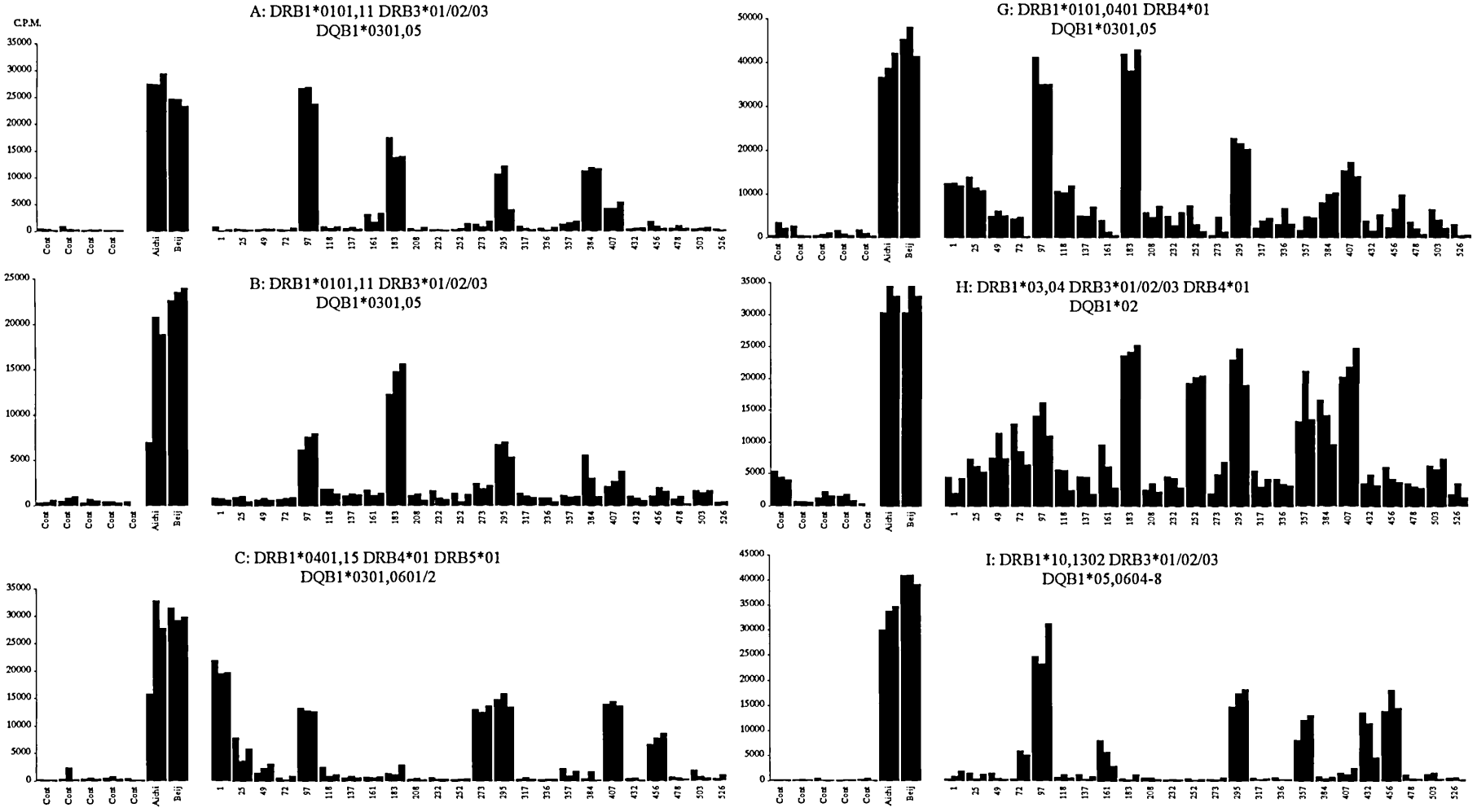
Two conservative immunodominant regions were identified (residues 295-328, and 407-442) which induced responses in every donor. The majority of HA responses are HLA-DR restricted.

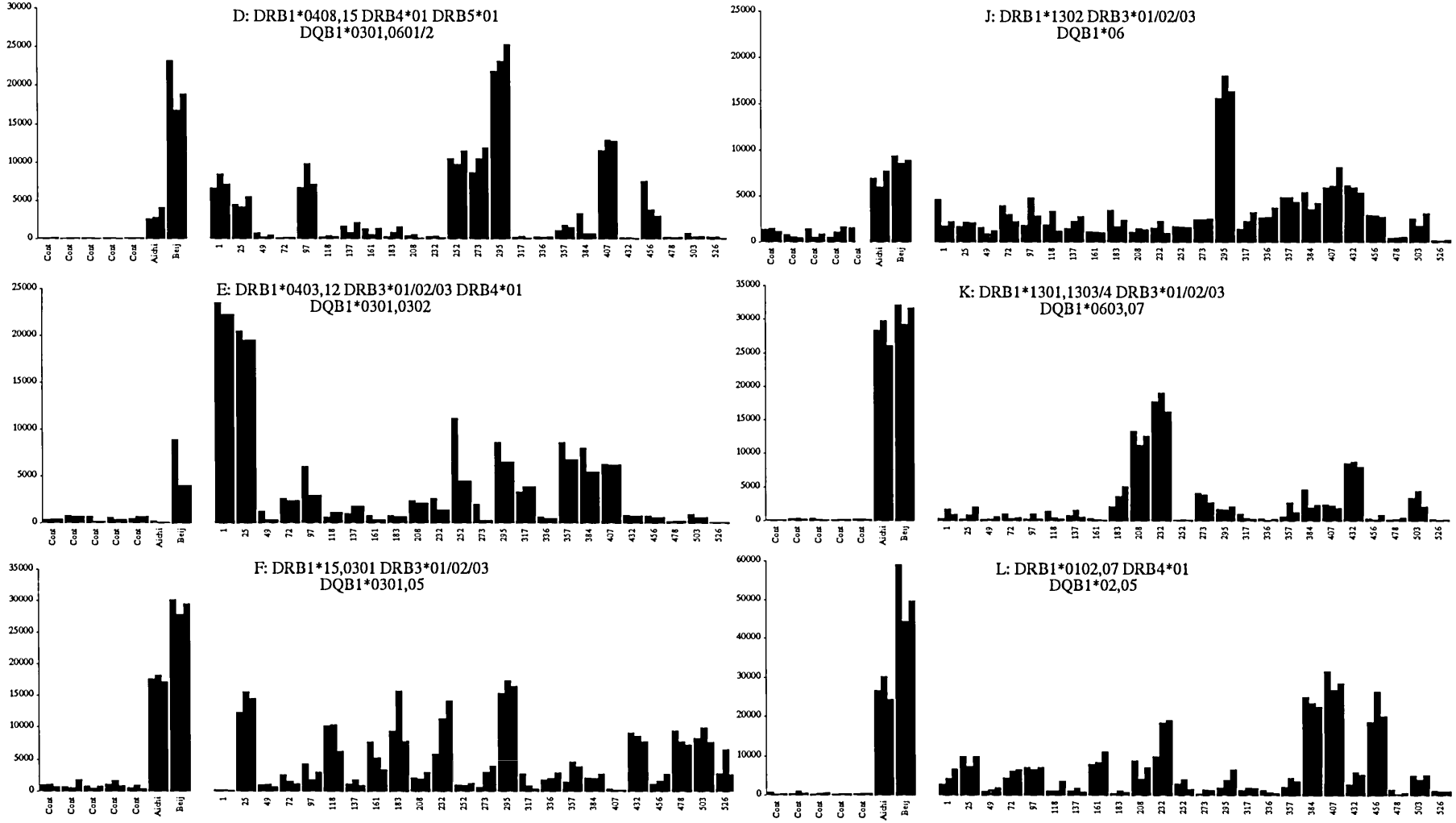
Table 5.1, Subject details

Donor	Age	Sex	Class I MHC Haplotype	Class II MHC Haplotype	Duration Illness Days	Cell Recovery
A	35	F	A2,68, B18,44, CW7,7/8V	DRB1*0101,11, DRB3*01/02/03, DQB1*0301,05	7	7.9
B	55	F	A1, B18,27, CW1,7	DRB1*0101,11, DRB3*01/02/03, DQB1*0301,05	14	4.5
C	35	M	A2,3, B7,44 CW5,7	DRB1*0401,15, DRB4*01, DRB5*01, DQB1*0301,0601/2	10	13.3
D	24	F	A1,72, B7,37, CW6,7	DRB1*0408,15, DRB4*01, DRB5*01, DQB1*0301,0601/2	10	4.7
E	24	F	A11,24, B52,75, CWND	DRB1*0403,12, DRB3*01/02/03,DRB4*01,DQB1* 0301,0302	14	8.1
F	24	M	A1,2, B8,52, CWND	DRB1*15, 0301, DRB3*01/02/03, DQB1*02,0601/2	7	3.6
G	25	F	A2, B44/63, CW1,5	DRB1*0101,0401 DRB4*01, DQB1*0301,05	10	4.5
H	21	F	A2,3, B7,44, CW5,7	*"DRB1*03,04, DRB3*01/02/03, DRB4*01, DQB1*02"*	14	4.4
I	40	F	A2, B15,65, CW3,10	DRB1*10,1302, DRB3*01/02/03, DQB1*05,0604-8	14	13.4
J	25	M	A24, B55,62, CW9	DRB1*1302, DRB3*01/02/03, DQB1*02,06	7	4.4
K	25	F	A30,68, B41,51, CW(1501-3),(41-42)	DRB1*1301,1303/4, DRB3*01/02/03, DQB1*0301,603	5	7.0
L	25	M	A2, B14, CW5	DRB1*0102,07, DRB4*01, DQB1*02,05	14	10.4
M	38	M	A2,3, B7,51, CW1,7	DRB1*0101, DQB1*05	Nil	1.2
N	30	M	A2,66, B27,51, CW1,7	DRB1*0103,08, DQB1*0301,04	Nil	0.7
O	42	M	A2,31, B51,60, CW4,10	DRB1*0101,0402, DRB4*01, DQB1*0302,05	Nil	0.6
P	34	M	A2,28, B7,17, CW6,7	DRB1*15,7, DRB4*01, DRB5*01, DQB1*0303,601/2	Nil	0.6
Q	36	F	A2,19, B14,18, CWND	DRB1*0101,12, DRB3*01/02/03, DQB1*0301,05	Nil	2.1
R	28	M	ND	DRB1*0101,07, DRB4*01, DQB1*02,05	Nil	1.5

Subjects A-L had history of influenza during November/December 1992, subjects M-L had no history of influenza during the preceeding 4yr. Subject F was MHC Class II typed serologically only, typing reported using molecular typing nomenclature for continuity. ND= Not done. Cell recovery is a ratio of cells recovered at d21 in culture to number PBMC initially seeded. Cell recovery = (Number cells recovered d7/ number PBMC originally seeded) x (number recovered d14/ number seeded d7) x (number recovered d21/ number seeded d14).

Figure 5.1 Repertoire of CD4+ T cell responses to HA/Beijing/32/92, HA Aichi/68, and panel of HA Beijing/32/92 specific peptides, by 12 unrelated adult donors with history of recent influenza





CD4+ T cell lines from 12 unrelated donors (A to L), with a history of recent influenza, were selected for 2-3 wks with HA A/Beijing/32/92 (H3N2). Response to HA and peptides tested at d14 or d21 in triplicate wells of round bottomed 96 well tissue culture plates: 4 x 10^4 irradiated autologous PBMC (3000 rads) were preincubated with HA or peptides for 1h prior to the addition of an equal number of T cells. T cell proliferation in response to HA A/Aichi/68 (1.0 μg/ml = Aichi), HA Beijing/32/92 (0.1 μg/ml = Beij), or peptide pools (10μg/ml individual peptides: Peptides labelled by n-terminal residue, and pools labelled by N-terminal peptide),was measured by pulsing at 48h with [3]TdR and harvesting 16h later. Control (Cont) is response of T cells to APC and medium. X-axis represents c.p.m.. Results from individual wells rather than means of triplicate wells shown.

Figure 5.2, Number of donors responding >5x G.M. background proliferation to HA peptide pools after 2nd and /or 3rd in vitro stimulation

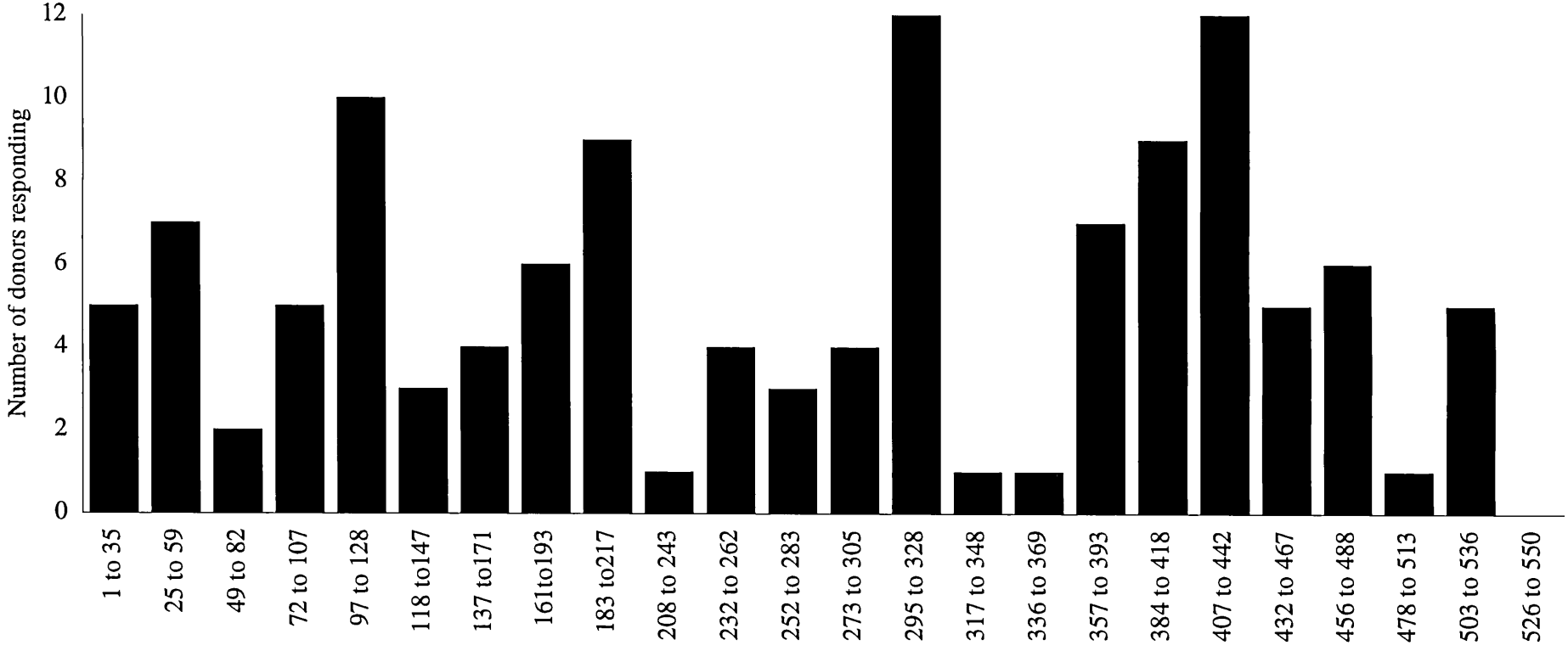
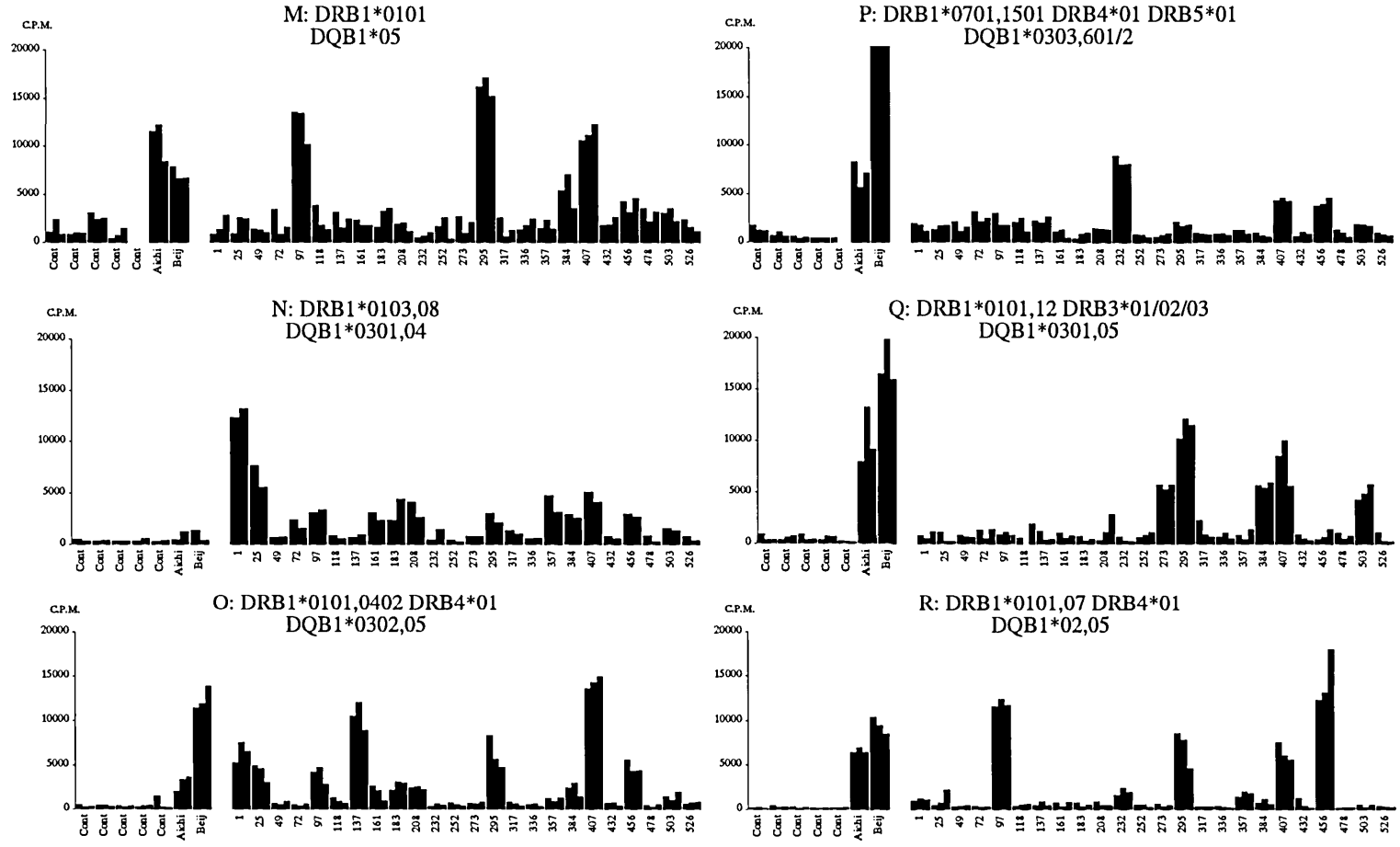
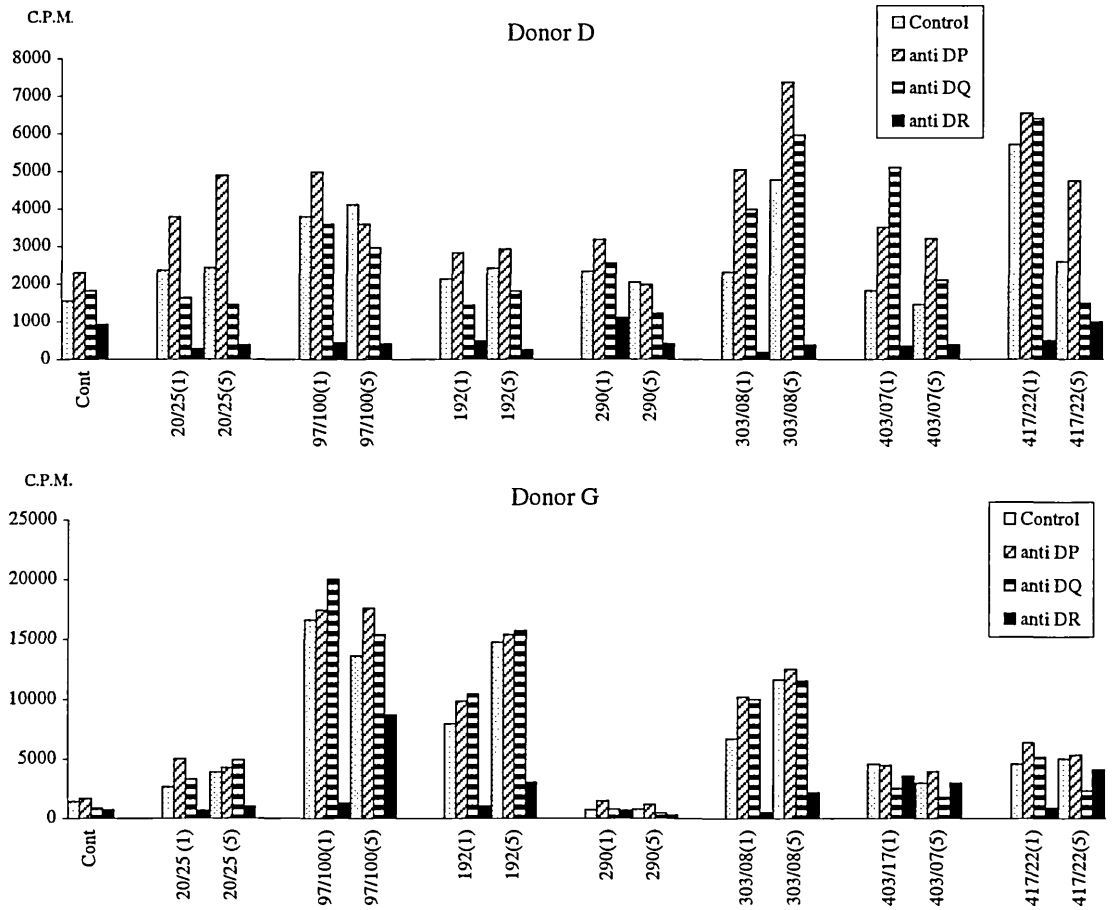


Figure 5.3 Response of CD4+ T cells from 6 donors with no recent history of influenza to HA Beijing/32/92, HA Aichi/68, and HA Beijing/32/92 specific peptides



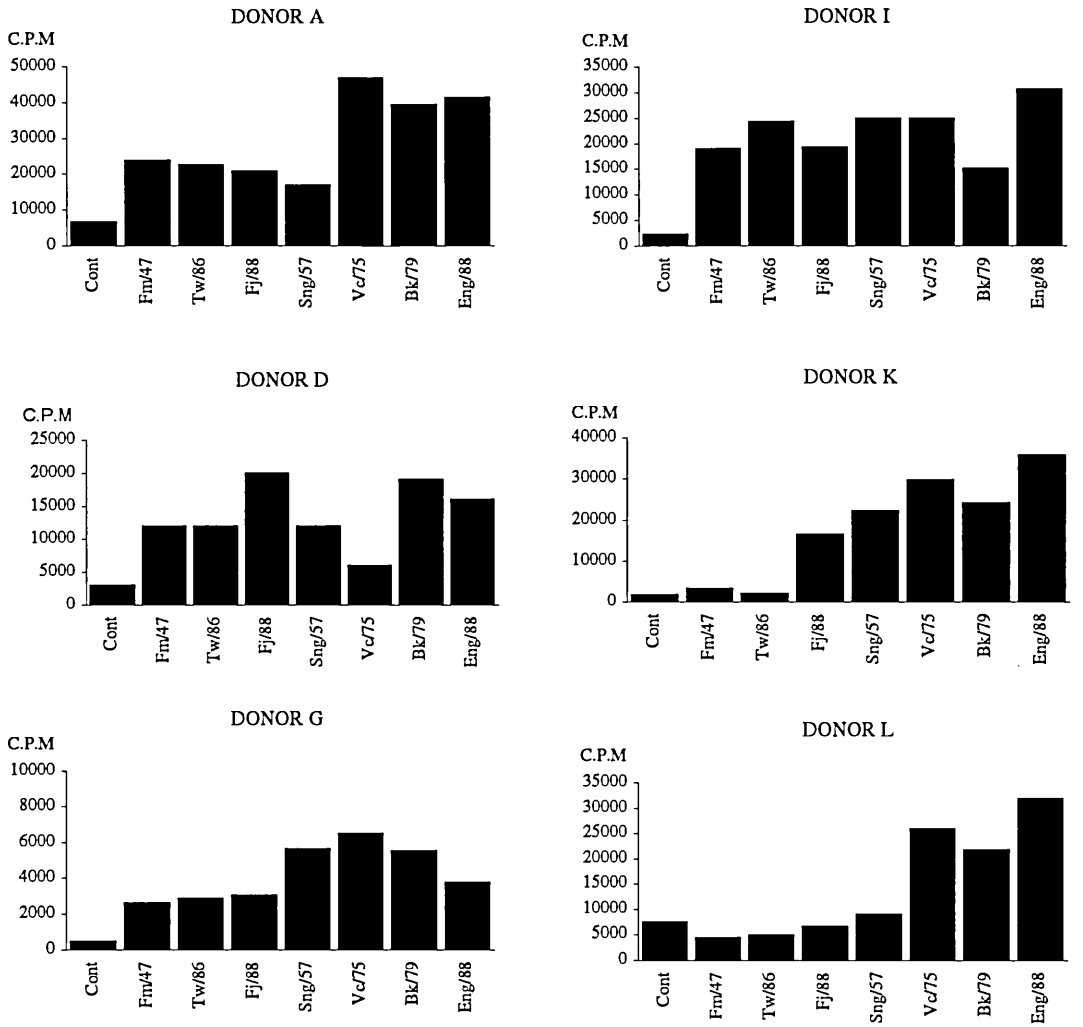
HA responses of CD4+ T cell lines from 6 donors with no history of influenza during the last 4y. For details, see Fig. 5.1. Three donors had previously been frequently exposed to influenza virus, donors N and P, who were practicing physicians, and donor O, who had worked extensively with H3 influenza A viruses 10y previously. Details as Fig. 5.1.

