

HELPER T CELL EPITOPES OF HUMAN RHINOVIRUS

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by

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Abstract

Little is known about the cellular immune response generated towards human rhinovirus (HRV), the major causative agent of the common cold. There are over one hundred HRV serotypes which can be assigned to two groups, major and minor, depending on the cellular receptor they bind. Minor receptor group serotype 1A was selected for further study as the three dimensional structure of the virus is known. A HRV specific CD4+ helper T cell (Th) response was demonstrated in mice immunised with HRV. The HRV 1A specific Th cells responded in vitro to eight other HRV serotypes. This heterotypic response occurred in three different haplotypes. However, serotypes belonging to the two receptor groups gave different response profiles, and this was attributed to the demonstration that minor, but not major group viruses bound to cells of the murine immune system. The specific protein regions involved in the generation of Th cell responses were identified using synthetic peptides representing the entire sequences of the four structural proteins of HRV 1A. Only a small number of peptides were identified as stimulating a positive response in T cells primed towards intact virus and the repertoire of the Th response was different in each MHC haplotype tested. When the peptides identified as positive were used as immunogens, they elicited Th cell responses towards peptide, HRV 1A and heterotypic serotypes. These peptides were also able to provide help for virus specific antibody responses and to provide T cell help for antibody production towards a non-virus B cell epitope. Finally, the location of the Th cell epitopes in relation to the known three dimensional structure of the virus was examined.

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List of Abbreviations

2ME	2-mercaptoethanol
³ HT	[³