Figure 5.4 Two examples of studies with anti-MHC class II antibodies



Effect of MHC class II blocking antibodies on HA specific CD4+ T cell responses to dominant peptides. Autologous PBMC were incubated with 10μg/ml anti HLA-DP, -DQ, or -DR antibodies, or with medium alone (control). Individual peptides or pairs of overlapping peptides (1 and 5μg/ml) were added, and the PBMC incubated for 1h prior to the addition of responder HA Beij selected CD4+ T cells. g.m. of triplicate wells shown. Further experimental details are described in Fig. 5.1. Numbers on the X-axis refer to the individual peptide or pair of peptides; numbers in parentheses refer to the concentration of peptide in μg/ml.

Figure 5.5 Response of 6 CD4+ T cell lines to panel of influenza A viruses



Responses of 6 CD4+ T cell lines to a panel of influenza A viruses. Autologous irradiated PBMC were prepulsed for 1h with 1-10 HAU of virus prior to the addition of 3 wk CD4+ T cell lines selected by A/Beijing/32/92. Control (Cont) = APC, T cells and medium. Viruses FM/47 = A/Formosa/1/47, Tw/86 = A/Taiwan/1/86, Fj/88 = A/Fiji/2/88 (all H1N1); Sng/57, A/Singapore/1/57 (H3N2); Vc/75 = A/Victoria/3/75, Bk/79 = A/Bangkok/1/79, A/England/427/88 (all H3N2). g.m. of triplicate wells shown. Further experimental details as Fig.1.

Table 5.2-Summary of CD4+ T cell response to individual HA peptides within dominant peptide pools, and HLA restriction of response following natural infection

Donor	DRB1*	DRB3*, DRB4*, DRB5*	DQB1 *	Dominant peptide Pools	cpm	Dominant peptides within pools	cpm	Sequence	Restn.	Comments on HA region
A	0101	3*0X	0301	97	25000	97-100-105	6668/177-54/6067	CYPYDVPDYASLRSLVASSGTLEF	100=DR	Conserved amongst H3N2
	110X		050X	183	15000	192-8	10249 /4894	TSLYVRASGRVTVSTKRSQQTV	DR	193 S to N (conservative) in some H3N2 viruses, 197 Q to R (conservative), 213 I to V (conservative)
				295	10000	303-8	252 6/8086	GACPRYVKQNTLKLATGMRNV	DR	K to R 307 (conservative)
				384	12000	388	5259	TNEKFHQIEKSEVEGR	DR	Conserved amongst H3N2
				407	5000	407	2248	QDLEKYVEDTKIDLWS		
B	0101	3*0X	0301	97	7000	97-100	23868 /24903	CYPYDVPDYASLRSLVASS		Conserved amongst H3N2
	110X	0301	050X	183	15000	192-8	4049 6/29856	TSLYVRASGRVTVSTKRSQQTV		193 S to N (conservative) in some H3N2 viruses, 197 Q to R (conservative), 213 I to V (conservative)
				273	2000	277-282	14954 /12425	CSSECITPNGSIPNDKPFQNV		278 I to S (non-conservative)
				295	6000	303-8	6313 /8765	GACPRYVKQNTLKLATGMRNV		307 K to R (conservative)
G	0101	5*01	0301	1, 25	12,000/ 12,000	20-25	8408 /4919	VPNGTLVKTITNDQIEVTNAT	DR	Conserved amongst H3N2
	0401		0601/2	97	35000	97-100	22075 /31087	CYPYDVPDYASLRSLVASS	DR	Conserved amongst H3N2
						118	6052	LEFINEDFNWTGVAQD		124 G to D (conservative), 126 T to N (non-conservative), 131 T to A (conservative), 133 N to S to D (both conservative)
						129	6459	GVAQDGGSYACKRGSV		131 T to A (conservative), 133 N to S to D (both conservative) 135 G to K (non-conservative some Beijing/92 like strains, 137 N to Y (non-conservative), region 140-146 is antibody site A
				183	42000	192	30487	TSLYVRASGRVTVSTK	DR	On edge of AB site B, 193 S to N (conservative), 197 Q to R (conservative)
				295	24000	303/8	12587 /8632	GACPRYVKQNTLKLATGMRNV	DR	307 K to R (conservative)
				384	10000					
				407	17000	403/7	7030	EGRIQDLEKYVEDTKI	?DQ	Conserved amongst H3N2

C	0401	4*01	0301	1,25	20,000/ 5,000	20-25	13925 /6988	VPNGTLVKTITNDQIEVTNAT		Conserved amongst H3N2
	1501	5*01	0601/2	25	5000	39	1259	ATELVQSSSTGRICD		53 N to D (conservative)
				97	12000	100	5358	YDVPDYASLRSLVASS		Conserved amongst H3N2
				273	13000	290	10031	NDKPFQNVNRITYGAC		299 K to R (conservative)
				295	14000	303-8	1633 /8122	GACPRYVKQNTLKLATGMRNV		307 K to R (conservative)
						357	2921	NSEGTGQAADLKSTQA		
				407	14000	407	1628	QDLEKYVEDTKIDLWS		Conserved amongst H3N2
				407	14000	422	2009	SYNCELLVALENQHTI		Conserved amongst H3N2
				456	8000	463-8	5811 /9252	GNGCFKIYHKCDNACIGSIRN		479 E to G (non-conservative)
						508	1375	KSGYKDWILWISFAI		
D	0408	4*01	0301	1,25,	7,000/ 5,000	20-25	7000	VPNGTLVKTITNDQIEVTNAT		Conserved amongst H3N2
	1501	5*01	0601/2	97	7000	100	10000	YDVPDYASLRSLVASS		Conserved amongst H3N2
				295	22000	303-8	3300 /9800	GACPRYVKQNTLKLATGMRNV	DR	307 K to R (conservative)
				407	11000	417/22	13500 /15000	KIDLWSYNCELLVALENQHTI	DR	Conserved amongst H3N2
				456	4000	463	18000	GNGCFKIYHKCDNACI		479 E to G (non-conservative)
E	0403	3*0X	0301	1	23000	20/25	18500 /16500	VPNGTLVKTITNDQIEVTNAT		Conserved amongst H3N2
	12	4*01	0302	25	21000					
H	03	3*0X	02	97	15000	100	25,000	YDVPDYASLRSLVASS		Conserved amongst H3N2
	04	4*01		183	25000	192	25000	DRDQTSLYVRASGRVTVSTK		On edge of AB site B, 193 S to N (conservative), 197 Q to R (conservative)
				252	22000	257	29000	IAPRGYFKIRNGKSSIMRSDAP		262 T to N (non-conservative,)
				295	24000	303/8	13000 /22000	GACPRYVKQNTLKLATGMRNV		307 K to R (conservative)
				407	22000	407	1535	QDLEKYVEDTKIDLWS		Conserved amongst H3N2
				407	22000	417	23000	KIDLWSYNCELLVALE		Conserved amongst H3N2

Continued overleaf

Table 5.2 (Continued)

Donor	DRB1*	DRB3*, DRB4*, DRB5*	DQB1 *	Dominant peptide Pools	c.p.m.	Dominant peptides within pools	c.p.m.	Sequence	Restn.	Comments on HA region
F	150X		02	25	14000	25	2500	LVKTITNDQIEVTNAT		Conserved amongst H3N2
	0301		0601/2	25	14000	44	8500	QSSSTGRICDSPHRIL		50 K to R (conservative), 53 N to D (conservative), 54 N to S (conservative)
						97/100	5000 /5000	CYPYDVPDYASLRSLVASS		Conserved amongst H3N2
						110/113	9000 /7500	SLVASSGTLEFINEDFNWT		124 G to D (conservative), 126 T to N (non-conservative),
				232	11000	232	10000	IYWTIVKPGDILLINS		242 V to I (conservative), 244 V to L (conservative)
				295	17000	303/8	5000 /7000	GACPRYVKQNTLKLATGMRNV		307 K to R (conservative)
				407		407	8000	QDLEKYVEDTKIDLWS		Conserved amongst H3N2
						417	2500	KIDLWSYNCELLVALE		Conserved amongst H3N2
				432	8000	436	5000	TIDLTDSEMNKLFEKTR		Conserved amongst H3N2
I	100X	3*0X	05	97	25000	97/100	20000 /27000	CYPYDVPDYASLRSLVASS	DR	Conserved amongst H3N2
	1302		0604-8	97	25000	113	2500	ASSGTLEFINEDFNWT		122 T to N (conservative) 124 G to D (conservative), 126 T to N (non-conservative)
				161	6000					
				295	17000	303/308	6000 /16000	GACPRYVKQNTLKLATGMRNV	DR	307 K to R (conservative)
				295	17000	313	7500	TLKLATGMNNVPEKQT	DR	Conserved amongst H3N2
				357	10000	378	9000	NGKLNRLIEKTNEKFG	?	384 V to L (conserved)
				432	10000	442/447	16000 /15500	SEMNKLFEKTRKQLRENAEDM	DR	453 R to K (conserved)
				456	15000	463	6000	GNGCFKIYHKCDNACI	?DR	Conserved amongst H3N2
J	1302	3*0X	02	295	17000	308	24412	YVKQNTLKLATGMRNV		Conserved amongst H3N2
			06	407	6000	407	3945	QDLEKYVEDTKIDLWS		Conserved amongst H3N2
				407	6000	417	5048	KIDLWSYNCELLVALE		Conserved amongst H3N2
				432	6000	442	6860	SEMNKLFEKTRKQLRE		453 R to K (conservative)

K	1301	3*0X	0301			20	1217	VPNGTLVKTITNDQIE		Conserved amongst H3N2
	1303/4		0603	183	4000	198/202	17800 /2190	ASGRVTVSTKRSQQTVSTKRSQ	DR	207 R to K(conservative), 213 I to V(conservative), 214 I to T (non-conservative),
				208/232	11000	228/32	34500 /43000	SRISIYWTIVKPGDILLINS	DR	242 V to I (conservative), 244 V to L (conservative)
				232	16000	243/247	1407 /1354	LLINSTGNLIAPRGYFKIRN		244 V to L (conservative),248 N to T (non-conservative)
				273	4000	277/82	21500 /22200	CSSECITPNGSIPNDKPFQNV	DR	278 I to S (non-conservative)
				295	2000	303	3190	GACPRYVKQNTLKLAT	DR	307 K to R (conservative)
						378	2550	NGKLNRLIEKTNEKFH		384 V to L (conservative)
				384	2,000	388	3300	TNEKFHQIEKEFSEVE		Conserved amongst H3N2
				384	2000	403	6900	EFSEVEGRIQDLEKYVEDTKI	DR	Conserved amongst H3N2
				407	2000					
				432	9000	442/7	18360 /15400	SEMNKLFEKTRKQLRENAEDM	DR	453 R to K (conservative)
				503	3000	508/13	2370 /3117	KSGYKDWILWISFAISCFLL		Conserved amongst H3N2
L	0102	4*01	02	232	16000	232/236	10000 /7500	YWTIVKPGDILLINSTGNLI		242 V to I (conservative), 244 V to L (conservative), 248 N to T (non-conservative)
	0701		05	384	25000	384	7900	LIEKTNEKFHQIEKSE		384 V to L (conservative)
				384/407	25,000/ 30,000	403/407	9000 /10500	EGRIQDLEKYVEDTKIDLWS		Conserved amongst H3N2
				407	30000	422/427	3500 /8500	SYNCELLVALENQHTIDLTDS		Conserved amongst H3N2
				456	5000	463/8	7000 /9500	GNGCFKIYKHKCDNACI		479 E to G (non-conservative)
M	101		5	97	12000	97/100/105	19000/95 00/20000	CYPYDVPDYASLRSLVASSGTLEF	DR	Conserved amongst H3N2
				295	16000	303/8	15000 /15000	GACPRYVKQNTLKLATGMRNV	DR	K to R 307 (conservative)
				407	10000	403	17000	EGRIQDLEKYVEDTKI	DQ	Conserved amongst H3N2

Chapter 6, Human CD4$^+$ T Cell Responses to HA A/Beijing/32/92 Following Influenza Subunit Vaccination

6.1 Introduction

Influenza subunit vaccines are widely prescribed and produce satisfactory levels of neutralising antibody to the vaccine strains (Palache 1992). However the protection provided by these antibodies is generally strain specific, and therefore short lived. Influenza subunit vaccines are also poor inducers of CD8$^+$ cytotoxic responses (McMichael 1994). Surprisingly there has never been a systematic examination of human CD4$^+$ T cell recognition of HA following any form of influenza vaccination. The finding that natural infection in adult boosts and /or induces CD4$^+$ T cell recognition of HA regions that are conserved in structure amongst H3N2 influenza A viruses, prompted the examination of the CD4$^+$ T cell HA response following vaccination. In particular we wished to discover whether a similar pattern of HA epitope selection occurred following vaccination, as this might provide some protection against future antigenic shift or drift. In addition we hoped to be able to compare the immunogenetics of the CD4$^+$ T cell recognition of HA following both immunological challenges. If similar patterns of epitope selection were seen following infection and vaccination, strong evidence would be provided that the observations regarding epitope recognition made following natural infection were not simply due to my donors being infected with an influenza A strain that was unrelated to A/Beijing/32/92.

A panel of normal adult donors willing to be vaccinated with a subunit influenza vaccine were selected. In order to allow a direct comparison between natural infection and vaccination, the 1993/4 vaccine containing A/Beijing/32/92 (the strain of H3N2 influenza A responsible for infecting the donors in Chap. 5) was used.

6.2 Aims

(1) To examine human $CD4^+$ T cell HA recognition in an outbred adult population following subunit vaccination. With particular reference to the balance of recognition between conserved regions and regions subject to drift mutation amongst human H3N2 influenza A strains.

(2) To compare the observed pattern of $CD4^+$ T cell HA epitope selection to that seen following natural infection with the same strain of influenza (A/Beijing/32/92)

(3) To investigate the immunogenetics of the $CD4^+$ T cell HA recognition following vaccination, and compare to the immunogenetics of HA recognition following natural infection.

6.3 Methods

Subjects details

Eight unrelated healthy adult volunteers were investigated prior to and 3-4 mo following vaccination (details in Table 6.1) Except for Donor S, who was selected as a known weak responder to HA, the donors were chosen at random. Donor I had a history of influenza during November/December 1993 (when A/Beijing/32/92 like strains were circulating), the others had no recollection of influenza like ill-nesses during the previous 5 yr. 3 subjects had been previously vaccinated against influenza: Donor I received annual influenza vaccines between 1985-1990, donor O received a trivalent subunit vaccine 5yr previously, and donor T aged 34yr received influenza vaccination on a nearly annual basis between the ages of 12 and 18. Subjects received 0.5ml of Influvac, containing 15μg each of HA from 3 viruses: A Beijing/32/92 (H3N2), A/Singapore/6/86 (H1N1), B/Panama/45/90 (influenza B) according to the manufacturers instructions. The vaccine was kindly donated by Dr R. Brands, Solvay Duphar, Weesp, The Netherlands.

Class I and II MHC types, of the donors, are shown in Table 6.1. Subjects S and V shared DRB1*0701,1501 DRB4*01, DRB5*01, DQB1*02,0601/2 haplotypes. Three donors shared the HLA DRB1*0101, DQB1*05 haplotype. Responses of donors with DRB1*0103, -0301, -0402, -080X, -100X, -1301, -1302 containing haplotypes were also examined.

Experimental methods

All experimental details are described in Chap. 2.

6.4 Results

The investigation of adult $CD4^+$ T cell responses to HA following infection (Chap. 5), revealed a marked difference in the relative *in vitro* expansion of $CD4^+$ T cell lines derived from donors recently exposed to influenza (mean expansion 7.2) compared to those from adults with no recent history of influenza (mean expansion 1.1)(data in Table 5.1). Differences in the level of proliferative responses to HA and peptides in the 2 groups of donors were also observed(see figs 5.1 and 5.3). In the current study $CD4^+$ T cell lines derived from donors S, T, N, R, U, and O (standard donors), prior to vaccination, expanded less than 3 fold in the presence of antigen (Table 6.1), and had HA Aichi and HA Beijing specific proliferative responses of less than 12,000 cpm (Fig. 6.1). Two donors (N and U) mounted negligible responses to HA. In marked contrast $CD4^+$ T cell lines derived from donors I and V, prior to vaccination, expanded at least 8 fold *in vitro* during culture, and had higher HA specific proliferation (Figure 6.2). Donor H had a history of influenza during November and December of 1993, whereas donor V had no recollection of an influenza like illness during the past 5yr. However on the basis of my previous data this donor is likely to have had a fairly recent subclinical influenza infection.

HA recognition by standard donors

Following vaccination $CD4^+$ T cell lines derived from the standard donors showed increases in both *in vitro* cell yield (Table 6.1), and in HA Beijing specific proliferation (Fig. 6.1). Generally T cell lines with the largest increase in HA specific expansion *in vitro* also demonstrated the largest increases in HA Beijing specific proliferation. Every donor responded more strongly to HA Aichi following vaccination with HA Beijing, indicating the cross reactivity of the $CD4^+$ T cell response amongst H3N2 influenza A viruses.

Vaccination clearly boosted the proliferative responses of every standard donor to the HA peptide pools (Fig. 6.1). A strong response was observed to at least one of the pools representing the highly conserved HA2 subunit (which comprises residues 329-550). Furthermore, as discussed in detail below, the majority of responses to the HA1 subunit localised to conserved regions (data in Table 6.2), with only two major exceptions: donor O responded to antibody site A, and donor U to residues 146-61 which included antibody site B. (Detailed description of responses given below).

HA recognition by 2 donors with evidence of recent exposure to influenza A

The 2 subjects with sizeable HA responses pre-vaccination (donor I had influenza like symptoms, and V who on the basis of our data had probably been recently infected) showed no marked increase in cell yield during culture of their $CD4^+$ T cell lines after vaccination. The magnitude of their anti-HA Aichi and -HA Beijing specific proliferative responses did increase, but this was associated with a much smaller overall rise in HA specific peptide response. This may in part reflect a shift in the dose response curve to some of the individual peptides following vaccination. A stronger response was mounted by donor V to 5μg/ml of peptide 232 (50,000 cpm) than to 10μg/ml of the same peptide within peptide pool 232-

247 (20,000 cpm). Interestingly there were other examples of stronger responses being mounted to single peptides than peptide pools, this may reflect partial antagonism amongst overlapping peptides within a peptide pool (see Tables 5.2 and 6.2). Both donors responded strongly to the HA2 subunit and the great majority of their responses to HA1 were again directed to conserved regions. Once again when the response within the pools was examined most responses were directed to conserved regions of HA.

Detailed localisation of HA responses

Donor N responded strongly to peptide pools 183-202, 208-228, 232-247, 295-313, 384-403. Within these pools the response localised to residues 192-207, 228-247, 303-323, 384-399. Residues 192-207 are located on the edge of antibody site B though only includes 3 conservative drift mutation (residue 193 drifted $193^{S\ to\ N}$, and then recently reverted to the A/Aichi sequence, residue $197^{Q\ to\ R}$, and $207^{R\ to\ K}$). Residues 228-247 which includes 2 conservative ($242^{V\ to\ I}$, and $244^{V\ to\ L}$) and one non-conservative ($248^{N\ to\ T}$) substitutions, though the non-conservative drift mutation is on the extreme C-terminus of this region and therefore may not be significant. Residues 303-323 include a single highly conservative substitution ($307^{R\ to\ K}$). Residues 384-399 include a conservative substitution at their N-terminal (residue $384^{V\ to\ L}$) but are otherwise highly conserved. Interestingly this donor mounted a moderate $CD4^+$ T cell proliferative response which appeared to be HLA-DP restricted (see below) to residues 173-188 which includes 4 conservative substitutions, the last of which is on the edge of antibody site B ($172^{D\ to\ G}$, $173^{N\ to\ K}$, $182^{I\ to\ V}$, $188^{N\ to\ D}$).

Donor O responded strongly to peptide pools 97-113, 137-156, 183-202, 295-313, 384-403, 407-427, and 456-473. Within these pools the responses localised to residues 97-115, 137-157, 192-207, 303-323, 403-422, and 463-478. Residues 97-115 are conserved amongst human H3N2 strains. Residues 137-157 spans

antibody site A and includes 4 conservative (143 $^{S\ to\ P}$, 145 $^{S\ to\ N}$, 146 $^{G\ to\ S}$, 155 $^{T\ to\ H}$) and 3 non-conservative substitutions (137 $^{N\ to\ Y}$, 144 $^{G\ to\ V}$, 156 $^{E\ to\ K}$). Residues 192-207 and 303-323 are discussed above. Residues 403-422, and 463-478 are conserved amongst human H3N2 influenza A strains.

Donor R recognised peptide pools 97-113, 183-202, 208-228, 232-247, 295-313, 384-403, 407-427, 456-473. Within these pools the responses were localised to residues 97-115, 192-207, 228-247, 303-323, 388-403, 407-422, 463-483. Residues 97-115 are conserved amongst human H3N2 influenza A strains. Residues 192-207, 228-247, 303-323, and 407-422 are discussed above. Residues 388-403 are conserved amongst human H3N2 influenza A strains. Residues 463-483 include one non-conservative substitution (479 $^{E\ to\ G}$). Donor S responded to peptide pools 72-92, 232-247, 295-328, 456-473, 503-521. Within these pools responses localised to residues 87-107, 232-247, 303-323, 463-483, 513-528. Residues 87-107 include one conservative substitution (94 $^{F\ to\ Y}$). Residues 232-247, 303-323, 463-483 are discussed above. Residue 513-528 are conserved amongst human H3N2 influenza A strains.

Donor T responded strongly to only one peptide pool 432-452. The response within the pool was localised to residues 442-457 which are generally highly conserved amongst H3N2 human influenza A viruses with the exception of a single highly conservative substitution (453 $^{R\ to\ K}$).

Donor U responded moderately to a number of peptide pools, the strongest recognition was of peptide pools 97-113, 183-202, 295-313, 384-403, 407-427, and 432-452. Within these pools responses were localised to regions 198-213, 303-328, 403-422, , 442-457 which are all discussed above and residues 422-437 which are conserved amongst human H3N2 influenza A viruses. This donor responded less strongly to residues 146-61 which encompasses antibody site B and includes

one conservative substitution between Aichi/68 and Beijing/32/92 (146 $^{G\ to\ S}$), and 5 non-conservative substitutions (155 $^{T\ to\ H}$, 156 $^{E\ to\ K}$, 158 $^{G\ to\ E}$ 159 $^{S\ to\ Y}$ 160 $^{T\ to\ K}$).

Donor I responded strongly to 6 peptide pools. Within the pools the responses localised to residues 97-115, 303-323, 313-328, 378-393, 442-462, 463-478 which are conserved (see above for details). In addition this donor mounted a moderate response to residues 113-128 which has undergone one conservative (124 $^{G\ to\ D}$) and two non-conservative substitutions (122 and 126 $^{T\ to\ N}$).

Donor V recognised peptide pools 72, 97, 295, 357, 432, 456. within these pools responses were localised to residues 87-107, 232-247, 303-323, 412-427, 463-483, 513-528. Regions 87-107, 232-247, 303-323, 463-483, are discussed above. Residues 412-427 and 513-528 are highly conserved amongst H3N2 influenza A viruses.

MHC class II restriction

The results of experiments using anti-MHC class II blocking antibodies are shown in Table 6.2. Where inhibition of responses could be demonstrated this was usually with anti-HLA-DR antibodies. A number of responses appeared to be restricted by more than one class II molecule: In particular the recognition of peptides 303 and 308 by donor N appeared to contain both an HLA-DP and -DR restricted component, and the response to peptide 384 by donor R appeared to contain an HLA-DQ and -DR restricted component.

6.5 Discussion

Comparison to natural infection

Vaccination clearly boosts $CD4^+$ T cell responses to HA Beijing in a very similar manner to natural infection: The selection of conserved epitopes dominates, with every donor recognising at least one peptide pool representing the conserved HA2 subunit. At present the sequence of the HA2 subunit of circulating influenza A strains is examined less frequently than the much more variable HA1 subunit. Some residue substitutions do occur in HA2 (Appendix 1) and it would be interesting to know whether these changes have any relationship to influenza severity.

Responses to the HA1 subunit were directed to regions of HA which are highly conserved amongst H3 influenza A viruses. Following natural infection two regions of HA were frequently recognised in an outbred adult population, despite differences in their class II MHC haplotypes (Chap. 5). In this study all 8 donors responded to the first region (peptide pool 295-328), and 6 to the second (peptide pool 407-442), though the magnitude of individual donors' responses varied considerably (Fig. 6.1 and 6.2).

As the subunit vaccine contained HA derived from an H1 influenza A virus, it was not possible to directly examine cross reactivity of anti-H3 response with H1 and H2 influenza A viruses. However because donors O, R, S, I, V responded to residues 463-473 and donors T and U responded to residues 442-462, and both regions are highly conserved amongst human influenza A viruses, such cross-reactivity is likely.

The demonstration that the subunit vaccine can induce $CD4^+$ T cell responses to regions of HA conserved within a subtype means that there would appear to be less need to further supplement current subunit vaccines with conserved internal viral proteins, such as nucleoprotein, in order to boost influenza A virus cross

reactive $CD4^+$ T cell recognition. (See below for discussion of impications for influenza vaccine administration policy.)

Influence of MHC class II on HA epitope selection

MHC class II haplotype associated recognition patterns of HA were striking: Donors S and V who expressed identical DRB1* and DQ1* alleles responded to the same peptide pools, despite marked differences in their pre-vaccination responses. Furthermore donors O, R ,and U who share the DRB1*0101, DQB1*05 MHC class II haplotype all responded to peptide pools 97-128, 183-217, 295-328, and 407-442, and their responses localised to identical peptides or pairs of peptides within the pools. The study of natural infection included several donors with this haplotype allowing a direct comparison (Chap.5 Fig. 5: Donors A, B, G,). The two groups recognised identical peptides pools, furthermore the responses within these pools localised to identical individual peptides or pairs of peptides (Table 5.2 and Table 6.2), and overall the level of proliferative response by the two groups was similar.

The observation in Chap. 5, that individual allelic forms of certain DRB1* types are associated with different peptide recognition patterns, has been confirmed: Thus the HA epitope recognition pattern of donor N (DRB1*0103) differed from that of donors R, O, and U (DRB1*0101); and the responses of donors T (HLA DRB1*1302) and U (HLA DRB1*1301) were very different. This clearly illustrates the importance of detailed class II MHC typing. See Chap 10 for comparison of observed patterns of HA epitope selection to published HLA-DR binding motifs.

Two interesting donors

The response of donor T, who had been repeatedly vaccinated as an adolescent, was of interest: Prior to vaccination he mounted a relatively weak response to HA and a trivial response to the peptide pools. Following vaccination his response to

HA was boosted but was associated with the recognition of only peptide pool 432-467 (strongly) and peptide pool 295-328 (weakly). Following infection I observed an HA response restricted only to these two pools in a HLA DRB1*1302 homozygous donor (donor J, Fig 5.1). Interestingly this allele is associated with relative protection from both malaria (Hill *et al.,* 1991) and hepatitis B (Thursz *et al.,* 1995). It is not apparent why this donor does not have a response associated with his rare DRB1*0301/4 DQB1*06 haplotype (Dr K.I.Welsh, personal communication). Possible explanations include primary vaccine failure, or the presence of a non-functional DRB1* allele. A third possibility that repeated vaccination has interfered with his HA response seems unlikely given the strong responses mounted by donor V who also had been repeatedly vaccinated.

The response of donor R was also of interest. Prior to vaccination he responded to 4 peptide pools (97-128, 295-328, 407-442, 456-488). The first 3 responses are associated with the DRB1*0101 DQB1*05 MHC class II haplotype (see Chap. 5: Fig. 5.1 donors A, B, G,)). Following vaccination new responses to 3 pools (183-217, 208-243, 232-262) were seen, two of which are DRB1*0701 associated (208, 232; see Chap. 7), demonstrating a shift in the balance of MHC class II haplotype associated epitope recognition pattern following vaccination. A marked change in HA recognition also occurred in donor N which may be a more extreme example of the same phenomenon, though unfortunately as no other donor expressing DRB1*0103 or DRB1*08 was examined this is difficult to prove. A number of donor N's responses declined particularly to peptide pools 1, 25, 407, 456, and a strong response occurred to peptide pools 208 and 232 which localised on the region 228-247. Residues 228-247 are very close to a side-wall of HA's sialic acid binding site, formed by residues 224-228 (Weiss *et al.,* 1988). Within this wall is a non-conservative drift mutation $226^{L\ to\ Q}$ which is very unusual in mammalian H3N2 influenza A viruses. Interestingly this substitution is very common in influenza strains passaged through eggs and thought to be an adaptive change.

Both the vaccine and the HA Beijing used to drive the lines contain 226^{Q}. There is no evidence that donor N had influenza in the preceding 4 yr and he has never been vaccinated against influenza. He is therefore unlikely to have been exposed to an influenza HA with this substitution. However the marked difference in peptide recognition pattern before and after vaccination might be explained by the presence of 226^{Q} altering antigen processing allowing the recognition of the region 228-247 by $CD4^{+}$ T cells. The decline in response to several pools by this donor is more difficult to explain.

Future studies

Influenza vaccination is currently offered to high risk groups on an annual basis. There is little evidence that annual influenza vaccines affords greater protection to normal individuals than intermittent vaccination, furthermore the relative increase in HI titres acheived by vaccination declines with annual administration (reviewed by Nicholson 1993). Given that the adult $CD4^{+}$ T cell response is directed towards consered HA epitopes, and that influenza vaccines are poor inducers of CD8+ T cell responses (McMichael, 1994), there is need for a detailed study of the effects of regular annual vaccination: It will be important to find out the effect of repeated vaccination on the $CD4^{+}$ T cell repertoire and level of HA response, as well as establishing the optimal interval between vaccination (see Chap.10). This will depend on the duration of $CD4^{+}$ T cell memory to influenza type A HA following subunit vaccination.

6.6 Conclusions

$CD4^{+}$ T cell HA epitope selection following subunit vaccination is dominated by the recognition of conserved HA epitopes. Every donor recognised at least one region of the HA2 subunit. The pattern of HA epitope selection following

vaccination is very similar to that observed following natural infection, and the immunogenetics of both responses appear to be identical.

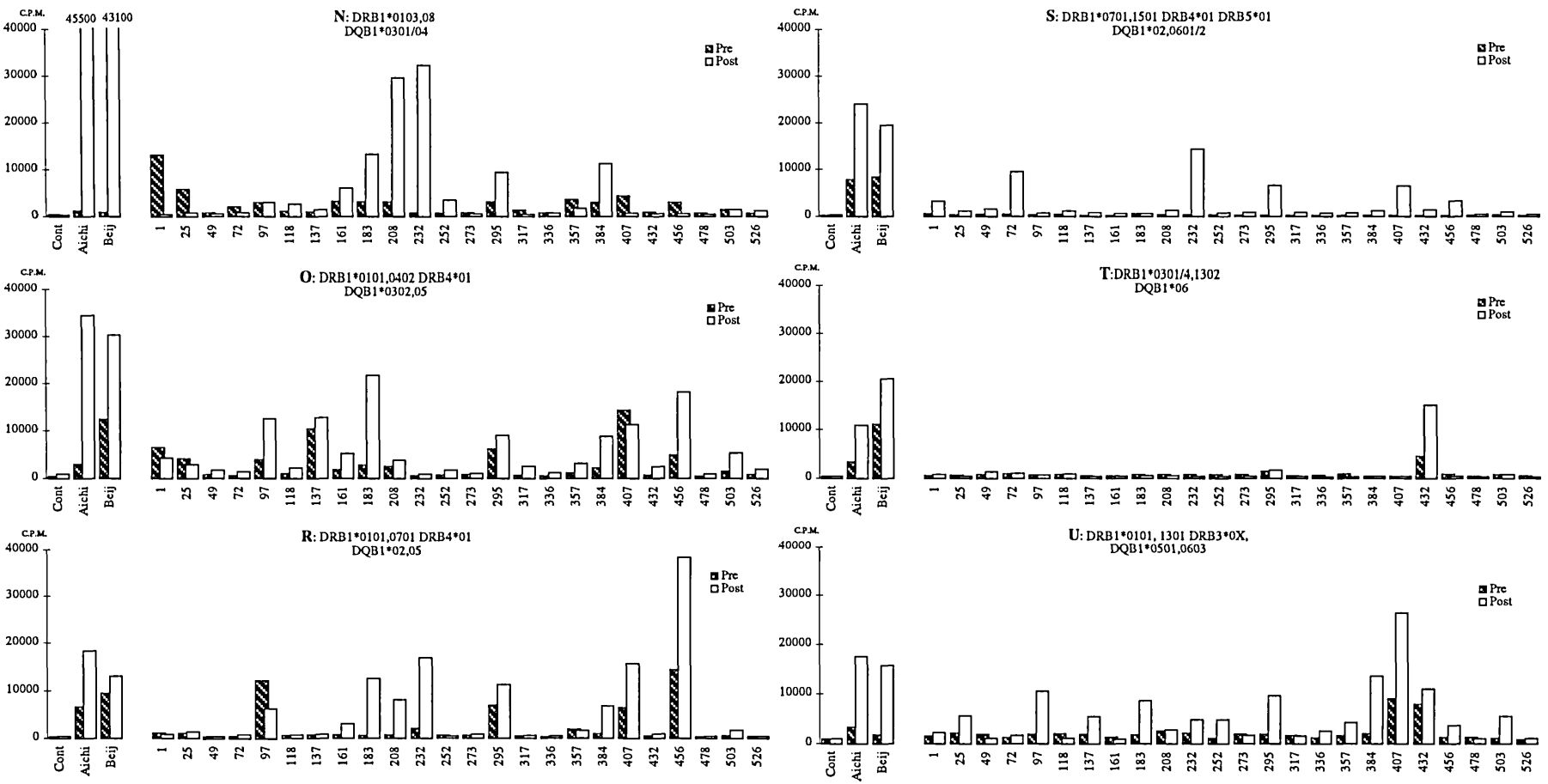
Sub-unit vaccination induces $CD4^+$ T cell responses which are cross reactive with the original H3N2 influenza strain HA A/Aichi/68, and may be cross reactive with H1 and H2 influenza A strains. There is therefore less need to supplement current vaccines with conserved internal viral proteins in order to obtain cross-reactive $CD4^+$ T cells.

Table 6.1, Donor details and expansion of CD4+ T cell lines *in vitro*

Donor	Age	Sex	MHC CLASS I	MHC CLASS II	Expansion of line pre-vaccination	Expansion of line post-vaccination
I	40	F	A2 B15,65 C3,10	DRB1*100X,1302 DRB3*01/02/03 DQB1*05,0604-8	13.4	17.4
N	31	M	A2,66 B27,51 C1,7	DRB0103,080X DQB1*0301,04	0.7	17.5
O	42	M	A2,31 B51,60 C4,10	DRB1*0101,0402 DRB4*01 DQB1*0302,05	0.6	5.5
R	28	M	A2,29 B44,35 C4,1601	DRB1*0101,0701 DRB4*01 DQB1*02,05	1.7	10.7
S	31	F	A3,23 B7,44 C4,0702	DRB1*1501,0701 DRB4*01 DRB5*01 DQB1*02,0601/2	2.6	4.4
T	34	M	A26,31 B38 C0701,1203	DRB1*0301/4,1302 DQB1*06	1.2	7.2
U	33	M	A2,3 B65,51 C5,0802	DRB1*0101,1301 DRB3*01/02/03 DQB1*0501,0603	1.7	10.7
V	55	M	A2,30 B44,51	DRB1*1501,0701 DRB4*01 DRB5*01 DQB1*02,0601/2	8.1	7.4

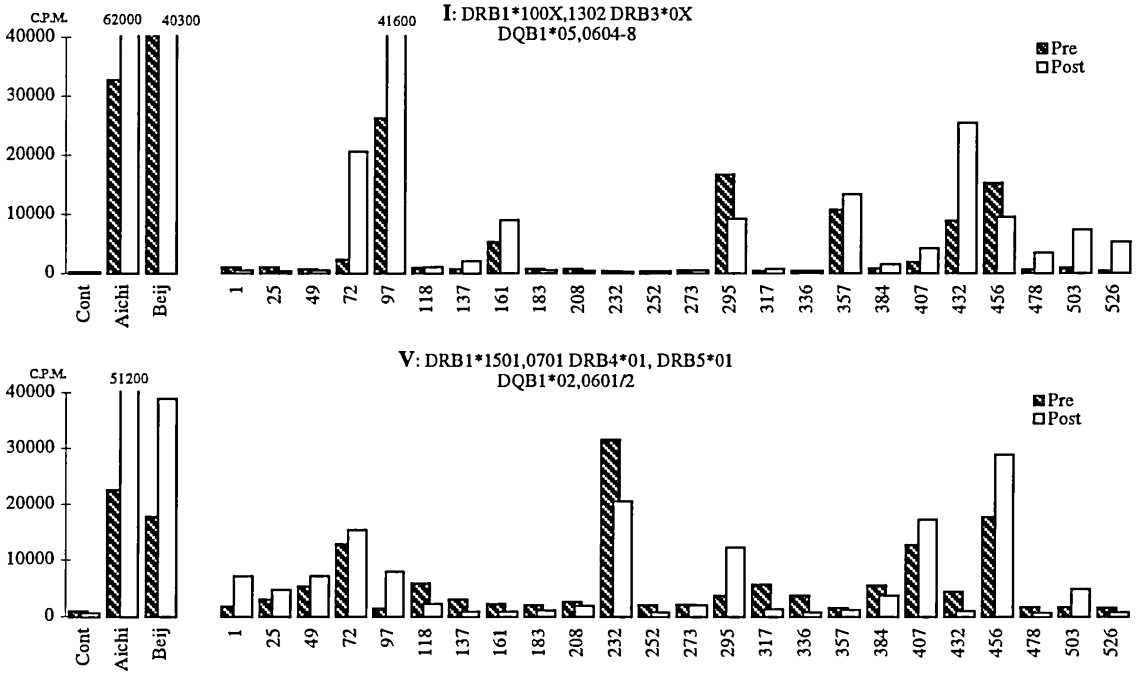
All donors, except I and V, had no evidence of influenza A infection during preceeding 5yr. Donor I has a history of recent influenza. Donor V is likely to have been recently infected (see results).
Donors O, T, and V received influenza vaccines more than 5yr previously (see subject details).
Expansion of line is a ratio of cells recovered at 21d in culture, to number PBMC initially seeded = (Number cells recovered 7d/ number PBMC originally seeded) x (number recovered 14d/ number seeded 7d) x (number recovered 21d/ number seeded 14d).

Figure 6.1, Responses of HA selected CD4+ T cell lines derived from six donors with no evidence of recent exposure to H3 influenza A



CD4+ T cell lines were derived before (dark shaded column), and 3 mo following vaccination with Influvac (white column). Lines were selected with full length HA A/Beijing/32/92 (HA Beijing). T cell proliferation to HA A/Aichi/68 (1.0 μg/ml), HA Beijing (0.1 μg/ml), and peptide pools (5 peptides per pool, 10 μg/ml each peptide), were tested at 14d, or 21d of culture using irradiated autologous prepulsed PBMC as APC. At 48h T cell lines were pulsed with [3]TdR, and harvested 16h later. X-axis represents g.m. of triplicate wells: Control "Cont"= proliferative response of T cell lines to complete medium and autologous APC, "Aichi" = response to HA A/Aichi/68, "Beij" = response to HA Beijing (0.1 μg/ml). Peptide pools labelled by n-terminal residue of n-terminal peptide in each pool, 10/μg/ml each peptide). Y-axis represents c.p.m..

Figure 6.2, HA response by HA selected CD4+ T cells derived from donors with evidence of recent exposure to influenza A



Shows results obtained from CD4+ T cell lines derived from donors I and V before (dark shaded columns) and 3-4 mo following vaccination with Influvac (white columns). Details as in Fig. 6.1.

Table 6.2, Summary of CD4+ T cell response to individual HA peptides within dominant peptide pools, and HLA restriction of response following vaccination

Donor	DRB1*	DRB3* DRB4* DRB5*	DQB1*	Dominant peptide Pools	CPM	Dominant peptides	CPM	Sequence	Restriction	Comments on HA region
I	100X	3*0X	05	97	25000	97/100	20000 /27000	CYPYDVPDYASLRSLVASS	DR	Conserved amongst H3N2
	1302		0604-8	97	25000	113	2500	ASSGTLEFINEDFNWT		122 T to D 1972 (non-consevative0, 124 G to D (conservative), 126 T to N (non-conservative)
				295	17000	303/308	6000 /16000	GACPRYVKQNTLKLATGMRNV	DR	K to R 307 (conservative)
				295	17000	313	7500	TLKLATGMRNVPEKQT	DR	Conserved amongst H3N2
				357	10000	378	9000	NGKLNRLIEKTNEKFG		384 V to L (conservative)
				432	10000	442/447	16000 /15500	SEMNKLFEKTRKQLRENAEDM	DR	453 R to K (conservative)
				456	15000	463	6000	GNGCFKIYHKCDNACI	?DR	Conserved amongst H3N2
N	0103		0301	161	5000	173	4700	KFDKLYIWGVHHPSTD	DP?	172 D to G ((conservative), 173 N to K (conservative) 182 I to V (conservative) from 1972. 188 N to D (conservative) from 1975
	080X		0401	183	11000	192	5700	TSLYVRASGRVTVSTK	DR	193 N to S 1975 (conservative) = reversion to original H3N2 sequence.197 Q to R (conservative) 1979. 207 K to R (conservative) 1971. 213 I to V (conservative) since 1979 with exceptions. 214 I to T (non-conservative) new.
				208	30000	228	21000	SRISIYWTIVKPGDIL	DR	242V to I (conservative) 1972
				232	31000	232	47500	IYWTIVKPGDILLINI	DR	242 V to I (conservative) 1972. 244 V to L (conservative) 1977. 248 N to T (non-conservative) 1986
				295	9000	303/8	4000 /4000	GACPRYVKQNTLKLATGMRNV	DP + DR	K to R (conservative) 1986
				384	10000	403	9000	EGRIQDLEKYVEDTKI	DR	384 V to L (conservative)
O	0101	4*01	0302	1	4000	20/25	6300	VPNGTLVKTITNDQIEVTNAT		31 D to N (conservative) 1971.
	0402		05	97	13000	97/100	13500	CYPYDVPDYASLRSLVASS	DR	Conserved amongst H3N2
				137	13000	137/42	18000	YACKRGSVNSFFSRLNWLHKS	DR	137 N to Y (non-conservative) 1977. 143 P to S (conservative) 1977. 144 G to V (non-conservative) 145 S to N (conservative) 1977 also 145 some Beij like strains S to K (non-conservative)
				183	20000					
				295	9000	303/8	10500 /9300	GACPRYVKQNTLKLATGMRNV	DR	307 K to R (conservative) since 1980
				384	9000					
				407	10000	407	11000	QDLEKYVEDTKIDLWS	DQ	Conserved amongst H3N2
				456	17000	463/8	23700 /24500	GNGCFKIYHKCDNACIGSIRN	DR	479 E to G (non-conservative) 1975

R	0101	4*01	02	97	5000	97/100	34000 /45000	CYPYDVPDYASLRSLVASS	DR	Conserved amongst H3N2
	0701		05	183	10000	192	12000	TSLYVRASGRVTVSTK	DR	193 reversion to S (conservative) had been N 1979-91. Q to R (conservative) 1979. 207 R to K (conservative) 1972
				208	7000	208	13000	RSQQTVTPNIGSRPWV		213 I to V (conservative) 1980. 214 I to T (non-conservative) new. 217V to I (conservative) 1972-1979
				208/232	6000/ 12,000	228/32	18000 /18500	SRISIYWTIVKPGDILLINSTG	DR (+DP??)	242 V to I (conservative) 1972. 244 V to L (conservative) 1977. 248 N to T (non-conservative) 1986
				295	10000	303/8	19000 /25000	GACPRYVKQNTLKLATGMRNV	DR	K to R (conservative) 1986
				384	5000	388	6500	TNEKFHQIEKSEVEGR		Conserved amongst H3N2
				384/407	5,000 / 11,000	403	6300	EGRIQDLEKYVEDTKI	?DR AND ?DQ	Conserved amongst H3N2
				456	38000	463/8	15000 /17000	GNGCFKIYHKCDNACIGSIRN	DR	479 E to G (non-conservative) 1975
S	1501	4*01	02	72	10000	87/92	12000 /6000	FVERSKAYSNCYPYDVPDYAS		94 F to Y (conservative)
	0701	5*01	0601/2	232	14000	232	14000	IYWTIVKPGDILLINS		242 V to I (conservative) 1972. 244 V to L (conservative) 1977. 248 N to T (non-conservative) 1986
				295	7000	303/8	6000 /15000	GACPRYVKQNTLKLATGMRNV		R to K 307 (conservative)
				456	3000	463/8	4000/4000	GNGCFKIYHKCDNACIGSIRN		479 E to G (non-conservative) since 1975
				503	5000	513	2000	DWILWIWISFAISCFL		Conserved amongst H3N2 strains
T	0301/4		06	432	15000	442	16500	SEMNKLFEKTRKQLRE		453 R to K (conservative)
	1302									
U	0101	3*01	0501	97	1800	97/100	2000/2100	CYPYDVPDYASLRSLVASS		Conserved amongst H3N2
	1301		0603	183	8000	198	12000	ASGRVTVSTKRSQQTV		207 R to K (conservative) since 1971. 213 I to V (conservative) since 1979 with exceptions. 214 I to T (non-conservative) new.
				295	6500	303/308/313	2500/2570 /2750	GACPRYVKQNTLKLATGMRNV		R to K 307 (conservative)
				384/407	22000	403/407	12000 /18000	EGRIQDLEKYVEDTKI		Conserved amongst H3N2 strains
				432	10000	442/7	6500	SEMNKLFEKTRKQLRENAEDM		453 R to K (conservative) 1986. 461 E to D (conservative) 1971
				503	2500	513	2200	KSGYKDWILWISFAI		Conserved amongst H3N2 strains
V	1501	4*01	02	72	10000	87/92	12500 /4000	FVERSKAYSNCYPYDVPDYAS		94 F to Y (conservative),otherwise conserved amongst H3N2 strains
	0701	5*01	0601/2	232	20000	232	50000	IYWTIVKPGDILLINSTG		242 V to I 1972. 244 V to L 1977. 248 N to T 1986
				295	12000	303/8	15500 /19500	GACPRYVKQNTLKLATGMRNV		K to R 307 (conservative)
				407	17000	412/417	9000 /29000	QDLEKYVEDTKIDLWS	15	Conserved amongst H3N2 strains
				456	28000	463/8	32000 /42000	GNGCFKIYHKCDNACIGSIRN	7	479 E to G (non-conservative) since 1975
				503	5000	513	6500	KSGYKDWILWISFAI	7 or DRW	Conserved amongst H3N2 strains

Chapter 7, $CD4^+$ T Cell Lines Derived From 6 Unrelated Adults Sharing HLA-DR and -DQ Alleles Recognise Identical Regions of Influenza A Haemagglutinin Following Subunit Vaccination

7.1 Introduction

Investigation of human $CD4^+$ T cell recognition of influenza A HA following natural infection (Chap. 5) and subunit vaccination (Chap. 6), revealed that both the majority of adult $CD4^+$ HA responses, and the dominant ones, were directed towards "conserved regions" of HA, which have not been subject to frequent drift mutation amongst human H3N2 influenza A strains. By chance both studies included pairs of donors sharing class II MHC haplotypes. Each pair responded to identical HA peptides, and though variations were observed in strength of responses, the overall similarity of their HA repertoires was remarkable, especially considering their differences in age and exposure history to influenza, a virus noted for continual antigenic variation (Webster *et al.,* 1992, Bean *et al.,* 1992). Furthermore identifiable patterns of HA peptide recognition were also observed amongst donors who shared single MHC class II alleles.

Recently a remarkable conservation has been demonstrated in the human HLA-A2 restricted CTL response to influenza matrix protein (MP) (Moss *et al.,* 1991, Lehner *et al.,* 1995), its structure shows considerably less variation than HA (reviewed by Lamb 1989), and the requirements for peptide binding to MHC class I are thought to be much more exacting than for MHC Class II (Rammensee, 1995, Hammerling *et al.,* 1995). Thus a less restricted $CD4^+$ T cell repertoire of HA epitope selection might be expected, particularly in an outbred population who are likely to differ in previous exposure to influenza.

To extend the initial observations the $CD4^+$ T cell HA repertoires of 6 unrelated individuals who expressed identical HLA-DR, and -DQ alleles, were examined. Three of these individuals were originally found by chance (subjects A, B, F), and

remaining donors were kindly identified by Dr K. Welsh. Short term $CD4^+$ T cell lines were selected with HA Beijing/32/92(H3N2), epitope recognition has been examined prior to and 3 mo following vaccination with a trivalent subunit influenza vaccine containing HA A/Beijing/32/92.

7.2 Aims

1) To extend previous observations on the influence of MHC class II over HA peptide recognition, by examining before and after influenza subunit vaccination, the HA responses of 6 unrelated adults, who express identical MHC class II alleles, but differ in exposure to influenza A.

7.3 Methods

Subjects and vaccination

The responses of 6 unrelated healthy adult volunteers were examined prior to and 3-4 mo following vaccination (donor details in Table 7.1). The responses of subjects A + B to the HA peptide pools following vaccination was described in Chap. 6. Donors C-E were specifically examined because they were known to have a very closely matched MHC class II type to donor A. HLA class I and II types of the donors are shown in Table I. Five subjects express identical DRB1*, DRB4*01, DRB5*01, DQA1*, and DQB1* alleles. Donor F differs in DQB1* type, and has a non-expressed DRB4*01 allele.

Subjects received 0.5ml of Influvac, containing 15μg each of HA Beijing/32/92 (H3N2), HA A/Singapore/6/86 (H1N1), HA B/Panama/45/90 (influenza B) as previously described (Chap.6).

MHC Class II restriction studies

MHC restriction studies were performed at 21d of culture, by using irradiated (3000 rads) PBMC from 2 partially MHC class II matched donors to present HA Beijing/32/92 and HA peptides to developing T cell lines. One of these donors had a DRB1*0101,0701 DRB4*01 DQB1*02,05 haplotype (DR 07 donor), and the other a DRB1*0408,1501 DRB4*01 DRB5*01 DQB1*0301,0601/2 haplotype (DR 15 donor)

Short term CD4+ T cell lines were selected with HA A/Beijing/32/92 as before, and all other experimental methods are described in Chap. 2.

7.4 Results

HA and HA peptide recognition

Prior to vaccination HA and HA peptide recognition was variable in magnitude (Fig. 7.1): Donor A made a weak proliferative response to HA and no significant response to the HA peptide pools. In contrast donors C, D, and F recognised HA more strongly, and mounted proliferative responses to peptide pools 232, 407, and 456.

Despite differences in $CD4^+$ T cell recognition of HA and HA peptides pre-vaccination, following subunit vaccination $CD4^+$ T cell lines derived from every donor mounted strong proliferative responses to HA A/Beijing/32/92, HA A/Aichi/68, and several HA peptide pools. Though the degree of boosting of individuals responses were variable (see discussion), their post vaccination HA peptide recognition repertoires were remarkably similar to each other: Every $CD4^+$ T cell line recognised peptide pools 72, 232, 407, and 456. There was also a similarity in their weaker HA responses with 5/6 donors recognising peptide pools 1, 208, 295, and 503.

When the $CD4^+$ T cell recognition of individual peptides within the dominant pools was examined (Fig. 7.2), the donors responses were found to localise to identical peptides or pair of overlapping peptides, with the exception of donor A's response to pool 456, which was not clearly localised. It is interesting that this donor, who had the weakest pre-vaccination HA response, demonstrated the most variability in HA recognition following vaccination.

HLA Restriction of the $CD4^+$ T cell HA Response

In order to examine the balance of HA peptide recognition between the DRB1*0701 and DRB1*1501 containing haplotypes of these donors, HA recognition experiments were repeated using PBMCs from two donors with a single MHC class II haplotype match as APCs (Fig. 7.3). The majority of HA and HA peptide responses were associated with the DRB1*0701 haplotype, with only the response to 417 being largely restricted by the DRB1*1501 haplotype. Interestingly the response to peptide 232, though clearly most powerful induced on a DRB1*0701 background, also appeared to contain a smaller DRB1* 1501 haplotype restricted component. The alternative explanation, that an element of the response is HLA-DRB4*01 restricted, is unlikely because we have examined HA epitope selection by 5 other donors expressing this allele and no significant recognition of this region was observed (see Chap. 5 and Chap.6). Studies using anti-framework MHC class II antibodies (not illustrated) supported the conclusion that the responses to peptide 232 were predominantly HLA-DR, rather than HLA-DQ restricted.

Peptide 232-247, which represents a region close to a side-wall of the HA sialic acid binding site (formed by residues 224-228)(Weiss *et al.,* 1988), is interesting as $CD4^+$ T cell recognition of this peptide occurred in the context of both HLA-DRB1*0701, and -*1501. There are major differences in structure between these alleles, including differences in their P1 pockets, and it is surprising that they can

both present a single HA peptide to $CD4^+$ T cells, particularly as we have not observed strong recognition of this peptide by $CD4^+$ T cells in the context of many other HLA-DRB1* alleles (DRB1*0101, -030X, -0401, -0402, -0408, -080X, -110X,-120X, -1302)(Chap.5 and Chap.6).).

7.5 Discussion

$CD4^+$ T cell response to influenza vaccination

Influenza vaccination had a variable effect on the $CD4^+$ T cell response of the above donors to HA A/Beijing/32/92, HA A/Aichi/68, and the HA peptides: The largest boosting was seen in the donors with the weakest pre-vaccination HA peptide responses (subjects A, and F), whereas the donors with the strongest HA peptide responses showed less boosting (subjects B and D). Interestingly the response of 4 donors to HA A/Aichi/68 was boosted more by subunit vaccination than their response to HA A/Beijing/32/92 (subjects B, C, D, F). This may relate to the presence of impurities in the bromelain produced HA A/Aichi/68 and the need to use higher concentrations: HA A/Beijing/32/92 was not bromelain cleaved, is highly purified, and contains only a trace of NP, whereas HA A/Aichi/68 is less pure containing NA and NP. The greater boosting of the HA A/Aichi/68 response may reflect a response to NA which has been boosted by influenza vaccination (which contains NA). Alternatively vaccination may have boosted a response to NP which is present in the vaccine and HA A/Beijing/32/92 preparation in trace quantities, and in larger amounts in the HA A/Aichi/68 preparation. The pre-vaccination differences in $CD4^+$ T cell recognition of the bromelain cleaved HA A/Aichi/68, and HA A/Beijing/32/92 reflect the differences in immunogenicity of these preparations originally described in vaccine experiments and trials (Jennings *et al.,* 1974, Tyrrell 1974).

Influence of MHC class II on HA peptide recognition

The immunogenicity of a peptide antigen for $CD4^+$ T cells is dependant on the generation of an appropriate peptide fragment, the fragment's ability to bind MHC class II molecules, and the presence of T cells capable of binding the peptide - MHC complex. Instances of unresponsiveness have been recorded which are due to inappropriate antigen processing (Brett *et al.,* 1988, Bodmer *et al.,* 1989), and because of the deletion of specific T cells (Vidovic and Matzinger, 1989). The 6 donors investigated in this study were unrelated, and are very likely to have differed in exposure history to influenza A, a virus noted for its continual antigenic change, because they differ in age and originate from different parts of the UK. It was therefore remarkable that their HA specific $CD4^+$ T cell repertoires, displayed such marked similarity in HA peptide recognition. The observed pattern of epitope selection is HLA DRB1*0701, 1501 specific, as we have observed different patterns of HA peptide recognition in the context of other HLA-DR alleles expressed by other donors (see Chap.5 and Chap.6).

Although it might be expected that syngeneic mice would recognise identical $CD4^+$ T-cell epitopes following nasal infection, variability in epitope selection of T cell clones between individual mice has been reported; With clear MHC class II associated response patterns emerging only when the results from several mice were combined (Smith *et al.,* 1994). This finding may, however, reflect the limitations in sampling size imposed by the use of $CD4^+$ T-cell clones rather than short term lines.

Once again as described in Chap.5 and Chap.6 the regions of HA which induced the strongest responses from these donors (residues 87-107, 232-247, 303-323, 417-432, and 463-483) have been remarkably free of drift mutation since the emergence of human H3N2 influenza A strains in 1968. Between the original H3N2 influenza virus A/Aichi/68 and the immunising strain A/Beijing/32/92 the following substitutions have occurred: Residues 87-107 one conservative drift

mutation (94 $^{F\ to\ Y}$); residues 232-247 two conservative drift mutations (242 $^{V\ to\ I}$, 244 $^{V\ to\ L}$); residues 303-323 one conservative drift mutation (307 $^{K\ to\ R}$); residues 417-432 are conserved; residues 463-483 contain one non-conservative mutation (479 $^{E\ to\ G}$). In addition residues 303-323, 417-32, and 463-483 all have partial homology with H1N1 and H2N2 influenza A viruses (see appendix 1). The observed similarity in response patterns might therefore be a consequence of repeated exposure to different strains of influenza A boosting $CD4^+$ T cell recognition of a limited number of conserved HA epitopes (see Chap. 10).

This precise definition of epitope recognition by MHC class II, offers an explanation for the recent associations of relative resistance to malaria (Hill *et al.*, 1991) and hepatitis B (Thursz *et al.*, 1995) with certain HLA-DRB1* alleles: In these diseases the recognition of stable immunodominant epitopes might be strongly associated with protective HLA alleles.

Chap. 9 describes an investigation into the binding affinity of the HA peptides for the HLA-DRB1*0701 allele, and in Chap. 10 there is a detailed discussion of the relative importance of peptide binding affinity for MHC class II alleles, and drift mutation, in the establishment of a hierarchy of immunodominance.

Conclusion

HA recognition by 6 unrelated adult donors who express identical HLA-DR and -DQ alleles has been examined following influenza subunit vaccination. Despite clear differences in exposure to influenza A, after vaccination, these donors recognised identical HA peptides. CD4+ T cell HA epitope selection is therefore precisely defined by MHC class II.

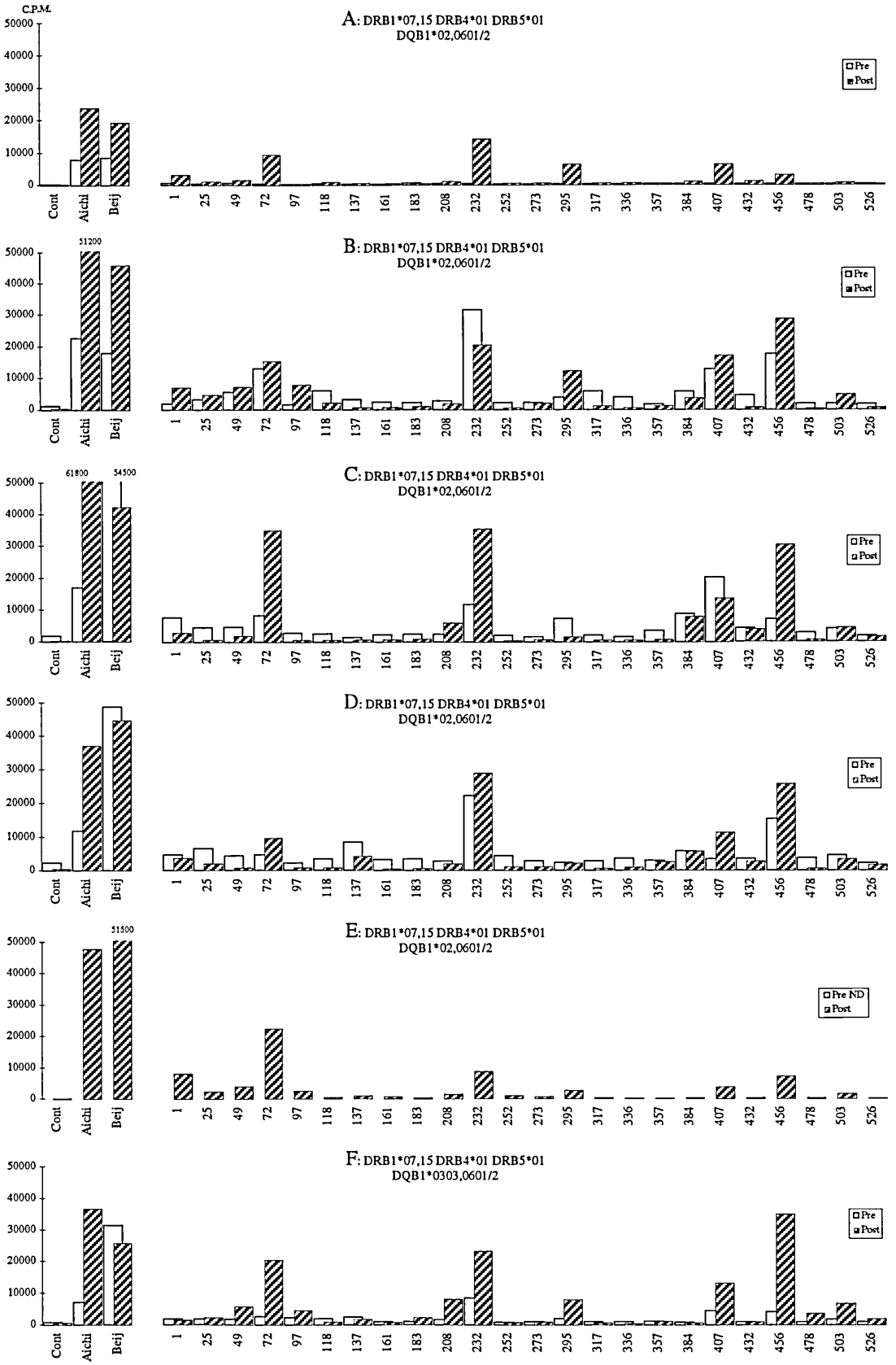
Surprisingly the majority of HA responses were found to be restricted by a single MHC class II allele (DRB1*0701). One peptide (232-247), which was not frequently recognised following natural infection, was found to be recognised in

association with both HLA DRB1*-0701 and -1501, despite considerable differences in structure between these alleles.

Table 7.1, Donor Details

DONOR	AGE	SEX	MHC CLASS I	MHC CLASS II
A	31	F	A3,23 B7,44 C4,702	DRB1*1501,0701 DRB4*01 DRB5*01 DQA1*0101/4,0501 DQB1*02,0601/2
B	55	M	A2,30 B44,51	DRB1*1501,0701 DRB4*01 DRB5*01 DQA1*0101/4,0501 DQB1*02,0601/2
C	34	M	A1,2 B62,64 BW6,3 CW8,304	DRB1*1501,0701 DRB4*01 DRB5*01 DQA1*0101/4,0501 DQB1*02,0601/2
D	38	M	A3B7,13 CW4,6	DRB1*1501,0701 DRB4*01 DRB5*01 DQA1*0101/4,0501 DQB1*02,0601/2
E	28	M	A30,31 B7,13 CW6,7	DRB1*1501,0701 DRB4*01 DRB5*01 DQA1*0101/4,0501 DQB1*02,0601/2
F	34	M	A2,28 B7,17 CW6,7	DRB1*1501,0701 DRB4*01 DRB5*01 DQA1*0102/3,0201 DQB1*0303,0601/2

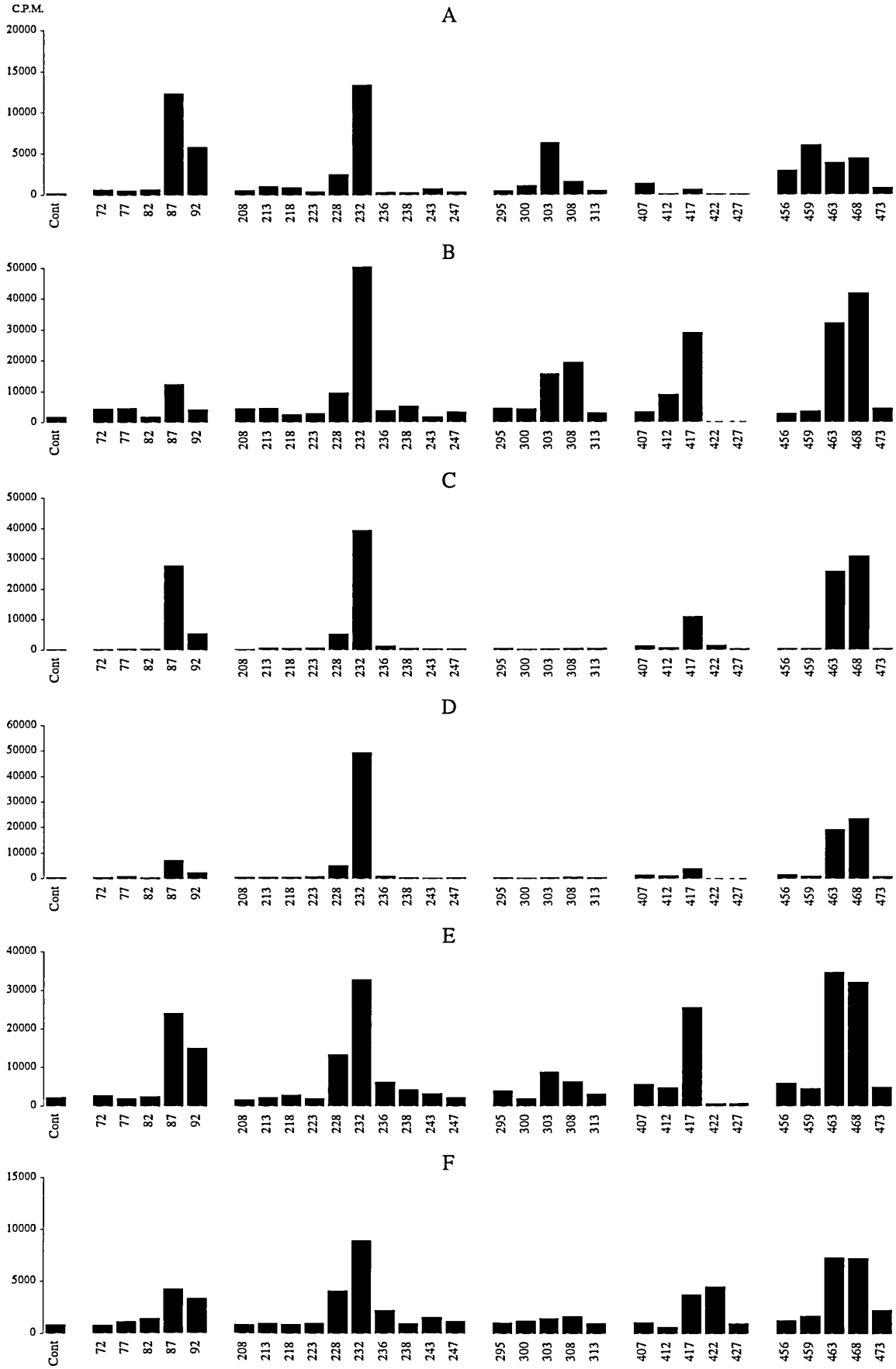
Fig.7.1, CD4+ T Cell Recognition Of HA A/Beijing/32/92, HA A/Aichi/68, And HA Beijing Specific Peptides



CD4+ T cell lines were derived from donors A-F before (white columns) and 3 mo following vaccination with Influvac (dark shaded columns). Lines were selected with full length HA A/Beijing/32/92. T cell proliferation to HA A/Aichi/68 (1.0μg/ml), HA A/Beijing/32/92 (0.1μg/ml) and peptide pools (5 petides per pool, 10μg/ml each peptide), were tested at 14d or 21d of culture using irradiated autologous prepulsed PBMC as APC. At 48h T cell lines were pulsed with [3]TdR, and harvested 16h later.

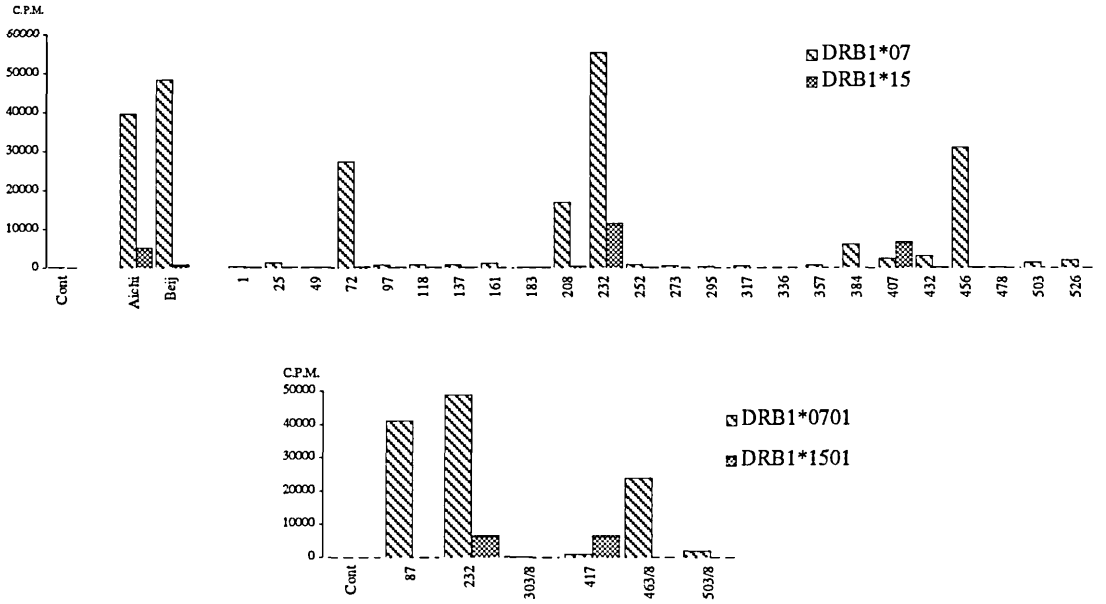
X-axis represents geometric mean of triplicate wells: Control, "Cont"= proliferative response to complete medium and autologous APC, "Aichi"= response to A/Aichi/68 HA 1.0μg/ml, "Beij"= response to HA A/Beijing/32/92 0.1μg/ml. Peptide pools labelled according to number of the first residue of first peptide in pool (5 peptides per pool, 10μg/ml each peptide). Y-axis represents cpm

Fig. 7.2, CD4+ T cell Responses To HA Peptides Within Dominant Peptide Pools



T cell proliferation to individual peptides (5μg/ml) within dominant peptide pools was tested at 14d or 21d of culture using irradiated autologous PBMC as APC. Details as Fig.7.1.

Fig. 7.3, CD4+ T Cell Responses To HA And HA Peptides Using Single MHC class II Haplotype Matched PBMC As APC



Donor C's CD4+ T cell proliferation to HA A/Aichi/68, HA A/Beijing/32/92, and HA Beijing specific peptide pools (upper panel), and dominant single peptides (lower panel) was tested at 21d using PBMCfrom 2 partially MHC class II matched donors as APC. Donor "DR7"= DRB1*0101,0701 DRB4*01, DQB1*02,05, "DR15" donor = DRB1*0408, 1501, DRB4*01, DRB5*01, DQB1*0301,0601/2. Details otherwise as in Fig 7.1.

Chapter 8, CD4+ T Cell Memory Following Influenza A Virus Infection

8.1 Introduction

Natural influenza infection, and subunit vaccination induce adult human $CD4^+$ T cell responses in adults to HA A/Beijing/32/92, which are dominated by the recognition of HA regions which are not subject to frequent drift mutation (Chap.5 and Chap.6). This recognition of conserved HA epitopes offers some protection against future antigenic shift and drift by influenza A viruses, as the production of neutralising antibodies, immunoglobulin class switching, and affinity maturation are all $CD4^+$ T cell dependent (Burns *et al.,* 1975, Anders *et al.,* 1979). In addition $CD4^+$ T cells amplify $CD8^+$ T cell cytotoxic responses (Biddison *et al.,* 1981), mainly directed towards highly conserved internal viral proteins (reviewed by McMichael 1994). $CD4^+$ T cells may also participate in viral clearance more directly by the secretion of interferon γ (reviewed by Askonas, 1988).

It is surprising therefore that there is no information regarding the duration of $CD4^+$ T cell memory following natural infection or vaccination. It is important to obtain this data in order to optimise influenza vaccination protocols. Though annual vaccination is recommended for high risk groups there is little evidence that it affords greater protection than intermittent vaccination, furthermore the relative increase in HI titres achieved by vaccination declines with annual administration (reviewed by Nicholson, 1992). Given that the adult $CD4^+$ T cell response is directed towards conserved HA epitopes, and that influenza vaccines are poor inducers of $CD8^+$ T cell responses (reviewed by McMichael, 1994), it is possible that intermittent influenza vaccination will prove to be as effective as annual vaccination (see Chap. 10).

In order to examine more precisely the decline in $CD4^+$ T cell HA memory after natural infection, 5 donors were re-examined 12-24 mo. following infection.

8.2 Aim

To examine CD4+ T cell memory 12-24 mo. following natural infection, and compare results to those obtained 3-6 mo. after infection.

8.3 Subjects

The responses of 5 subjects (donors D, G, H, K, L) were re-examined between 12-24 mo. following their original influenza infections. Donor details are given in Table 8.1, which also shows the *in vitro* cell yields at the time of the original and follow up experiments. Donors D, G, L were non-responders to HA prior to influenza in Nov./Dec. 1993 (see Chap. 5)

8.4 Results

Cell yield

On repeat culture with HA A/Beijing/32/92 12-24 mo following infection the *in vitro* cell yield, which is an index of cell expansion during 3 wks of culture (see Chap 4.), declined in 4/5 donors (Table 8.1). Donor L, whose cells had previously expanded particularly well in culture, demonstrated the most marked fall in cell yield, declining within 17 mo to 1/7 of the *in vitro* expansion originally observed following infection. Interestingly donor K's cell yield was unchanged.

HA and HA peptide proliferative responses

Response to HA and HA peptides is shown in Fig 8.1. Donor G showed a marked decline in HA and HA peptide response 12 mo following infection (and 6 mo following the first assay) to about 1/3 of her original response. The HA specific proliferation by donor L also declined but by a smaller amount. However the

peptide proliferation assays are carried out using the same number of cells, hence on the basis of the original number of cells found in culture, the memory $CD4^+$ T cell repertoire of this donor appears to have dropped more than five fold. Interestingly the majority of donor L's decrease in $CD4^+$ T cell response occurred in response to the HA1 subunit. The response of donor D showed a decline in HA and HA peptide recognition, except to peptide pools 97 and 295, the two original dominant epitopes. The HA peptide recognition pattern of donor H was more clearly defined on re-examination, due to the loss of weak responses. Given the fall in *in vitro* cell yield by donors D and H, their $CD4^+$ T cell specific memory appears to have fallen by about 50% on re-examination. Most surprisingly the HA peptide specific response of donor K was found to have increased on re-examination, with no decrease in cell yield after culture (see discussion).

8.5 Discussion

The HA specific responses of 5 donors has been re-examined 12-20 mo. following initial infection. Two donors D, L were known to be non-responder to HA prior to their infection. In the majority of donors re-investigated 12-20 mo. following infection the HA specific CD4+ T cell yield *in vitro* has declined. However the cell yield in the majority of donors remains above the level of that observed for the "memory" donors in Chap. 5 (donors who responded to HA but who had no history of influenza for at least 4yr). In contrast the proliferative responses to HA and HA peptides using the same number of cultured cells for the assay remain more stable. The decline *in vitro* cell yield is probably a reflection of a falling number of HA specific precursor $CD4^+$ T cells with time. There was some association between the magnitude of the decline in individual donors cell yield and the decline in their HA peptide proliferative responses. Donor K's CD4+ T cell line showed neither a decline *in vitro* cell recovery or a fall in HA peptide recognition. A possible explanation of this finding is that this donor was re-

infected with an H3 influenza A virus, though this must have been either mild or sub-clinical, as there was no history of a typical influenza like illness.

In 2/5 donors HA2 peptide responses declined, possibly indicating a loss of cross reactive protection against H1 influenza A infection. It will be interesting to see whether these donors remain free of influenza over the next winter.

In order to complete this preliminary investigation donors will have to be re-examined on an annual basis, probably for at least another 3 yrs. An attempt was made to investigate the decline in $CD4^+$ T cell T memory following influenza subunit vaccination. Unfortunately this study was severely affected by the transfer of tissue culture facilities to a new laboratory, and a series of laboratory infections, and the results are difficult to interpret and therefore not illustrated.

8.6 Conclusions

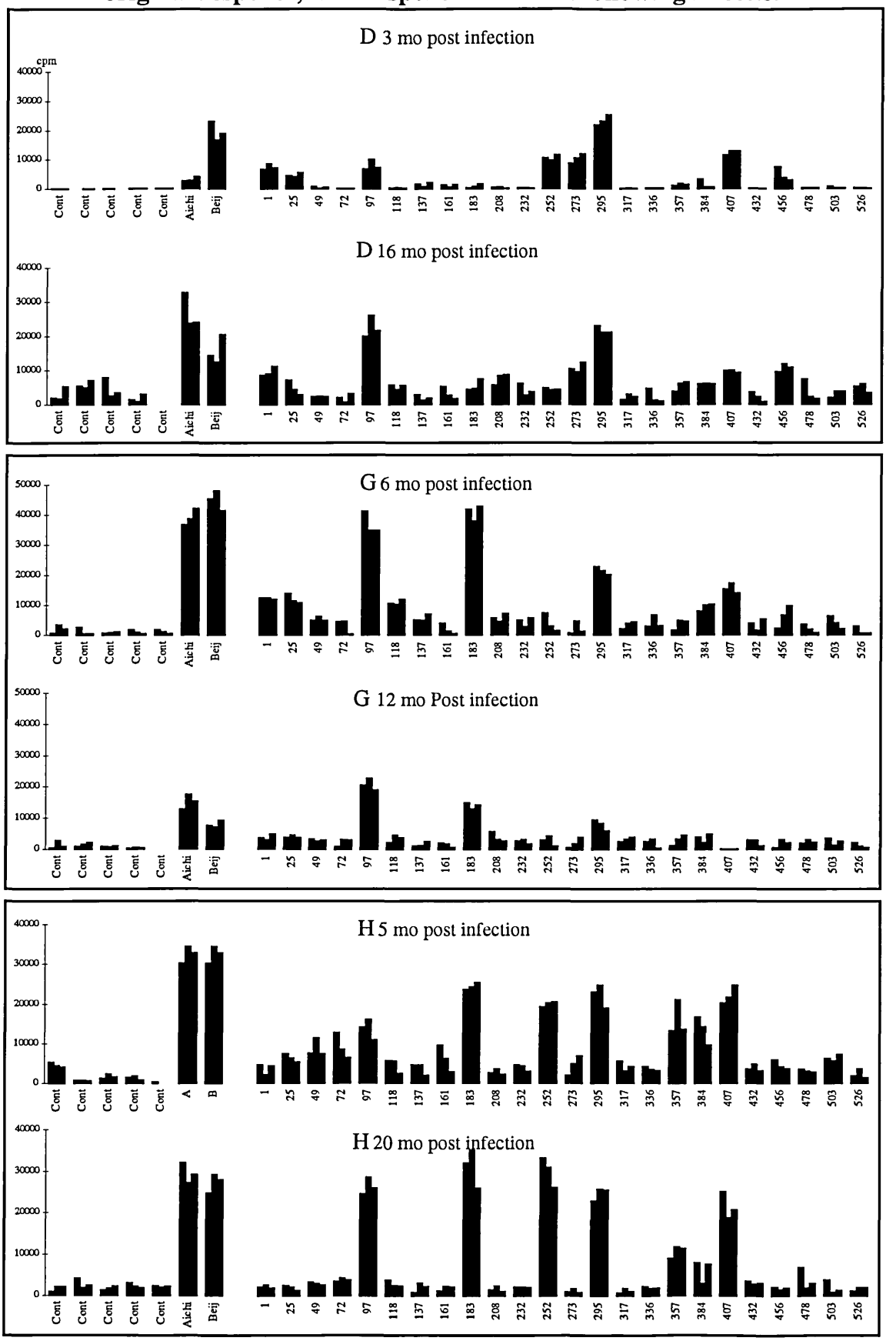
CD4+ T cell recognition of HA has been examined 12-20 mo following natural influenza infection. In the majority of adult donors a decline in cell recovery *in vitro* was observed, which probably reflects a declining HA specific CD4+ T cell precursor frequency. In contrast the HA peptide repertoire after 3 *in* vitro restimulations remained more stable. This apparent discrepancy may reflect the use of a constant number of $CD4^+$ T cells, which have been selected over 3 wks *in vitro*, in the two sets of proliferation assays, indicating that though the number of circulating $CD4^+$ T cells may have declined with time following infection, the cells which remain have retained there ability to respond to HA and HA peptides.

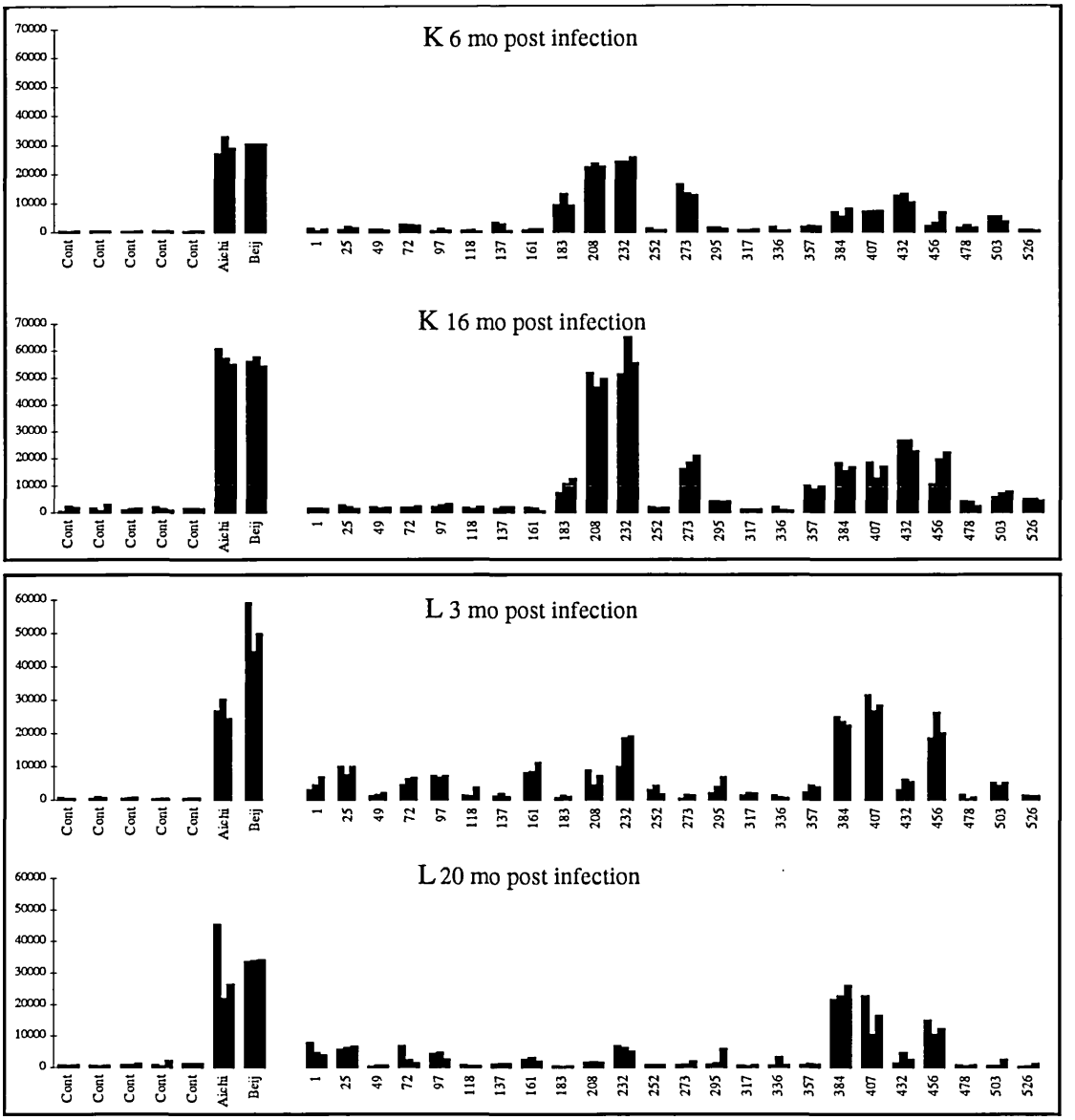
Table 8.1, CD4+ T cell memory following natural infection: Donor details, times intervals, and cell yields

Donor	MHC class II	Mo. after infection first examined	Mo. after infection re-examined	Initial cell yield	Memory cell yield
D	DRB1*0408, -1501 DRB4*01, DRB5*01, DQB1*0301, -0601/2	3	16	4.7	2.86
G	DRB1*0101,-0401 DRB4*01, DQB1*0301,-05	6	12	4.5	2.68
H	DRB1*030X,-040X, DRB3*0X, DRB4*01, DQB1*02	5	20	4.4	2.36
K	DRB1*1301,-1303/4, DRB3*0X, DQB1*0603,-07	6	16	7	7.2
L	DRB1*0102,-0701 DRB4*01 DQB1*02,-05	3	20	10.4	1.56

Cell yield, is an index of the expansion of the $CD4^+$ T cell lines *in vitro* = (Number cells recovered d7/ number PBMC originally seeded) x (number recovered d14/ number seeded d7) x (number recovered d21/ number seeded d14).

Table 8.1, CD4⁺ T cell memory following natural infection: Comparison of original response, with response 12-20 mo. following infection





CD4+ T cell lines were derived from 5 unrelated donors 3-6 mo. and 12-20 mo. following natural infection with A//Beijing/32/92 (H3N2) like strains, and selected for 3 wks in vitro using full length HA beijing/32/92. X-axis is proliferative response to medium and autologous APC="Cont", 1.0µg/ml HA Aichi/68 ="Aichi", 0.1mg/ml HA A/Beijing/32/92 ="Beij", or peptide pools (5 individual peptides at individual concentration of 10µg/ml). Peptide pools labelled according to N-terminal residue of N-terminal peptide. Y-axis represents cpm [3]TdR incorporation. Individual data points of triplicate reactions, rather than means illustrated.

Chapter 9, MHC Class II-HA Peptide Binding Studies

9.1 Introduction

Following influenza subunit vaccination $CD4^+$ T cell lines derived from 6 unrelated adults differing in exposure history to influenza A were shown to recognise identical HA peptides, and the response was found to be identical to that observed after natural infection (Chap 7). The majority of the $CD4^+$ T cell response was associated with DR0701. $CD4^+$ T cell recognition of an antigen is dependant on many factors (see Chap 1 and discussion), one of which is the ability of processed peptides to bind to MHC class II. In order to explore the relationship between HA peptide-MHC class II binding affinity, and the observed HA recognition patterns, the relative binding affinity of purified DR0701 for all of the 118 synthetic HA peptides has been examined.

Following influenza A infection, 2 regions of HA were recognised by $CD4^+$ T cells from 18/18 unrelated donors (residues 295-328, and 407-442, Chap.5). Within the first region responses were focused on residues 303-323. The second HA region contained at least 2 immunogenic sequences, residues 407-412, and 417-437. In addition a further 4 important HA regions (residues 97-115, 192-212, 403-422, 417-432) were identified following infection, and 2 were identified following subunit vaccination (residues 228-247, and 463-483). A few peptides have been reported to have the ability to bind to many MHC class molecules (Sinigaglia *et al.,* 1988, Pania-Bordignon *et al.,* 1989, Sette *et al.,* 1995),but were surprised that so many HA regions could be recognised by $CD4^+$ T cells in the context of more than one MHC class II allele. We were naturally curious therefore to discover whether the MHC class II promiscuity of the frequently recognised HA peptides was positively correlated with their relative binding affinity for a range of common MHC class II molecules, or whether promiscuity might relate to other factors (see discussion). MHC class II-HA peptide binding assays have

therefore been performed using 5 purified HLA-DR molecules (DR-0101, 0301, 0401, 0701, and -1302) and 13 HA peptides (6 pairs of overlapping peptides and one individual peptide) representing the 7 frequently recognised regions of HA A/Beijing/32/92.

These experiments were performed in Dr Adrian Hill's laboratory (Institute of Molecular Medicine, Oxford, UK), in collaboration with Dr Miles Davenport.

9.2 Aims

(1) To examine the relative binding affinity of the 118 HA peptides, which had been used for HA epitope mapping, for purified HLA-DR0701, and to explore the relationship between relative binding affinity and $CD4^+$ T cell antigen recognition *in vitro* following natural infection and subunit vaccination.

(2) To examine the relative binding affinity of 13 peptides representing 7 frequently recognised regions of HA for 5 commonly expressed HLA-DR molecules, with particular reference to the relationship between promiscuity and MHC class II relative binding affinity.

9.3 Methods

Peptides

The peptides used in this study were synthesised in order to examine $CD4^+$ T cell recognition of HA. ~~There~~ design was not influenced by the results of any previous study of $CD4^+$ T cell recognition of HA or any published MHC class II binding motif. The concentration of HA peptides was standardised using a micro BCA assay (see Chap.2). The purity of 5 peptides chosen at random was tested using reverse phase HPLC (see Chap.2). Peptide purity varied between 60-95% (see discussion).

Relative Binding affinity of HA peptides for DR0701

The relative binding affinity of every HA peptide for DR0701 was examined in a competition assay using a biotinylated human invariant chain peptide (Ii), containing the CLIP peptide (residues 82-107, sequence: LPKPPKPVSKMR-MATPLLMQ) and purified DR0701, using the experimental methods established by Miles Davenport and Adrian Hill (Davenport *et al.,* 1995a). Details are given in Chap. 2.

HA peptides frequently recognised in the context of different MHC class II haplotypes

The binding affinities of purified DR-0101, -0301, -0401, -0701, -1301, for HA peptides 97-112, 100-115, 192-207, 198-213, 232-247, 303-318, 308-323, 403-418, 407-422, 417-432, 422-437, 463-478, and 468-493, was examined as described above. As a negative control the relative binding affinity of a polyalanine-lysine repeat peptide (AAAAKAAAAA) for these DR molecules was also examined.

9.4 Results

HA peptide binding to HLA-DR0701

Measurable inhibition of Ii chain binding to HLA DR-0701 occurred with 58 of the 118 HA peptides. Of these 9 had an IC_{50} ≤1.0 μM, 31 had an IC_{50} of ≤10 μM (Table 9.1). The strongest *in vitro* $CD4^+$ T cell proliferative response, following influenza subunit vaccination, was associated with a peptide with an IC_{50} ≤1 μM (peptide 232, Figs. 7.1). Overall however there was no direct relationship between relative binding affinity and magnitude of secondary $CD4^+$ T cell responses; in particular the second strongest T cell response was to 2 overlapping

peptides, one with an IC_{50} of 8.1μM (peptide 463) and the other with an IC_{50} of >100μM (peptide 468); and the third strongest response was to two peptides with an IC_{50} of 20μM. Only 5 peptides representing the HA2 subunit (residues 329-550) had an IC_{50}<100μg, with the highest relative affinity belonging to peptide 508 (2.1μM). In addition the peptide with the highest relative affinity for DR0701 (peptide 192-207) was not recognised by $CD4^+$ T cells following sub unit vaccination, despite being recognised in the context of DRB1*0101 -0401, -0408 alleles (see below and Chap.5).

The majority of peptides with an IC_{50} for DR0701 <10μM which were not recognised by the $CD4^+$ T-cell lines were subject to drift mutation, which was frequently non-conservative in nature (Table 9.1). In contrast the peptides to which $CD4^+$ T cell responded in the context of DR-0701 contained a maximum of 2 highly conservative drift mutations. The exceptions were peptides 100-115 and 105-120 which are conserved in structure but were not recognised, despite being recognised in the context of DR-0101 (Chap. 5 and Chap.6); and peptides 349-364, 369-384, and 513-528 which are again conserved in structure, but to which no strong $CD4^+$ T cell responses have been observed in association with any MHC class II allele.

We then examined whether the observed pattern of HA peptide binding matched known HLA DR-0701 motifs. Unfortunately no binding studies using phage display libraries have yet been published, though a possible motif has been derived from pool sequencing of peptides eluted from DR0701, by Chicz and co-workers (1993). Some of the HA peptides can be aligned with their reported motif, however a new motif P1 I, L, V, (W, or Y) and P9 V, I, L, (Y or F) fitted the observed pattern of HA peptide binding more exactly (Table 9.2, see Chap.10 for discussion), and there appears to be a preference (though not an absolute requirement) for hydrophobic residues at P2 and P7, polar residues at P4 and P6,

and small residues at P5. Interestingly this new motif suggests that though the P1 and P9 pockets are the most important for peptide selection, most peptide residues interact with DR-0701, and that the final peptide affinity for DR-0701 is the combination of several pocket specificities. Furthermore the few peptides which do not fit the P9 requirements have suitable residues located at P10 or P8.

Frequently recognised peptides

The results of binding studies using the frequently recognised peptides are shown in Fig. 9.2. Because of the varying affinity of CLIP for individual HLA-DR molecules (Sette *et al.,* 1995), results from different MHC class II molecules cannot be directly compared.

Peptides 303-318 and 308-323 represent the first HA regions recognised by every donor following natural infection. Peptide 303-318 bound to every HLA-DR molecule tested. Interestingly, with the exception of DR-0701, the position of peptide 303-318 within the observed peptide binding hierarchy for an individual HLA-DR molecule, was directly related to the strength of $CD4^+$ T cell recognition of the peptide in the context of the molecule. The binding of the overlapping peptide 308-323 was more variable. This peptide is associated with strong $CD4^+$ T cell responses in the context of DR-0101, -0401, and -1302, and this was reflected by significant binding to all 3 molecules.

The second frequently recognised region residues 403-442 was represented by two pairs of HA peptides: 403-418 + 407-422, and 417-432 + 422-437. No significant binding of peptide 403-418 was detected and peptide 407-422 bound only to DR0301. As this molecule bound the control peptide weakly (a polyalanine-lysine repeat peptide AAAAKAAAAA), this result must be interpreted with caution. Detectable binding of at least one of the second pair of peptides occurred to every DR molecule tested. The strongest binding occurred to DR-0101, -0401, and -0701.

HA region 97-115 was recognised by $CD4^+$ T cells in the context of DR-0101, and -0401, and this was reflected by the significant binding of both peptide 97-112, and 100-115 to DR0101, and the binding of peptide 100-115 to DR-0401. Furthermore 4 donors expressing DRB1*0101 (A, B, G, and M, see Table 5.2) mounted $CD4^+$ T cell responses to both peptides, whereas 3 donors expressing DRB4* alleles (C, D, H, see table 5.2) recognised only peptide 100-115.

The correlation between binding and $CD4^+$ T cell recognition was less strong for peptides 192-207 and 197-212. $CD4^+$ T cell proliferative responses to these peptides were seen in the context of DR-0101, -04, and possibly -03 (donor H who expressed DR-03, and -04 recognised these peptides). Peptide 192-207 bound strongly to DR-0101, -0401, and -0701, whereas it was only recognised in the context of the first 2 molecules. In addition peptide 192-207, which was recognised in association with DR-0101, -0401, and possibly -0301 bound strongly to only DR-0301.

Peptide 232-247 was recognised in the context of DR-0701, -1301, and -0103 and/or -08 (see Table 6.2, donor N). This peptide bound strongly to DR-0701, and not to -0301, and -0401 in keeping with the $CD4^+$ T cell proliferation data. However peptide 232-237 also bound strongly to both DR-0101, and -1302, though it was not recognised in either context.

Finally peptides 463-478 and 468-483 bound strongly to DR-0701 which is again in keeping with the observed $CD4^+$ T cell response in the context of this allele. Intermediate binding was also detected to DR-0101, and -0401, and this also correlated with moderate $CD4^+$ T cell responses.

With the exception of DR-0101, and -1302, where $CD4^+$ T cell responses of donors who expressed only DRB1 alleles were examined, it is difficult to explore the relationship between relative binding affinity and immunodominance. Donor M, who expressed DR-0101, responded strongly to peptide pools 97, 295, 407, and 432 (Fig 5.2, Table 5.2). Within the pools the response localised to regions

97-115, 303-323, and 403-422. This donor failed to respond to peptide 198-213, however the presence of one conservative drift mutation in this region may account for this observation (207 $^{R\ to\ K}$). The hierarchy of response was 295 (strongest), 97, 407, 432. Thus two responses were associated with strongly binding peptides, and 2 strongly binding peptides failed to induce a response, though they induce $CD4^+$ T cell responses in the context of other MHC class II alleles (198-213, and 232-247). The response to residues 403-422 (encompassing response to pools 384 and 407) was believed to be DQ5 restricted on the basis of anti-class II MHC antibody studies, and the lack of binding of peptides 403-418 and 407-422 supports this conclusion.

Donor J, who expresses only DR-1302, recognised only peptide 308-323 strongly and mounted an intermediate response to peptides 407-422, and 417-432. These responses were in keeping with the results of this binding assay (assuming response to 407-422 is DQ restricted), though $CD4^+$ T cell recognition of peptide 303-318 might have been expected.

9.5 Discussion

Relative HA peptide binding affinity for DR0701

We have examined the relationship between $CD4^+$ T cell epitope recognition following natural viral infection or vaccination (which induces $CD4^+$ T cell responses which we have shown to be very similar to those induced by natural infection - see Chap 7), and relative MHC class II binding affinity. We have found that though peptide 232-247, which was associated with the strongest $CD4^+$ T cell response, has a high relative binding affinity for DR0701, peptides with intermediate (463-478, 468-483, 87-102), and relatively low affinities (92-107) for DR0701 were also associated with strong proliferative responses. Furthermore no response was seen to the HA peptide (192-207) with the highest relative binding affinity for DR0701.

$CD4^+$ T cell recognition of antigen, is dependant on many factors, including antigen processing (Ziegler and Unanue, 1981, 1982), the ability of processed peptide to bind to MHC class II molecules (Buus *et al.,* 1986), competition amongst peptides for MHC binding (Babbitt *et al.,* 1986, Buus *et al.,* 1987), and the presence of an appropriate repertoire of TCR (Vidovic and Matzinger, 1988, Schaeffer *et al.,* 1989). The binding affinity of an antigenic peptide to bind MHC class II molecules is an important factor, and a clear relationship between immunodominance and MHC class II binding affinity has been reported (Liu *et al.,* 1993, Nikcevich *et al.,* 1994).

There are a number of potential technical problems, related to the synthetic HA peptides, which might explain some of our results. The possibility of synthesis error needs to be considered. However several peptides with high binding affinities for DR0701 which were not recognised in the context of this allele, were associated with strong responses in the context of other alleles, making this explanation unlikely at least in these instances (peptides 192-207, 105-120, 100-115, 20-35, see Chap. 5 and Chap.6). The purity of the majority of HA peptides is not quantitated, and it is also conceivable that truncated peptides may have interfered with peptide-MHC binding. The presence of truncated peptides might also have ~~lead~~ led to an underestimate of peptide binding affinity if they were too short to bind to MHC class II molecules. Though this would be relatively insignificant: Even if the purity of a given HA peptide was 50% this would only double its IC_{50}.

Some differences between the observed patterns of $CD4^+$ T cell HA recognition and relative peptide binding affinity may be due to antigen processing. Also there could be substantial differences in the quantity of the "natural equivalent epitopes" of the HA peptides produced, because of inherent differences in their resistance to proteolytic cleavage (for example > 1% of a strongly binding

peptide may become available for MHC class II, whereas all of a moderately binding peptide might survive). As the HA peptides used in these studies have not been optimised, there are likely to be differences in the length of the N and C terminals of the HA peptides and their naturally processed equivalents.

It is possible that some peptides with intermediate relative binding affinities may not have been recognised by $CD4^+$ T cells in the context of DR0701 because of competition with other MHC class II alleles for peptide binding.

Failure of some of the HA peptides to be recognised may be due to the absence of $CD4^+$ T cell expressing appropriate TCRs; "holes in the TCR repertoire"(Vidovic and Matzinger, 1988). One reason for this might be tolerance due to regions of sequence identity between HA and "self antigens". Most diversity between individuals occurs in their MHC. As our donors differed in class I MHC alleles, Dr K. Welsh kindly used his sequence subtractor program to look for regions of sequence identity between their shared DRB1*-0701, -1501, DRB5*-01, DQB1*-05 molecules and HA peptides 192-207, 100-120, and 20-35 which represent HA regions which were recognised in the context of other DR alleles. None was found (personal communication K. Welsh).

Antibody binding to an antigen has been reported to modulate $CD4^+$ T cell responses (Watts and Lanzavecchia, 1993, Simitsek *et al.,* 1995). Several HA peptides which were not recognised by $CD4^+$ T cells, including peptide 192-208 which displayed the highest relative binding affinity for DR0701, were located in or close to antibody neutralising sites (summarised in Table 9.2).The neutralising antibody response to HA is very variable between individuals, and is largely influenced by exposure history (Oxford, 1981). As the pre-vaccination exposure of these 6 donors varied, a more diverse pattern of $CD4^+$ T cell HA recognition

might be expected, if significant antibody modulation of $CD4^+$ T cell recognition occurred in this system.

Finally viral drift mutation may have influenced $CD4^+$ T cell HA epitope recognition. The regions associated with the strongest $CD4^+$ T cell responses by these donors, have been relatively free of drift mutation, and the changes that have occurred are highly conservative in nature (summarised in Table 9.2- see Chap.7 and appendix 1 for details). In contrast the HA peptides which bound to DR0701 but were not recognised, were generally subject to more extensive drift mutation. These findings contrast recent murine models of primary H3 influenza A infection, where $CD4^+$ T cell recognition of variable regions of HA was prominent (Barnett *et al.,* 1989b, Burt *et al.,* 1989). They also contrast a study of T cell recognition of *Plasmodium falciparum* circumsporozoite protein, where immunodominant epitopes have been identified which are clustered in highly variable regions of the molecule (Good *et al.,* 1988).

One explanation of our results is that the precisely defined DR1*0701 associated recognition pattern of HA epitopes has emerged as a consequence of repeated exposure to different influenza A strains, boosting the recognition of conserved regions of the molecule, which were not necessarily major components of the primary T cell response due to their intermediate relative binding affinity (see Chap 10 for full discussion).

Promiscuous HA peptides

In general the degree of promiscuity of a given HA peptide was reflected in the number of HLA-DR molecules it could bind to. However the ability of a DR molecule to bind a peptide was not always associated with significant $CD4^+$ T cell recognition. For example peptide 232 bound strongly to DR0101, and peptides 100 and 192 bound strongly to DR0701 but no T cell responses were observed.

As discussed in detail above there are many reasons for this lack of $CD4^+$ T cell recognition including differences in antigen processing and the lack of an appropriate repertoire of $CD4^+$ T cells.

Sinigaglia and co-workers have described a "universal T cell epitope", a 21 residue modified *Plasmodium falciparum* circumsporozoite peptide, which was recognised by $CD4^+$ T cells in the context of at least 7 MHC class II alleles, in both malaria-exposed, and unexposed donors. The authors reported that T cell responses in association with different HLA-DR alleles localised to different regions within the peptide (Sinigaglia *et al.,* 1988). Pania-Bordignon and colleagues have described 2 tetanus toxoid peptides, p2 and p30, 15 and 21 residues in length respectively, which are also recognised by $CD4^+$ T cells in the context of several MHC class II alleles. The shorter p2 peptide appeared to contain a universal epitope, whereas the longer p30 peptide was recognised in different ways in the context of several MHC class II molecules (Panina-Bordignon *et al.,* 1989). The CLIP fragment of the human invariant chain binds to many (if not all) HLA-DR molecules using a supermotif containing methionine residues at P1 and P9, and alanine at P4 and a proline at P6 (Malcherek *et al.,* 1995).

In order to examine whether the promiscuous HA peptides contained a supermotif or alternatively contained several common motifs, their sequences were aligned with those of CLIP and the *P. falciparum* sporozoite epitope, and compared to known class II MHC motifs (Fig.9.3). It appears that HA peptides 192-207, and 417-422 contain more than one motif rather than a universal motif in the fashion of the CLIP peptide. The malaria sporozoite universal peptide which is thought to contain more than one MHC class II binding motif (Sinigaglia *et al.,*1988), does not match the described DR-0301, -0701, or -1302 motifs, when placed in the same register as the DR0101, and -0401 motifs. In contrast, the HA

306-318 peptide would appear to bind to all the DR molecules examined using a single motif.

The published DR0301 binding motifs for the for P4, P6, and P9 specificities do not match the observed binding of HA peptides. Similarly the P4 and P6 specificities for DR0401, and the P9 specificity for DR1302 also do not fit the observed pattern of peptide binding (see Table 9.4, and Chap.10).

The ability of the conserved peptide 303-313 to both bind and be recognised in association with a large number of HLA-DR molecules might suggest that it has the potential to be used as a $CD4^+$ T cell specific peptide vaccine against influenza. However though this peptide is recognised by every donor, the magnitude of specific response varies considerably, and recognition of this region is frequently a minor component of many donors' overall HA repertoire. Furthermore the use of such a small region of HA as a vaccine has the potential to drive antigenic drift within the region. Its also seems futile to expend a considerable amount of time and resources on the design of a peptide vaccine simply to induce a response to a region which is obtained by using the currently available subunit vaccines.

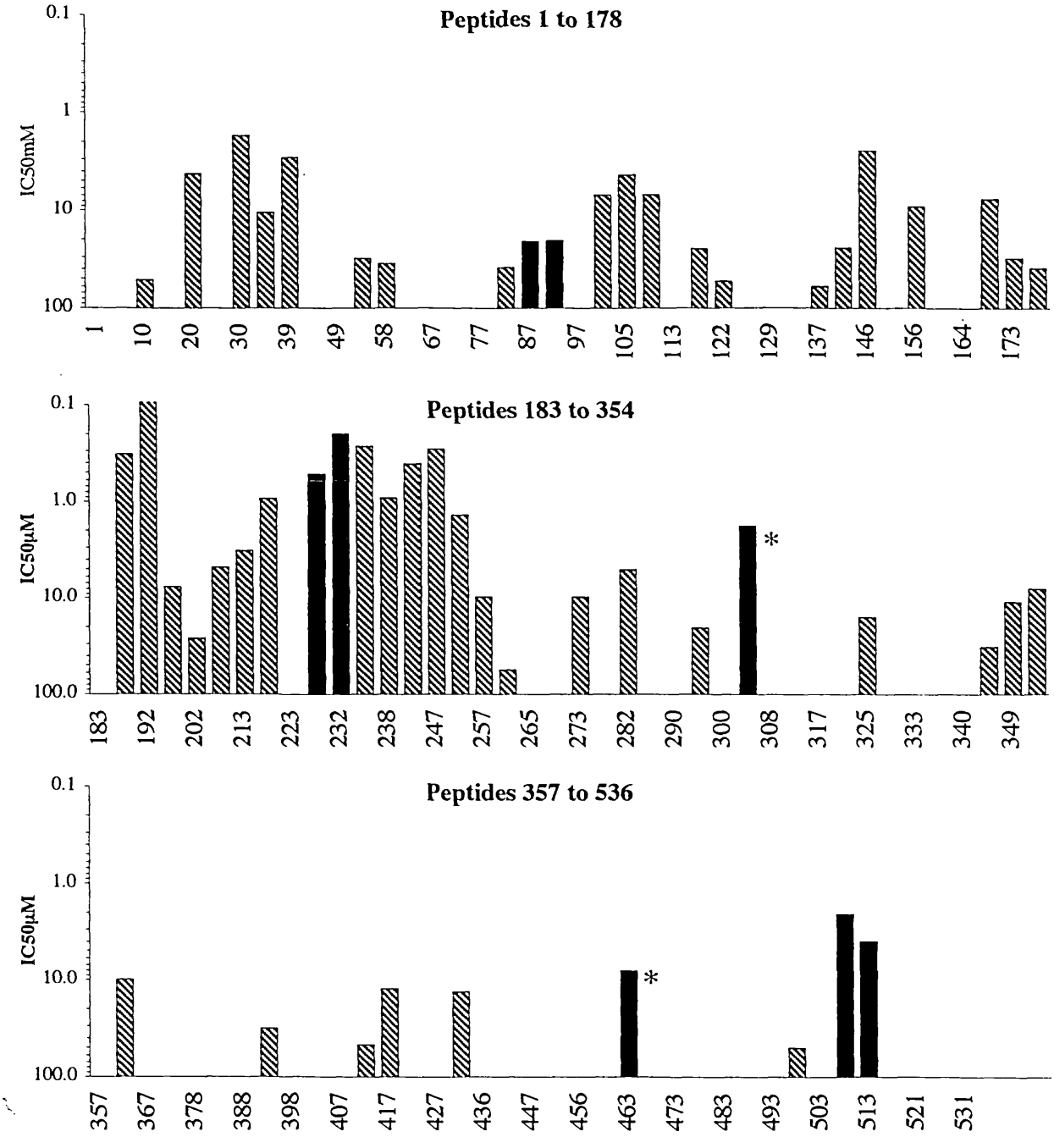
9.6 Conclusions

The relative binding affinity of the HA peptides for purified HLA-DR0701 has been examined using biotinylated invariant chain peptide and a competition assay. Though $CD4^+$ T cell responses were directed towards HA peptides with measurable binding affinity for DR-0701, the relationship between binding affinity and T cell recognition was not direct. There are several reasons for this finding such as intracellular processing, holes in the TCR repertoire, drift mutation in TCR residues or their flanking regions, and the selection of conserved HA

epitopes by repeated exposure to influenza. A putative HLA DR0701 binding motif has been identified.

The relative binding affinity of promiscuous HA peptides for DR-0101. -0301, -0401, -0701, and -1302 was also examined. The number of DR molecules these peptides could associate with was related to the degree of their promiscuity, although MHC binding did not necessarily result in $CD4^+$ T cell recognition. The binding of peptides 192-207, and 417-432 is due to the presence of more than one MHC class II motif within the peptides rather than the presence of a supermotif. In contrast peptide 306-318 appears to bind to several DR molecules using the same motif.

Figure 9.1 HA peptide binding to purified HLA-DR0701



Binding affinity of peptides for DR0701 was determined by their ability to inhibit the binding of biotinylated invariant chain peptide (LPKPPKPVSKMRMATPLLMQ) to purified DR0701: 1µg of HLA-DR0701 was incubated with biotinylated peptide with or without inhibitor peptide at 37°C for 24h at pH5. Solutions were neutralised with Tris-HCl (pH 7.5) before transfer to wells pre-coated with L-243 antibody. Binding of biotinylated peptide was detected with Avidin-horseradish peroxidase conjugate and developed with o-phenylenediamine. Results are presented as IC_{50} = concentration in µM, of peptide causing 50% inhibition of invariant chain binding.
Results from every HA peptide shown on X-axis, though only alternate peptides are labelled for clarity. Y-axis represents IC_{50} and is logarithmic, and in reverse order. Thus peptides with the highest affinity (and therefore lowest IC_{50}) for DR0701 are represented by the largest columns. Filled columns indicates CD4+ T cell recognition of HA peptide in the context of DR0701. * indicates CD4+ T cell reognition in the abscence of measurable peptide binding.

Table 9.1, Rank order of HA peptide relative binding affinity for DRB1*0701

Order	Sequence	IC 50 µM	Mean Prolif.	HA Sequence Variations between A/Aichi/68 and A/Beijing/32/92	Notes
1	192 to 207	0.0868		R to K 207	Antibody site B
2	232 to 247	0.2	30900	V to I 242, V to L 244	Radical change 226 L to Q
3	236 to 251	0.2688		V to I 242, V to L 244, **N to T 248**	
4	247 to 262	0.2868		**N to T 248**, M to I 260, **T to N 262**	
5	188 to 203	0.3258		N to D 188, Q to R 189, E to D 190, S to N 193, Q to R 197	
6	243 to 258	0.4048		V to L 244, **N to T 248**	
7	228 to 243	0.522	6680	V to I 242, V to L 244	
8	238 to 253	0.9196		V to I 242, V to L 244, **N to T 248**	
9	218 to 233	0.9306		**L to Q 226**	Antibody site D
10	252 to 267	1.384		M to I 260, **T to N 262**	
11	30 to 45	1.7088		D to N 31	
12	303 to 318	1.8102	5600	K to R 307	
13	508 to 523	2.1096	3400	Conserved	
14	146 to 161	2.5122		G to S 146, **T to H 155, E to K 156, S to L 157, G to E 158, S to Y 159, T to K 160**	Antibody site A and B
15	39 to 54	2.9304		K to R 50, N to D 53, N to S 54	
16	213 to 228	3.237		**L to Q 226**	
17	513 to 528	4.0344		Conserved	
18	20 to 35	4.217		D to N 31	
19	105 to 120	4.2918		Conserved	
20	208 to 223	4.784		I to V 213, **I to T 214**	Antibody site D
21	282 to 297	5.126667		Conserved	
22	100 to 115	6.9344		Conserved	
23	300 to 315	7.0848		K to R 307	
24	198 to 213	7.74		R to K 207, I to V 213	
25	168 to 183	7.8516		D to G 172, N to K 173, I to V 182	
26	354 to 369	8.0406		Conserved	
27	463 to 478	8.06	20100	Conserved	
28	156 to 171	9.4		**E to K 156, S to L 157, G to E 158, S to Y 159, T to K 160, V to A 163**	Antibody site B
29	273 to 288	9.7331		D to G 275, **I to S 278**	Antibody site C
30	257 to 272	9.7907		M to I 260, **T to N 262**	
31	363 to 378	9.695978		Conserved	
32	36 to 51	10.5974		K to R 50	
33	349 to 364	11.04		Conserved	
34	417 to 432	12.3872	13500	Conserved	
35	432 to 447	13.25369		Conserved	
36	325 to 340	16.04158		L to I 331	
37	110 to 125	19.7352		**T to N 122**, G to D 124	
38	92 to 107	20.5482	7000	F to Y 94	
39	295 to 310	20.6784		K to R 299, K to R 307	
40	87 to 102	21.16	17000	F to Y 94,	
41	329 to 344	24.41376		L to I 331	
42	118 to 133	24.9		**T to N 122**, G to D 124, **T to N 126**, T to A 131, N to D 133	
43	142 to 157	24.64		P to S 143, **G to V 144**, S to N 145, G to S 146, **T to H 155**, E to K 156, **S to L**/S 157	
44	202 to 217	26.44		207 R to K, I to V 213, **I to T 214**	
45	54 to 69	31.12667		**I to K 62**, D to N 63	
46	173 to 188	31.41333		I to V 182, **N to D 188**	
47	393 to 408	31.43147		Conserved	
48	344 to 359	32.3		I to V 347	
49	58 to 73	35.09333		**N to S 54, I to K 62**, D to N 63	
50	82 to 97	38.8		**E to K 82**, T to E 83, F to Y 94	
51	178 to 193	39.496		N to D 188, Q to R 189, E to D 190, S to N to S 193	
52	412 to 432	46.93333		Conserved	
53	498 to 513	50.70626		Conserved	
54	10 to 25	51.26667		Conserved	
55	122 to 137	52.88		**122 T to N, T to N 126**, T to A 131, N to D 133, **N to Y 137**	Antibody site E
56	262 to 277	56.25		**T to N 262**, D to G 275	
57	137 to 152	60		P to S 143, G to V 144, S to N 145, G to S 146, T to H 155E to K 156, S to L/S 157	
58	265 to 280	101.3067		D to G 275, **I to S 278**	

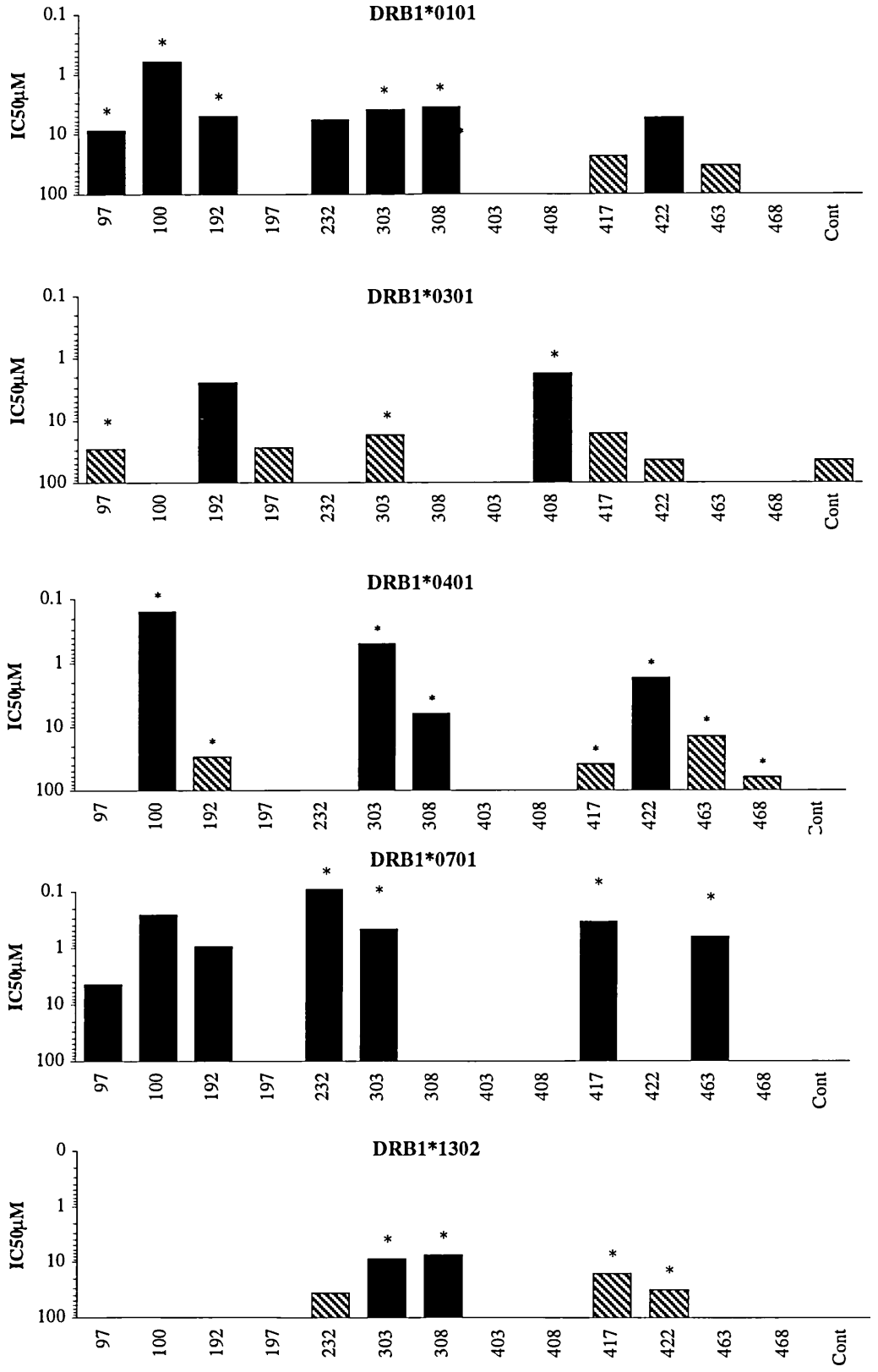
Table lists rank order of HA peptide binding affinity for purified DRB1*0701. Peptides with highest affinity for this allele have lowest IC50. Mean proliferation is geometric mean of $CD4^+$ T cell responses by 6 donors to individual peptides. Differences in HA structure between original human H3N2 influenza A virus A/Aichi/68 and A/Beijing/32/92 is given as a guide to sequence variation amongst human H3N2 influenza A viruses (see appendix 1 for more detailed analysis). Non-conservative substitutions are listed in **bold** type. Notes column indicates peptides which represent antibody neutralising sites.

Table 9.2, Putative DRB1*0701 Peptide Binding Motif

Rank	Sequence	IC 50 μM	MHC class II peptide binding pockets																							
										P 1			P 4		P 6			P 9							
	Putative DRB1*0701 motif (Chicz et al 1993)									F I L V Y					N S T											
1	192 to 207	0.0868						T	S	L	Y	V	R	A	S	G	R	V	T	V	S	T	K		
2	232 to 247	0.2				I	Y	W	T	I	V	K	P	G	D	I	L	L	I	N	S				
3	236 to 251	0.2688	I	V	K	P	G	D	I	L	L	I	N	S	T	G	N	L							
4	247 to 262	0.2868			S	T	G	N	L	I	A	P	R	G	Y	F	K	I	R	N					
5	188 to 203	0.3258		D	R	D	Q	T	S	L	Y	V	R	A	S	G	R	V	T						
6	243 to 258	0.4048								L	L	I	N	S	T	G	N	L	I	A	P	R	G	Y	F
7	228 to 243	0.522		S	R	I	S	I	Y	W	T	I	V	K	P	G	D	I	L						
8	238 to 253	0.9196		K	P	G	D	I	L	L	I	N	S	T	G	N	L	I	A						
9	218 to 233	0.9306				G	S	R	P	W	V	R	G	L	S	S	R	I	S	I	Y				
10	252 to 267	1.384								I	A	P	R	G	Y	F	K	I	R	N	G	K	S	S	I
11	30 to 45	1.7088				T	N	D	Q	I	E	V	T	N	A	T	E	L	V	Q	S				
12	303 to 318	1.8102			G	A	C	P	R	Y	V	K	Q	N	T	L	K	L	A	T					
13	508 to 523	2.1096		K	S	G	Y	K	D	W	I	L	W	I	S	F	A	I	S						
14	146 to 161	2.5122			S	F	F	S	R	L	N	W	L	H	K	S	E	Y	K	Y					
15	39 to 54	2.9304				A	T	E	L	V	Q	S	S	S	T	G	R	I	C	D	S				
16	213 to 228	3.237				V	T	P	N	I	G	S	R	P	W	V	R	G	L	S	S				
17	513 to 528	4.0344			D	W	I	L	W	I	S	F	A	I	S	C	F	L	L	C					
18	20 to 35	4.217		V	P	N	G	T	L	V	K	T	I	T	N	D	Q	I	E						
19	105 to 120	4.2918	Y	A	S	L	R	S	L	V	A	S	S	G	T	L	E	F							
20	208 to 223	4.784			R	S	Q	Q	T	V	T	P	N	I	G	S	R	P	W	V					
21	282 to 297	5.126667		I	T	P	N	G	S	I	P	N	D	K	P	F	Q	N	V						
22	100 to 115	6.9344						V	D	V	P	D	Y	A	S	L	R	S	L	V	A	S	S		
23	300 to 315	7.0848								I	T	Y	G	A	C	P	R	Y	V	K	Q	N	T	L	K
24	198 to 213	7.74		A	S	G	R	V	T	V	S	T	K	R	S	Q	Q	T	V						
25	168 to 183	7.8516		M	P	N	N	G	K	F	D	K	L	Y	I	W	G	V	H						
26	354 to 369	8.0406			R	H	Q	N	S	E	G	T	G	Q	A	A	D	L	K	S					
27	463 to 478	8.06	G	N	G	C	F	K	I	Y	H	K	C	D	N	A	C	I							
28	156 to 171	9.4					K	S	E	Y	K	Y	P	A	L	N	V	T	M	P	N	N			
29	273 to 288	9.7331							P	I	G	T	C	S	S	E	C	I	T	P	N	G	S	I	
30	257 to 272	9.7907					Y	F	K	I	R	N	G	K	S	S	I	M	R	S	D	A			
31	363 to 378	9.695978				Q	A	A	D	L	K	S	T	Q	A	A	I	D	Q	I	N				
	Suggested DRB1*0701 Motif									L I V W Y	H y d r o		P o l a r	S m a l l	P o l a r	H y d r o		V L I F M W							

See Fig. 9.1 and Table 9.1 for experimental details. A putative DRB1*0701 binding motif has been identified. The P1 and P9 pocket specificities appear to be the most stringent. 6/31 peptides do not fit this motif's P9 specificty, 5 of these have a suitable amino acid in the P10 position, and one has a suitable residue in the P8 position, which may reflect some flexibility in peptide binding.

Figure 9.2, Relative binding affinity of "frequently recognised" HA peptides for common DRB1* alleles



Binding affinity of promiscuous HA peptides to 5 common DRB1 alleles was investigated using a competition assay and biotinylated CLIP peptide. * indicates proliferative CD4+ T cell response to peptide in context of this allele. Y-axis is in reverse order and represents concentration of HA peptide in µM which causes 50% inhibition in CLIP binding to individual MHC class II alleles. Peptides with a high binding affinity have a low IC_{50}. A polyalanine peptide was used as a negative control. **Results are not directly comparable between DRB1 alleles because of differences in binding affinity of CLIP for individual alleles (see text).** Further details as in Fig 9.1.

Table 9.3, Relative binding affinity of "frequently recognised" HA peptides for common DRB1* alleles

Peptide	*0101	*0301	*0401	*0701	*1302
97	8.9	29.2	>100	>100	>100
100	0.6	>100	0.2	6.9	>100
192	5.0	2.5	29.8	0.1	>100
198	>100	28.0	>100	7.7	>100
232	5.9	>100	>100	0.2	36.6
303	3.9	17.2	0.5	1.8	8.9
308	3.6	>100	6.3	>100	7.6
403	>100	>100	>100	>100	>100
408	>100	1.8	>100	>100	>100
417	23.6	16.2	38.9	12.4	16.3
422	5.5	44.0	1.7	>100	31.6
463	34.5	>100	14.1	8.1	>100
468	>100	>100	63.1	>100	>100
PolyA	>100	43.7	>100	>100	>100

Binding affinity of promiscuous HA peptides to 5 common DRB1 alleles was investigated using a competition assay and biotinylated CLIP peptide. Affinities expressed as IC_{50} μM. Peptides with a high binding affinity have a low IC_{50}. A polyalanine peptide was used as a negative control. **Results are not directly comparable between DRB1 alleles because of differences in binding affinity of CLIP for individual alleles (see text).**

Table 9.4, Alignment of Promiscuous HA peptides and two "universal epitopes" to reported MHC class II binding motifs

Allele and Peptide	IC_{50} μM	MHC class II pocket specificity																			Notes
							P1				P4		P6			P9					
DRB1*0101							YV LF IA M W				LA IV MN Q		AG ST P			LA IV NF Y					Motif derived from pool sequencing and phage display data, described in Rammensee *et al.*, (1995).
192 to 207	5.0			T	S	L	Y	V	R	A	S	G	R	V	T	V	S	T	K		
303 to 318	3.9	G	A	C	P	R	Y	V	K	Q	N	T	L	K	L	A	T				
417 to 432	23.6		K	I	D	L	W	S	Y	N	A	E	L	L	V	A	L	E			
Malaria		E	K	K	I	A	K	M	E	K	A	S	S	V	F	N	V	V			
CLIP		K	P	V	S	K	M	R	M	A	T	P	L	L	M	Q	A	L	P	M	
							P1				P4		P6			P9					
DRB1*0301							L I F M V				D		K R E Q N			Y L F					Motif derived from pool sequencing and peptide binding studies, described in Rammensee *et al.*, (1995).
192 to 207	2.5				T	S	L	Y	V	R	A	S	G	R	V	T	V	S	T	K	
303 to 318	17.2	G	A	C	P	R	Y	V	K	Q	N	T	L	K	L	A	T				
417 to 432	16.2	K	I	D	L	W	S	Y	N	A	E	L	L	V	A	L	E				
Malaria		E	K	K	I	A	K	M	E	K	A	S	S	V	F	N	V	V			Not tested on DRB1*0301
CLIP		K	P	V	S	K	M	R	M	A	T	P	L	L	M	Q	A	L	P	M	
							P1				P4		P6	P7		P9					
DRB1*0401							FY WI LV M				FW IL VA DE no RK		NS TQ HR	Pol chg ali		Pol ali K					Motif derived from pool sequencing, peptide binding, and phage display data, described in Rammensee *et al.*, (1995).
192 to 207	29.8			T	S	L	Y	V	R	A	S	G	R	V	T	V	S	T	K		
303 to 318	0.5	G	A	C	P	R	Y	V	K	Q	N	T	L	K	L	A	T				
417 to 432	38.9	K	I	D	L	W	S	Y	N	A	E	L	L	V	A	L	E				
Malaria		E	K	K	I	A	K	M	E	K	A	S	S	V	F	N	V	V			
CLIP		K	P	V	S	K	M	R	M	A	T	P	L	L	M	Q	A	L	P	M	
							P1				P4		P6			P9					
DRB1*0701							L I V W Y	H y d r o			P o l a r	S m a l l	P o l a r	H y d r o		V L I F M W					Motif derived from data in Table 9.2. P4, P6, P7 positions specificities appear to be preferences rather than absolute requirements
192 to 207	0.1				T	S	L	Y	V	R	A	S	G	R	V	T	V	S	T	K	
303 to 318	1.8	G	A	C	P	R	Y	V	K	Q	N	T	L	K	L	A	T				
417 to 432	12.4			K	I	D	L	W	S	Y	N	A	E	L	L	V	A	L	E		
Malaria		E	K	K	I	A	K	M	E	K	A	S	S	V	F	N	V	V			
CLIP		K	P	V	S	K	M	R	M	A	T	P	L	L	M	Q	A	L	P	M	
							P1				P4		P6			P9					
DRB1*1302							I L F Y						P o s P o l			Y					Motif derived from pool sequencing and peptide binding studies, described in Davenport *et al.*, (1995).
192 to 207	>100				T	S	L	Y	V	R	A	S	G	R	V	T	V	S	T	K	
303 to 318	8.9	G	A	C	P	R	Y	V	K	Q	N	T	L	K	L	A	T				
417 to 432	16.3		K	I	D	L	W	S	Y	N	A	E	L	L	V	A	L	E			
Malaria		E	K	K	I	A	K	M	E	K	A	S	S	V	F	N	V	V			
CLIP		K	P	V	S	K	M	R	M	A	T	P	L	L	M	Q	A	L	P	M	

Promiscuous HA peptides, a previously described universal malaria T cell epitope (Sinigaglia *et al.* , 1988-see text), and the CLIP peptide, were aligned with reported MHC class II binding motifs. IC_{50} is concentration of peptide causing 50% inhibition in binding of biotinylated CLIP peptide, see Table 9.2 for details. ali = aliphatic, chg = charged, hydro = hydrophobic, pol = polar, pos = positive charge

Chapter 10, Discussion

In this thesis $CD4^+$ T cell recognition of HA A/Beijing/32/92 by 3 groups of adult donors has been examined. The first group of 12 subjects had a history of influenza during November and December 1993, a time when A/Beijing/32/92 like strains were circulating in London. The second group was comprised of 6 adults, who had no history of influenza infection for at least 4yr, but were known to mount polyclonal proliferative T cell responses to HA. The third group, of 12 donors, were vaccinated with a trivalent subunit vaccine containing HA A/Beijing/32/92.

It was striking that $CD4^+$ T cell recognition of HA of type A influenza by all 3 groups was found to be primarily directed towards HA regions, including parts of the HA2 subunit, that were not subject to frequent, or extensive, drift mutation (conserved regions). The HA2 subunit has been previously widely regarded as immunologically silent for $CD4^+$ T cells (Caton and Gerhard, 1992). Selection of conserved HA epitopes by $CD4^+$ T cells resulted in cross-reactive recognition of a panel of H1, H2, and H3 influenza A viruses.

Following natural infection 2 conserved regions of HA (residues 303-323, 407-432) induced $CD4^+$ T cell responses in 18/18 unrelated adult donors. This finding was unexpected as murine $CD4^+$ T cell HA epitope selection differs in the context of different MHC class II molecules (see Chap 1), and contrasts with $CD8^+$ T cell recognition of influenza A where epitope recognition is nearly always MHC class I allele specific (reviewed by Townsend and Bodmer, 1989). Their "universal" immunogenicity may relate to the presence of several different appropriate MHC anchor sequences within the peptides or it might be due to inherent chemical stability (see Chap 9).

Though the above mentioned HA regions were recognised by every donor, the data also clearly illustrates the powerful influence of MHC class II over HA

epitope selection: Following natural infection 3 pairs of unrelated donors, expressing identical low resolution HLA-DR and -DQ types, recognised identical HA peptides. In addition clear HLA-DR associated HA recognition patterns were evident amongst donor sharing single HLA-DR alleles. Furthermore 6 unrelated donors expressing the same HLA-DR and DQ alleles recognised identical HA peptides following influenza subunit vaccination.

Comparison of results to previous human studies of CD4$^+$ T cell recognition of HA

There has never previously been a systematic examination of human CD4$^+$ T cell recognition of HA in the polymorphic human population. Lamb and co-workers used an incomplete panel of peptides, spanning part of the HA1 subunit only, to examine the response of a single donor, and identified HA region 306-318 as a human CD4$^+$ T cell epitope (Lamb *et al.,* 1982b, 1983). A preliminary study by Rodda and co-workers describes human CD4$^+$ T cell recognition of the HA2 subunit, but donors HLA types were not investigated, and the study was marred by the use of 13mer peptides, which are probably sub-optimal for the identification of CD4$^+$ T cell responses (Rodda *et al.,* 1992, see Chap.1).

Comparison to human CTL response to influenza A

In contrast to the human CD4$^+$ T cell response to influenza A, CTLs recognition of HA is rare (reviewed by McMichael 1994), and the response is primarily focused on conserved internal viral proteins in the human and mouse (Townsend *et al.,* 1985, McMichael *et al.,* 1986, Gotch *et al.,* 1987, Gotch *et al.,* 1987, Biddison *et al.,* 1979), and is therefore almost always widely cross reactive between influenza A subtypes (Zweerink *et al.,* 1977, McMichael and Askonas 1978). CD8$^+$ T cell recognition of these internal viral proteins involves the selection of a limited number of highly allele specific epitopes, universal epitopes

are not found (reviewed by McMichael 1994). Interestingly an individual donor's CTL response, at least to matrix protein, involves only a small number of closely related T cell clones (Moss *et al.,* 1991), and the TCR usage of $CD8^+$ T cells from unrelated donors responding to matrix protein in the context of HLA-A2 is remarkably similar (Lehner *et al.,* 1995).

The CTL memory response to influenza A following infections declines over several yrs (estimated half life of 2-3 yr) (McMichael *et al.,* 1983b).

Comparison to murine models of influenza A infection

Several murine models of influenza virus infection have been investigated, and results of $CD4^+$ T cell recognition studies vary (discussed in detail in Chap. 1). The most detailed recent murine studies have been by Thomas and co-workers, who have investigated the recognition of HA A/Aichi/68 following primary nasal infection with live X-31 virus (containing A/Aichi/68 surface glycoproteins). In this system the murine $CD4^+$ T cell response to HA has been shown to include strong recognition of both variable epitopes closely related to antibody neutralising sites, as well as some recognition of more conserved regions of the HA1 subunit. Recognition of the HA2 subunit in this system has never been examined in detail, but is thought by this group to be rare (Barnett 1989, Burt 1989 and reviewed in detail in Chap 1).

How does $CD4^+$ T cell recognition of HA, compare to $CD4^+$ T cell recognition of other viral antigens?

There has only been limited analysis of human $CD4^+$ T cell specificities following other viral infections. Hepatitis B infection has been studied in most detail, though $CD4^+$ T cell cytotoxicity has primarily been examined: in this system much stronger $CD4^+$ T cell responses to core antigens than surface antigens are found,

and several immunodominant epitopes from core antigens have been described (reviewed by Jung *et al.,* 1994).

Polio Virus $CD4^+$ T cell epitopes have also been described in normal donors (who presumably will have received the Sabin vaccine). Interestingly these epitopes were closely related to antibody neutralising sites (Graham *et al.,* 1992). Similarly, human $CD4^+$ T cell epitopes located close to B cell epitopes have been reported in limited studies of Herpes Simplex Virus type 2 infection (Koelle *et al.,* 1994), Human Papilloma Virus 18 infection (Lehtinen *et al.,* 1992), Dengue virus infection (Livingston, 1994), and Rubella (McCarthy *et al.,* 1993).

Has repeated exposure to differing influenza A strains boosted $CD4^+$ T cell recognition of conserved HA epitopes?

Why does recognition of conserved HA regions dominate adult human $CD4^+$ T cell repertoire, whereas recognition of more variable regions closely related to antibody neutralising sites is prominent in murine models primary H3 influenza A infection, and also appears to be a feature of a number of other human viral infections (see above)? We have only examined $CD4^+$ T cell responses to HA by adult donors, who have almost certainly been repeatedly exposed to, and infected by, influenza. We do not yet know the effect of primary $CD4^+$ T cell exposure in man. The observed dominance of conserved epitope recognition may have resulted from repeated exposure to influenza A variants over many yrs boosting $CD4^+$ T cell responses to conserved regions of HA that were not necessarily major components of the primary $CD4^+$ T cell repertoire. This may have been exacerbated by the present co-circulation of H1 and H3 influenza A viruses. The MHC-HA-peptide binding studies presented in Chap. 9 offer some support to this explanation: Though $CD4^+$ T cell recognition was found to be restricted to peptides with measurable binding to DRB1*0701, the level of binding affinity and $CD4^+$ T cell recognition was not directly related. Many HA peptides which

bound strongly to this allele, but were not recognised by $CD4^+$ T cells, were subject to drift mutation, and several peptides which were highly conserved in structure but had only intermediate affinity for DRB1*0701 were recognised strongly by $CD4^+$ T cells. Interestingly selective boosting of conserved epitope recognition is a feature of the human antibody response to HA: Children primarily recognise variable regions, whereas adults generally respond more strongly to conserved regions of the molecule (Oxford *et al.,* 1978). It will be of great interest to discover whether the $CD4^+$ T cell response of children to primary influenza infection is directed to conserved or variable regions of HA.

Is there any evidence of viral escape from $CD4^+$ T cell recognition of HA?

Viral escape from CTL cell surveillance in HIV-1 patients has recently attracted much attention (Phillips *et al.,* 1991).Two categories of viral escape mutants have been proposed. The first includes viral strains with alterations in MHC contact residues within dominant CTL epitopes, or alterations in flanking residues which affect antigen processing. These would confer a selective advantage over the wild type virus within an individual host, but would normally not provide a selective advantage at the population level. This is because individuals expressing different MHC alleles which bound other viral epitopes would continue to respond normally to the variant strain. Interestingly however there is a report of possible viral escape by this mechanism in a population in which a high proportion of individuals express HLA-A11: Epstein Barr Virus (EBV) infection strains with mutations within the dominant HLA-A11 associated epitope of the EBNA4 antigen, affecting MHC binding, have been described (de Campos-Lima *et al.,* 1993).

The second category of potential viral escape mutants have alterations in TCR contact residues, leading to either loss of CTL recognition, or alternatively to CTL antagonism. CTL antagonist viral mutants would decrease response to wild type

virus, and would probably exist in a complex relationship with WT virus within a host (reviewed by Davenport, 1995). TCR escape mutants falling into this category would only be of advantage within an individual host. The first report of CTL escape mutants with alteration in TCR contact residues came from a transgenic mouse model of lymphocytic choriomenigitis virus (LCMV) infection (Pircher *et al.,* 1990). More importantly TCR cell escape mutants have been reported in human haemophiliacs with HIV infection (Phillips *et al.,* 1991). There is some evidence that TCR antagonist mutants may exist: Two studies, one examining CTL recognition of hepatitis B virus nucleoprotein (Bertoletti *et al.,* 1994), and the other the recognition of HIV-1 gag protein (Klenerman *et al.,* 1994), have reported that peptides corresponding to natural viral variants inhibit CTL responses to wild type virus.

In contrast to MHC class I associated escape, MHC class II escape has been suggested to confer no advantage to the pathogen, at least in part because of the increased diversity of the $CD4^+$ T cell response. Certainly within an individual host, once infection was established, viral escape from $CD4^+$ T cell recognition would provide little benefit because of the continued presence of a $CD8^+$ T cell response, unless MHC class II restricted cytotoxic activity was prominent (Potts and Slev, 1995).

One interpretation of the data presented in Chap 9 is that $CD4^+$ T cell recognition of variable regions might have been lost. If this is not the result of selection pressure by $CD4^+$ T cells, why have these regions undergone drift mutation? The cause of influenza A variation has been the cause of considerable debate over many years (e.g. Fitch *et al.,* 1991, Sugita *et al.,* 1991, Domingo *et al.,* 1993). Antibody would appear to be the cause of selection within the neutralising sites. Indeed *in vitro* selection of influenza viral strains can be demonstrated using monoclonal neutralising antibodies (Laver *et al.,* 1979, 1981, Newton *et al.,* 1983). If $CD4^+$ T cell recognition of antibody neutralising sites has been lost, this

would probably be a secondary consequence of antibody selection. There is also variation in regions of HA outside the neutralising antibody sites. Ina and Gojobori propose, on the basis of an analysis of coding and non-coding nucleotide mutations, that this variation is a result of viral RNA replication errors, though this study can be criticised as it was performed on H1N1 influenza A viruses, whose antibody neutralising sites are less well defined than H3N2 influenza A viruses (Ina and Gojobori, 1994). At present we cannot therefore exclude selective pressure by antibody or $CD4^+$ T cells outside the neutralising sites. So far such epitopes have only rarely been defined for $CD4^+$ T cells and primary $CD4^+$ T cell response have not yet been studied. $CD4^+$ T cell responses in adults are dominated by conserved epitope recognition and it will be interesting to establish whether this is indeed the result of boosting repeat exposure, and whether the recognition of variable regions is lost with time.

Other possible explanations for the predominant $CD4^+$ T cell recognition of conserved HA epitopes

Antibody binding to a T cell epitope has been reported to directly modulate epitope selection by altering antigen processing (Watts and Lanzavecchia, 1993, Simitsek *et al.,* 1995). Marked variations occur between individuals in the relative proportions of neutralising and cross reactive antibody produced (Oxford *et al.,* 1981), considerable individual variation might be expected in HA response, which was not observed (Chap. 7). (In contrast the indirect effect of antibody over T cell epitope selection, by driving viral drift mutation (discussed above), might alter $CD4^+$ T cell recognition at the host population level.)

Alternatively the differences in $CD4^+$ T cell HA epitope selection between adult humans and mice following primary nasal infection may reflect fundamental differences between I-A, which is the equivalent of HLA-DQ, and HLA-DR which is the equivalent of I-E. The majority of $CD4^+$ T cell clones described by Thomas

and colleagues (reviewed in detail in Chap. 1) are I-A restricted. In contrast the majority of responses observed in adult humans were HLA-DR restricted. Interestingly Thomas and co-workers described some I-E restricted responses directed towards conserved epitopes (Burt *et al.,* 1989).

Finally, though the strains of influenza A used for recent murine models of influenza are mouse adapted, they are not natural pathogens in mice, and this may relate to some of the apparent species differences.

Comparison of published MHC class II binding motifs with observed CD4$^+$ T cell HA epitope selection

The relationship between HA peptide binding affinity and CD4$^+$ T cell recognition was explored in detail in Chap. 9. Though dominant T cell epitopes were represented by HA peptides with measurable binding to DRB1*0701, a direct relationship between HA peptide binding affinity for this allele and CD4$^+$ T cell recognition was not found. The observed pattern of peptide binding was compared to a described DRB1*0701 peptide binding motif. It is apparent that this putative motif did not predict the observed pattern of HA peptide binding, and a new putative motif is proposed. This new model suggests that the majority of residues in contact with MHC have some influence over peptide binding, though the P1 and P9 pocket specificities are dominant.

Table 10.2 lists the HA peptides which were associated with dominant responses in the context of the following MHC class II alleles: DRB1*-0101, -0301, -0401, -0402, -0701, -0801, -1101, -1301, -1302, and -1501. An attempt has been made to fit these dominant HA peptides into published MHC class II binding motifs (details in Table 10.2). A variable degree of success was achieved: The most precise alignment was achieved for DRB1*1201. This was particularly interesting as this peptide very clearly dominated the response of donor E (DRB1*0403,-1201). In contrast the described motif for DRB1*0301,-1101, -1301, -1302 only

poorly matched the observed pattern of peptide binding. As discussed in detail in Chap. 9 there are several reasons why peptide binding may not accurately predict T cell epitopes. Firstly some of the HA peptides may have been recognised in association with DRW alleles, or in a few cases where restriction studies were either not carried out or were inconclusive with HLA-DQ or -DP alleles. The natural equivalents of the HA peptides which fit the putative motifs may not be produced by antigen processing. An appropriate TCR repertoire may not exist for these peptides. Drift mutation in HA may also have altered TCR contact residues, leading to loss of $CD4^+$ T cell recognition, and selective boosting of response to more conserved HA regions *in vivo*. Finally pool sequencing of self peptides eluted from MHC may detect peptides with a high abundance, but a low affinity, which may therefore not accurately predict "real" motifs, following infection or vaccination.

Comparison of observed pattern of HA recognition to a computer prediction of HA peptide processing and presentation

The observed pattern of peptide binding was then compared to a computer prediction of MHC class II peptide binding based on the results of phage display and peptide elution studies (Davenport *et al.,* 1995b). We compared predicted and observed T cell epitope selection for DRB1*0101, -0301, -1301, and -1302. The results are shown in Figure 10.1. The model accurately predicts the $CD4^+$ T cell epitopes for DRB1*0101 at residues 97-113, 192-207, 303-323, and 468-488. But this model also predicted that recognition of several other HA regions would occur. At least some of these are in regions subject to extensive drift mutation. In contrast the model for DRB1*1302 is very different to the observed pattern of HA epitope recognition which is focused primarily onto two regions 303-323, and 407-442. The success of the models predicting binding and processing for DRB1*0301 and DRB1*1301 appear to be intermediate between these two

extremes. This suggests that without performing detailed studies of $CD4^+$ T cell epitope recognition it may prove to be very difficult to accurately predict $CD4^+$ T cell epitopes.

Future studies

It will be particularly important to examine HA recognition by young children. Unless the $CD4^+$ T cell responses of children under 2 yr of age are examined it will be difficult to identify individuals who have only been exposed to influenza once, and some compromise may need to be made between the age of the donors and influenza exposure. Even so such a study would inevitably present a major technical challenge: At present 120ml of blood is routinely used to perform a detailed examination of HA responses. It seems unlikely that ethical permission to obtain more than 10ml of blood from small children would be granted. Various modifications in the experimental procedure are possible, all of which would decrease the number of T cells required: For example the number of responder T cells used in proliferation assays could be halved. The composition of the peptide pools could be altered so that rather than be made up of peptides in a linear sequence, the pools would comprised either variable or conserved peptides. In addition the number of peptides in each pool could be varied, so for example some pools representing relatively non-immunogenic regions of HA could contain 10 peptides, whereas others could contain fewer peptides. These measures would not be sufficient on there own, and antigen presenting cells would have to be obtained from other sources. One option would be to use EBV cells. Unfortunately these are not particularly efficient at expanding $CD4^+$ T cell lines, though they could be used for HA and HA peptide recognition assays. Another approach would be to select HLA-DR and DQ matched adult donors and use their PBMC as APC. Though this task would appear to be difficult, it is not impossible, as Dr K. Welsh was able to identify 6 donors with identical MHC class

II alleles. Indeed it would most interesting to examine the $CD4^+$ T cell responses of children who express MHC class II alleles where the adult HA repertoire is well defined (for example DRB1*0701,-1501). Less satisfactory alternatives include examining the responses of children who were infected with A/Beijing/32/92 one or two yr previously, however as repeat infection may well have occurred, interpretation might be difficult. Finally studies could be limited to HA peptides which demonstrate binding to purified MHC class II alleles expressed by the potential infant donors, however problems might arise if the balance of HA epitope recognition in association with HLA-DR, -DQ, and -DP varies between adults and children.

It will be important to complete the investigation of $CD4^+$ T cell memory following natural infection, and compare it to vaccination. This task may also prove to be a challenge as a severe influenza epidemic appears to be developing during the current winter and $CD4^+$ T cell responses will be boosted even if only sub-clinical infection of the donors occurs.

At present though influenza vaccination is recommended to high risk groups on an annual basis, there is little evidence that it affords greater protection to normal individuals than intermittent vaccination; furthermore the relative increase in HI titres achieved by vaccination declines with annual administration (reviewed by Nicholson 1992). Given that the adult $CD4^+$ T cell response is directed towards conserved HA epitopes, and that influenza vaccines are poor inducers of $CD8^+$ T cell responses (McMichael 1994), there is need for a detailed study of the effects of regular annual vaccination.

It will also be interesting to extend the peptide binding assays by examining the binding of HA peptides to other common HLA-DR alleles. It will be particularly interesting to discover if the observed low binding affinity of DRB1*0701 for HA2 peptides, is matched by other common DRB1* alleles as this may explain the observation that HA2 is non-immunogenic for murine $CD4^+$ T cell responses

(Caton and Gerhard, 1992), while inducing a strong response in all our donors- which may have been boosted by repeat infection.

Finally TCR usage by the 6 donors sharing MHC class II alleles will be of interest. Recently remarkable conservation of TCR usage in the human HLA-A2 restricted recognition of MP has been demonstrated (Lehner *et al.,* 1995) and it will be interesting if similar conservation occurs in the Class II restricted response to HA.

Table 10.1, Alignment of dominant HA peptides to reported MHC class II binding motifs

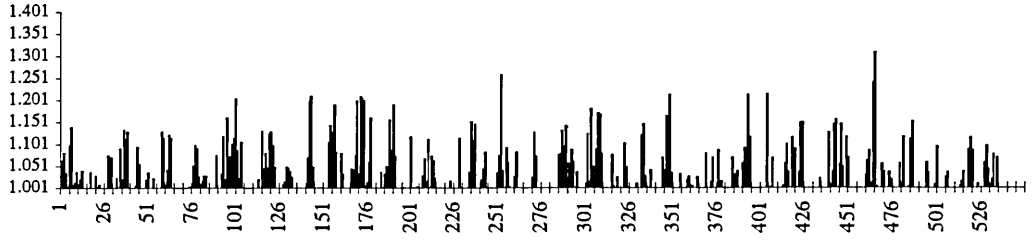
Allele	Identification of response	MHC class II Pockets																							
								P1			P4		P6		P9										
DRB1*0101		Consensus motif (Rammensee et al 1995).PS, SP, BS, TCE, PDL.						YV LF IA M W			LA IV M NQ		AG ST P		LA IV NF Y										
97 to 120*	HD, BA, RP							C	**Y**	P	Y	D	V	P	D	Y	**A**	S	L	R	S	L	V	A
97 to 120*	HD, BA, RP					C	Y	P	**Y**	D	V	P	D	Y	A	S	**L**	R	S	L	V	A	S	S
97 to 120*	HD, BA, RP	Y	P	Y	D	V	P	D	**Y**	A	S	**L**	R	**S**	L	V	**A**	S	S	G	T	L	E	F
192 to 207	BA, RP					T	S	L	**Y**	V	R	**A**	S	**G**	R	V	T	V	S	T	K				
303 to 318	HD, BA, RP			G	A	C	P	R	**Y**	V	K	Q	N	T	L	K	**L**	A	T						
417 to 432	HD, BA, RP				K	I	D	L	**W**	S	Y	N	A	E	L	L	V	A	L	E					
								P1			P4		P6		P9										
DRB1*0301		Consensus motif (Rammensee et al 1995). SP, TCE, IPS, BS						L I F M V			D		K R E Q N		Y L F										
100 to 115	RP, BA							Y	D	V	P	D	Y	A	S	**L**	R	S	L	V	A	S	S	
192 to 207	RP, BA				T	S	L	Y	**V**	R	A	S	G	**R**	V	T	**V**	S	T	K					
257 to 272	RP, BA	A	P	R	G	Y	F	K	**I**	R	N	G	K	S	S	I	**M**	R	S	D	A	P			
303 to 318	RP, BA			G	A	C	P	R	**Y**	V	K	Q	N	T	L	K	**L**	A	T						
407 to 422	RP, BA			Q	D	L	E	K	**Y**	V	E	**D**	T	**K**	I	D	**L**	W	S						
417 to 432	RP*, BA					K	I	D	**L**	W	S	Y	N	A	E	L	**L**	V	A	L	E				
								P1			P4		P6	P7	P9										
DRB1*0401		Consesus motif (Rammensee et al 1995). PDL, BS, IPS						FY W I LV M			FW IL VA DE no RK		NS TQ HR	Pol chg ali	Pol ali K										
20 to 40	RP, BA	V	P	N	G	T	L	**V**	K	T	**I**	T	N	**D**	Q	**I**	E	V	T	N	A	T			
192 to 207	RP, BA					T	S	L	**Y**	V	R	A	S	G	**R**	V	**T**	V	S	T	K				
303 to 318	RP, BA			G	A	C	P	R	**Y**	V	K	Q	N	**T**	L	K	**L**	A	T						
417 to 432	RP*, BA	K	I	D	L	W	S	**Y**	N	A	**E**	L	L	V	A	**L**	E								
								P1			P4		P6	P7	P9										
DRB1*0402		Consesus motif (Rammensee et al 1995). PDL.						VI LM			YF WI LM RN No DE		NQ ST K	RK HN QP rar- e D E	Pol ali H										
20 to 40	RP, BA	V	P	N	G	T	L	**V**	K	T	**I**	T	N	**D**	Q	**I**	E	V	T	N	A	T			
192 to 207	RP*, BA					T	S	L	**Y**	V	R	A	S	G	R	V	**T**	V	S	T	K				
303 to 318	RP*, BA			G	A	C	P	R	**Y**	V	K	Q	N	**T**	L	K	**L**	A	T						
417 to 432	RP*, BA					K	I	D	**L**	W	S	**Y**	N	A	**E**	L	**L**	V	A	**L**	E				
								P1			P4		P6		P9										
DRB1*0701		Motif derived from data in Table 9.2. P4, P6, P7 positions specificities appear to be preferences rather than absolute requirements						L I V W Y	H y d r o		P o l a r		S m a l l	P o l a r	H y d r o	V L I F M W									
192 to 207	RP, BA, SHM						T	S	**L**	Y	V	**R**	A	S	**G**	R	V	T	V	S	T	K			
232 to 247	RP, BA, SHM						W	T	**I**	V	K	P	**G**	D	**I**	L	**L**	I	N	S	T	G			
303 to 318	RP, BA, SHM			G	A	C	P	R	**Y**	V	K	Q	N	T	L	K	**L**	A	T						
417 to 432	RP, BA, SHM				K	I	D	L	**W**	S	Y	N	A	E	L	L	V	A	L	E					
								P1					P5												
DRB1*0801		Motif derived from IPS. (Chicz et al 1993).						F I L V Y					H K R												
173 to 188	RP*, BA	K	F	D	K	L	Y	**I**	W	G	V	**H**	H	P	S	T	D								
192 to 207	RP*, BA			T	S	L	Y	**V**	R	A	S	G	R	V	T	V	S	T	K						
228 to 247	RP*, BA			I	S	I	Y	W	T	I	V	**K**	P	G	D	I	L	L	I	N	S	T	G		
303 to 318	RP*, BA			G	A	C	P	R	**Y**	V	K	Q	N	T	L	K	**L**	A	T						

DRB1*1101		Consensus motif (Rammensee et al 1995). PDL, IPS							P1 W YF			P4 ML VI		P6 RK																	
97 to 120*	RP[a], BA							C	Y	P	Y	D	V	P	D	Y	A	S	L	R	S	L	V	F							
97 to 120*	RP[a], BA	Y	P	Y	D	V	P	D	Y	A	S	L	R	S	L	V	A	S	S	G	T	L	E								
192 to 207	RP[a], BA					T	S	L	Y	V	R	A	S	G	R	V	T	V	S	T	K										
303 to 318	RP[a], BA			G	A	C	P	R	Y	V	K	Q	N	T	L	K	L	A	T												
417 to 432	RP[a], BA				K	I	D	L	W	S	Y	N	A	E	L	L	V	A	L	E											
DRB1*1201		Consensus motif (Rammensee et al 1995). PS							P1 I L F Y V			P4 L M N V A		P6 V Y F I N A			P9 Y F M I V														
20 to 40	RP[d]	V	P	N	G	T	L	V	K	T	I	T	N	D	Q	I	E	V	T	N	A	T										
DRB1*1301		Motif from Davenport et al 1995. PS, BS.							P1 L I V			P5 P O S P O L					P9 Y														
198 to 213	RP, BA				A	S	G	R	V	T	V	S	T	K	R	S	Q	Q	T	V											
228 to 247	RP, BA	G		I	S	I	Y	W	T	I	V	K	P	G	D	I	L	L	I	N	S	T	G								
277 to 297	RP, BA	V	C	S	S	E	C	I	T	P	N	G	S	I	P	N	D	K	P	F	Q	N	V								
303 to 318	RP, BA		G	A	C	P	R	Y	V	K	Q	N	T	L	K	L	A	T													
442 to 492	RP, BA	M	S	E	M	N	K	L	F	E	K	T	R	K	Q	L	R	E	N	A	E	D	M								
DRB1*1302		Motif from Davenport et al 1995. PS, BS							P1 I L F Y					P6 Pos Pol			P9 Y														
303 to 318	HD, RP		G	A	C	P	R	Y	V	K	Q	N	T	L	K	L	A	T													
417 to 432	HD, RP				K	I	D	L	W	S	Y	N	A	E	L	L	V	A	L	E											
442 to 457	HD, RP	S	E	M	N	K	L	F	E	K	T	R	K	Q	L	E															
	HD, RP																														
DRB1*1501		Consensus motif (Rammensee et al 1995). SP, BS.							L V I			Q V I M					R K														
228 to 247	RP, SHM, BA							I	I	S	I	Y	W	T	I	V	K	P	G	D	I	L	L	I							
417 to 432	RP, SHM, BA						Q	D	L	E	K	Y	V	E	D	T	K	I	D	L	W	S									

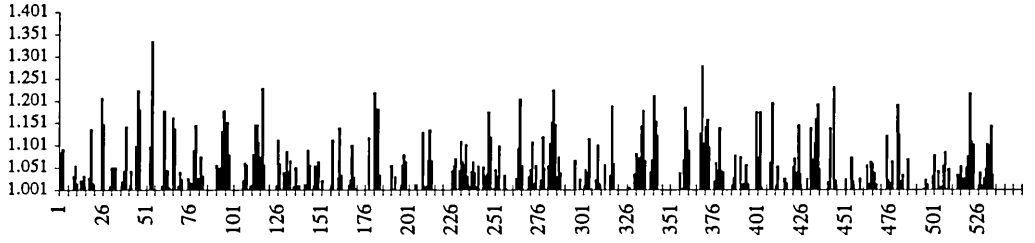
Table shows alignment of the HA peptides which were immunodominant in the context of individual DRB1 alleles. Source of motif indicated in outlined box: PS = pools sequencing of self peptides, IPS = individual peptide sequencing, BS = binding studies, TCE = T cell epitope, PDL = phage display library. Binding pocket specificities indicated. Preferences in bold type are strong. ali = preference for aliphatic residues, Hydo = preference for hydrophobic residues Pol= preference for polar residues, Pos = preference for positvely charged residues, small = preference for small residues. * indicates more than one possible alignment of peptide to putative motif.
Restriction of HA peptides determined as follows: RP = restriction determined by analysis of response patterns of more than one donor (see below), BA = MHC class II blocking antibodies suggest response is primarily HLA-DR or DRW restricted. SHM = HA response of donor who expressed only a single DRB1* allele examined. It was not always possible to prove response was restricted by an individual allele: [a] = response to this peptide may be DRB1*1501 restricted rather than allele indicated, [b] = response may be DRB1*0101 restricted, [c] = response may be DRB1*0103 restricted, [d] = response may be DRB1*0403 restricted

Figure 10.1, Predicted pattern of HA A/Beijing/32/92 Peptide Processing and Presentation by 4 DRB1* alleles

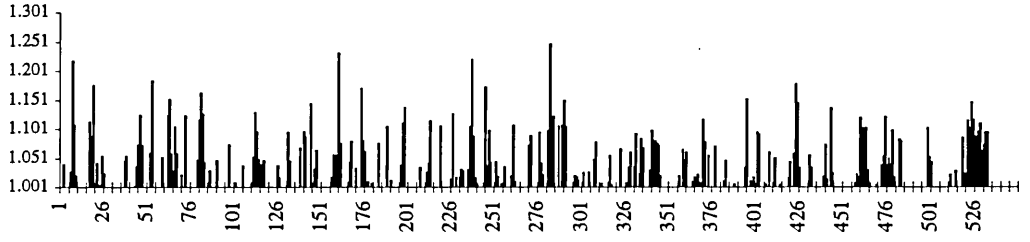
DRB1*0101



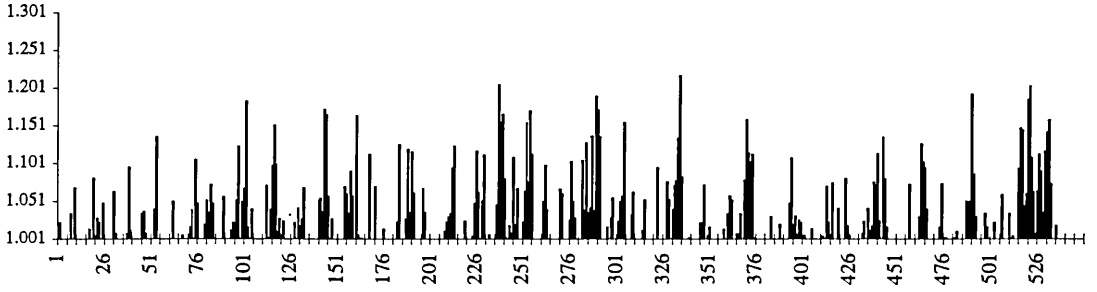
DRB1*0301



DRB1*1301



DRB1*1302



A computer analysis of HA peptide binding to 4 DRB1* alleles was performed using the method of Davenport et al (1995). X-axis represents HA A/Beijing/32/92 sequence. Y-axis shows PPP (Probability of being Processed and Presented), representing predicted probability that a 15mer peptide commencing at that position may be processed and presented on that DRB1* allele.

Appendix 1, Comparative H3N2 influenza A Haemagglutinin sequences

Comparative HA sequences of a panel of 14 H3N2 influenza A viruses, one H1 influenza A virus and one H2 influenza A virus. Sequence variations from HA1 subunit of Beijijng/32/92, and HA2 subunit of closely related A/Hong Kong/90 shown. * = absent residue.

Source of sequence information: A/BEIJING/92, personal communication N.Cox, (CDC, Atlanta, USA); A/MADRID/G252/93, A/ENGLAND/328/93, and A/ENGLAND/471/93 personal communication J.Ellis (PHLS, Colindale, London); A/SOUTH DAKOTA/01/91Entrez sequence number 422604; /SINGAPORE/12/89; Entrez sequence number 422598; A/MEMPHIS/6/86, Katz, and Webster 1988; A/LENNINGRAD/385/80 Migunova, *et al.,* 1990; A/BANKOK/79, Both, and Sleight 1981; A/ENGLAND/321/77, Hauptmann *et al.,* 1983; A/VICTORIA/3/75, Verhoeyen *et al.,* 1980; A/ENGLAND/72, Both *et al.,* 1983; A/MEMPHIS/1/71, Air 1981; A/AICHI/02/68, Verhoeyen *et al.,* 1980; A/Chile/157/83 (H1N1), Cox *et al.,* 1989; A/Singapore/1/57 (H2N2) Lin *et al.,* 1994..

Location of neutralising antibody combining sites of H3 influenza viruses

Site A: Protruding loop formed by amino acids 140-146; **Site B:** External residues 187-196 of an α helix and residues 155-60; **Site C:** A bulge in the tertiary structure at the disulphide bond between C 52 and C 277; **Site D:** A collection of substitution around the trimeric interface. Including residues 201, 205, 207, 217, 220, and also possibly residues 165, 174, 226, 242; **Site E:** Residues 122 and 126 (Wiley, *et al.,* 1981, Wharton, *et al.,* 1989).

HA sequences of a panel of human influenza A viruses

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
A/BEIJING/92	Q	K	L	P	G	N	D	N	S	T	A	T	L	C	L	G	H	H	A	V	P	N	G	T	L
A/MADRID/G252/93		K																							
A/ENGLAND/328/93		K																							
A/ENGLAND/471/93		K																							
A/DAKOTA/01/91		K																							
A/SINGAPORE/12/89		K																							
A/MEMPHIS/6/86		K											P												
A/LENNINGRAD/385/80		N																							
A/BANGKOK/1/79		N																							
A/ENGLAND/321/77		N														A									
A/VICTORIA/75		D																							
A/ENGLAND/72		D																							
A/MEMPHIS/1/71		Y																							
A/AICHI/02/68		D																							
A/Chile/157/83 (H1N1)		A		S	A	T	D	*	*	A	D		I		I		Y			N	N	S	T	D	T
A/SINGAPORE/1/57 (H2N2)	*	*	*	*	*	*	*	*	*	*	*	*	I		I		Y			N	N	S	T	E	K

	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
A/BEIJING/92	V	K	T	I	T	N	D	Q	I	E	V	T	N	A	T	E	L	V	Q	S	S	S	T	G	R
A/MADRID/G252/93																									
A/ENGLAND/328/93																									
A/ENGLAND/471/93																									
A/DAKOTA/01/91																									
A/SINGAPORE/12/89																									
A/MEMPHIS/6/86		E																							
A/LENNINGRAD/385/80										G						G									
A/BANGKOK/1/79																									
A/ENGLAND/321/77																									
A/VICTORIA/75																									K
A/ENGLAND/72																									K
A/MEMPHIS/1/71																									K
A/AICHI/02/68						D																			K
A/Chile/157/83 (H1N1)		D		V	L	E	K	N	V	T			H	S	V	N		L	E	D	N	H	N		K
A/Singapore/1/57 (H2N2)		D			L	E	R	N	V	T			H		K	D	I	L	E	K	T	H	N		
		{E}																							

	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
A/BEIJING/92	I	C	D	S	P	H	R	I	L	D	G	K	N	C	T	L	I	D	A	L	L	G	D	P	H
A/MADRID/G252/93																									
A/ENGLAND/328/93																									
A/ENGLAND/471/93																									N
A/DAKOTA/01/91																									
A/SINGAPORE/12/89																									
A/MEMPHIS/6/86																									
A/LENNINGRAD/385/80	L																								
A/BANGKOK/1/79																									
A/ENGLAND/321/77																									
A/VICTORIA/75			N	N								I	N												
A/ENGLAND/72			N	N								I	D												
A/MEMPHIS/1/71			N	N								I	D												
A/AICHI/02/68			N	N								I	D												
A/Chile/157/83 (H1N1)	L		K	K	G	I	A	P		Q	L	G	K		S	I	A	G	W	I		G	N		E
A/Singapore/1/57 (H2N2)	L		K	N	G	I	P	P		E	L	G	D		S	I	A	G	W			G	N		E

	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
A/BEIJING/92	C	D	G	F	Q	N	K	E	W	D	L	F	V	E	R	S	K	A	Y	S	N	C	Y	P	Y
A/MADRID/G252/93							K	E											Y						
A/ENGLAND/328/93							K	E											Y						
A/ENGLAND/471/93							K	E											Y						
A/DAKOTA/01/91							K	E											Y						
A/SINGAPORE/12/89							K	E											Y						
A/MEMPHIS/6/86							E	K											F						
A/LENNINGRAD/385/80							G	K						G					F						
A/BANGKOK/1/79							E	K											F						
A/ENGLAND/321/77							E	K											F						
A/VICTORIA/75							E	K											F						
A/ENGLAND/72							E	T											F						
A/MEMPHIS/1/71							E	T											F						
A/AICHI/02/68			V				E	T											F						
A/Chile/157/83 (H1N1)		ES	L		S		K	S		S	Y	I	A		T	P	N	SE	N	G	T			PG	
A/Singapore/1/57 (H2N2)		DR	L	L	S	V	P	E		S	Y	I	M		K	E	N	P	R	D	G				G
																						{E}			

	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125
A/BEIJING/92	D	V	P	D	Y	A	S	L	R	S	L	V	A	S	S	G	T	L	E	F	I	N	E	D	F
A/MADRID/G252/93																								D	
A/ENGLAND/328/93																								D	
A/ENGLAND/471/93																								D	
A/DAKOTA/01/91																								D	
A/SINGAPORE/12/89																								D	
A/MEMPHIS/6/86																								G	
A/LENNINGRAD/385/80																								E	
A/BANGKOK/1/79																								G	
A/ENGLAND/321/77																								G	
A/VICTORIA/75																								G	
A/ENGLAND/72																								G	
A/MEMPHIS/1/71																						T		G	
A/AICHI/02/68																						T		G	
A/Chile/157/83 (H1N1)	*	F	A			E	E			E	Q	L	S		V	S	S	F		FEI	F	P	K	E	S
A/Singapore/1/57 (H2N2)	S	F	N			E	E		K	H		L	S		V	K	H	F		I	L	P	K	D	*

	{E}														{A}	{A}	{A}	{A}	{A}	{A}	{A}					
	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
A/BEIJING/92	N	W	T	G	V	A	Q	D	G	G	S	Y	A	C	K	R	G	S	V	N	S	F	F	S	R
A/MADRID/G252/93						A		D		K										K					
A/ENGLAND/328/93						A		D		K										N					
A/ENGLAND/471/93						A		D												N					
A/DAKOTA/01/91						A		S		E										K					
A/SINGAPORE/12/89						A		S												N					
A/MEMPHIS/6/86						T		S												N					
A/LENNINGRAD/385/80						T		S											D	N					
A/BANGKOK/1/79						T		S											D	N					
A/ENGLAND/321/77						T		N										P	D	N					
A/VICTORIA/75						T		N				S						P	D	S	G				
A/ENGLAND/72	T					T		N				N						P	D	S	G				
A/MEMPHIS/1/71	T					T		N				N						P	G	S	G				
A/AICHI/02/68	T					T		N				N						P	G	S	G				
A/Chile/157/83 (H1N1)	S	W	P	HN	N	V	T	K		V	T	A	S		S	H	K	G	K	S	S		Y	R	N
A/Singapore/1/57 (H2N2)	R		Q	H	T	T	T					R			A	V	S	Q	N	P	S			R	N
					{B}	{B}	{B}	{B}	{B}	{B}					{D}										
	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175
A/BEIJING/92	L	N	W	L	H	K	S	E	Y	K	Y	P	A	L	N	V	T	M	P	N	N	G	K	F	D
A/MADRID/G252/93					H	K	L	E																	
A/ENGLAND/328/93					H	K	L	E																	
A/ENGLAND/471/93					H	K	L	E																	
A/DAKOTA/01/91					H	E	S	D																	
A/SINGAPORE/12/89					H	E	S	E																	
A/MEMPHIS/6/86					Y	E	S	E																	
A/LENNINGRAD/385/80					Y	E	S	E	S				V										N		
A/BANGKOK/1/79					Y	E	S	E	S				V										N		
A/ENGLAND/321/77					Y	E	S	E	S	T			V									D	N		
A/VICTORIA/75					Y	E	S	G	S	T			V	Q								D	N	S	
A/ENGLAND/72					Y	E	S	G	S	T			V									D	N		
A/MEMPHIS/1/71					T	E	S	E	S	T			V									D	N		
A/AICHI/02/68					T	E	S	G	S	T			V									D	N		
A/Chile/157/83 (H1N1)		L			T	E	K	N	G	S			N		S	K	S	Y	V			K	E	K	E
A/Singapore/1/57 (H2N2)	M	V			T		K	E	S	N			V	A	K	G	S	Y	N		T	S	G	E	Q
												{B}	{B}	{B}	{B}	{B}	{B}	{B}	{B}	{B}	{B}				
	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200
A/BEIJING/92	K	L	Y	I	W	G	V	H	H	P	S	T	D	R	D	Q	T	S	L	Y	V	R	A	S	G
A/MADRID/G252/93													D	S	D			S							
A/ENGLAND/328/93													D	S	D			S							
A/ENGLAND/471/93													D	S	D			S							
A/DAKOTA/01/91													D	R	E			N							
A/SINGAPORE/12/89											I		D	R	E			N							
A/MEMPHIS/6/86													E	K	E			N							
A/LENNINGRAD/385/80													D	K	E			N							
A/BANGKOK/1/79													D	K	E			N							
A/ENGLAND/321/77													D	K	E			K				Q			
A/VICTORIA/75													D	K	E			N				Q			
A/ENGLAND/72													N	Q	E			S				Q			
A/MEMPHIS/1/71													N	Q	E			S				Q			
A/AICHI/02/68							I						N	Q	E			S				Q			
A/Chile/157/83 (H1N1)	V		V	L			V					N	I	E	D		K	T	I		R	K	E	N	A
A/Singapore/1/57 (H2N2)	M		I				V				N	D	E	T	E		R	T			Q	N	V	G	T
	{D}						{D}										{D}								
	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225
A/BEIJING/92	R	V	T	V	S	T	K	R	S	Q	Q	T	V	T	P	N	I	G	S	R	P	W	V	R	G
A/MADRID/G252/93								K					V	I											
A/ENGLAND/328/93							R						V	I					F						
A/ENGLAND/471/93	K						R						V	T											
A/DAKOTA/01/91													V	I											
A/SINGAPORE/12/89													I	I											
A/MEMPHIS/6/86													V	I											
A/LENNINGRAD/385/80													V	I											
A/BANGKOK/1/79													I	I			V								
A/ENGLAND/321/77													I	I			V								
A/VICTORIA/75													I	I			V								
A/ENGLAND/72	K							G					I	I			V								
A/MEMPHIS/1/71							R						I	I											
A/AICHI/02/68							R						I	I											
A/Chile/157/83 (H1N1)	Y		S		V	S	S	H	Y	N	R	R	F	T		E		A	K			K			D
A/Singapore/1/57 (H2N2)	Y		S		G		S	T	L	N	K	R	S	T		D		A	T			K		N	
	{D}																								
	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250
A/BEIJING/92	Q	S	S	R	I	S	I	Y	W	T	I	V	K	P	G	D	I	L	L	I	N	S	T	G	N
A/MADRID/G252/93	Q																								
A/ENGLAND/328/93	L																								
A/ENGLAND/471/93	L																								
A/DAKOTA/01/91	L																								
A/SINGAPORE/12/89	L																								
A/MEMPHIS/6/86	L																								
A/LENNINGRAD/385/80	L																						N		
A/BANGKOK/1/79	L																						N		
A/ENGLAND/321/77	L																						N		
A/VICTORIA/75	L																		V				N		
A/ENGLAND/72	L																		V				N		
A/MEMPHIS/1/71	L																V		V				N	A	
A/AICHI/02/68	L																V		V				N		
A/Chile/157/83 (H1N1)	Q	E	G			N	Y				L	L	E				T	I	I	F	E	A	N		
A/Singapore/1/57 (H2N2)	L	G			M	E	F	S			L	L	D	M	W		T	I	N	F	E		T		

	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275
A/BEIJING/92	L	I	A	P	R	G	Y	F	K	I	R	N	G	K	S	S	I	M	R	S	D	A	P	I	G
A/MADRID/G252/93												N													
A/ENGLAND/328/93												N													
A/ENGLAND/471/93												N													
A/DAKOTA/01/91												T													
A/SINGAPORE/12/89												T													
A/MEMPHIS/6/86												T													
A/LENNINGRAD/385/80												T													
A/BANGKOK/1/79												T													
A/ENGLAND/321/77												T													
A/VICTORIA/75										M		T													
A/ENGLAND/72										M		T													
A/MEMPHIS/1/71										M		T													D
A/AICHI/02/68										M		T													D
A/Chile/157/83 (H1N1)					W	Y	A		A	L	S	R		G		G		I	T		N		S	M	D
A/Singapore/1/57 (H2N2)					*	E				I	K	R		S		G			K	T	E	G	T	L	E

	276	{C} 277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
A/BEIJING/92	T	C	S	S	E	C	I	T	P	N	G	S	I	P	N	D	K	P	F	Q	N	V	N	R	I
A/MADRID/G252/93	N																							R	
A/ENGLAND/328/93	N																							R	
A/ENGLAND/471/93																								R	
A/DAKOTA/01/91																								K	R
A/SINGAPORE/12/89																								K	R
A/MEMPHIS/6/86																								K	
A/LENNINGRAD/385/80																								K	
A/BANGKOK/1/79																								K	
A/ENGLAND/321/77																								K	
A/VICTORIA/75																								K	
A/ENGLAND/72			I																					K	
A/MEMPHIS/1/71			I																					K	
A/AICHI/02/68			I																					K	
A/Chile/157/83 (H1N1)	E		D	A	K		Q			Q		A		N	S	S	L						H	P	V
A/Singapore/1/57 (H2N2)	N		E	T	K		Q			L		A		N	T	T	L			H			H	P	L

	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325
A/BEIJING/92	T	Y	G	A	C	P	R	Y	V	K	Q	N	T	L	K	L	A	T	G	M	R	N	V	P	E
A/MADRID/G252/93																									
A/ENGLAND/328/93																									
A/ENGLAND/471/93																									
A/DAKOTA/01/91																									
A/SINGAPORE/12/89																									
A/MEMPHIS/6/86																									
A/LENNINGRAD/385/80					K		K					D													
A/BANGKOK/1/79							K																		
A/ENGLAND/321/77							K																		
A/VICTORIA/75							K																		
A/ENGLAND/72							K																		
A/MEMPHIS/1/71							K												R						
A/AICHI/02/68							K																		
A/Chile/157/83 (H1N1)		I		E			K			R	S	T	K		R	M	V			L			I		S
A/Singapore/1/57 (H2N2)		I		E							S	E	K		V					L					Q

	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350
A/BEIJING/92	K	Q	T	R	G	I	F	G	A	I	A	G	F	I	E	N	G	W	E	G	M	V	D	G	W
A/MEMPHIS/6/86																									
A/LENNINGRAD/385/80																									
A/BANGKOK/1/79																						I			
A/ENGLAND/321/77																						I			
A/VICTORIA/75																						I			
A/MEMPHIS/1/71						L																I			
A/AICHI/02/68						L																I			
A/Chile/157/83 (H1N1)	I		S			L										G			T			I	D		
A/Singapore/1/57 (H2N2)	I	E	S			L										G			Q			V	D		

	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375
A/HONG KONG/90	Y	G	F	R	H	Q	N	S	E	G	T	G	Q	A	A	D	L	K	S	T	Q	A	A	I	D
A/MEMPHIS/6/86																									
A/LENNINGRAD/385/80																									
A/BANGKOK/1/79																									
A/ENGLAND/321/77																									
A/VICTORIA/75																									
A/MEMPHIS/1/71																									
A/AICHI																									
A/Chile/157/83 (H1N1)			Y	H				E	Q		S		Y				Q					S			N
A/Singapore/1/57 (H2N2)			Y	H		S		D	Q		S		Y				K	E				K		F	

	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400
A/HONG KONG/90	Q	I	N	G	K	L	N	R	L	I	E	K	T	N	E	K	F	H	Q	I	E	K	E	F	S
A/MEMPHIS/6/86									L																
A/LENNINGRAD/385/80									V																
A/BANGKOK/1/79									V																
A/ENGLAND/321/77									V																
A/VICTORIA/75									V																
A/MEMPHIS/1/71									V																
A/AICHI									V																
A/Chile/157/83 (H1N1)	G		T	N		V		S	V				M		T	Q		T	A	V	G				N
A/Singapore/1/57 (H2N2)	G		T	N		V		S	V				M		T	Q		E	A	V	G				

	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425
A/HONG KONG/90	E	V	E	G	R	I	Q	D	L	E	K	Y	V	E	D	T	K	I	D	L	W	S	Y	N	A
A/MEMPHIS/6/86																									
A/LENNINGRAD/385/80																									
A/BANGKOK/1/79																									
A/ENGLAND/321/77																									
A/VICTORIA/75																									
A/MEMPHIS/1/71																									
A/AICHI																									
A/Chile/157/83 (H1N1)	K	L		K		M	E	N		E		K		D		G	F	L		I		T			
A/Singapore/1/57 (H2N2)	N	L		R		L	E	N		E		K	M			G	F	L		V		T			
	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450
A/HONG KONG/90	E	L	L	V	A	L	E	N	Q	H	T	I	D	L	T	D	S	E	M	N	K	L	F	E	K
A/MEMPHIS/6/86																									
A/LENNINGRAD/385/80																									
A/BANGKOK/1/79																									
A/ENGLAND/321/77																									
A/VICTORIA/75																									
A/MEMPHIS/1/71																									
A/AICHI																									
A/Chile/157/83 (H1N1)					L				E	R		L		F	H			N	V	K	N		Y		
A/Singapore/1/57 (H2N2)					L	M			E	R		L		F	H			N	V	K	N		Y	D	
	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475
A/HONG KONG/90	T	R	K	Q	L	R	E	N	A	E	D	M	G	N	G	C	F	K	I	Y	H	K	C	D	N
A/MEMPHIS/6/86			K																						
A/LENNINGRAD/385/80			R																						
A/BANGKOK/1/79																									
A/ENGLAND/321/77			R																						
A/VICTORIA/75			R																						
A/MEMPHIS/1/71			R																						
A/AICHI			R								E														
A/Chile/157/83 (H1N1)	V	K	S			K	N			K	E	I						E	F					N	
A/Singapore/1/57 (H2N2)	V		M				D		V	K	E	L						E	F						D
	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500
A/HONG KONG/90	A	C	I	G	S	I	R	N	G	T	Y	D	H	D	V	Y	R	D	E	A	L	N	N	R	F
A/MEMPHIS/6/86																									
A/LENNINGRAD/385/80								D																	
A/BANGKOK/1/79																									
A/ENGLAND/321/77																									
A/VICTORIA/75																									
A/MEMPHIS/1/71				E																					
A/AICHI				E																					
A/Chile/157/83 (H1N1)	E		M	E		V	K						Y	P	K		S	E		S	K	L			E
A/Singapore/1/57 (H2N2)	E		M	N		V	K						Y	P	K		E	E		S	K	L			N
	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525
A/HONG KONG/90	Q	I	K	G	V	E	L	K	S	G	Y	K	D	W	I	L	W	I	S	F	A	I	S	C	F
A/MEMPHIS/6/86																									
A/LENNINGRAD/385/80																									
A/BANGKOK/1/79																									
A/ENGLAND/321/77																									
A/VICTORIA/75																									
A/MEMPHIS/1/71																									
A/AICHI																									
A/Chile/157/83 (H1N1)	K		D			K		E		M	G	V	Y	Q			A		Y	S	T	V	A	S	S
A/Singapore/1/57 (H2N2)	E					K		S		M	G	V	Y	Q			A		Y	A	T	V	A	G	S
	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550
A/HONG KONG/90	L	L	C	V	V	L	L	G	F	I	M	W	A	C	Q	K	G	N	I	R	C	N	I	C	I
A/MEMPHIS/6/86																									
A/LENNINGRAD/385/80																									
A/BANGKOK/1/79																									
A/ENGLAND/321/77																									
A/VICTORIA/75																									
A/MEMPHIS/1/71																R									
A/AICHI																R									
A/Chile/157/83 (H1N1)		V	L	L		S			A		S	F	W	M	C	S	N	G	S	L	Q	C	R	I	C
A/Singapore/1/57 (H2N2)		S	L	A	I	M	M	A	G		S	F	W	M	C	S	N	G	S	L	Q	C	R	I	C

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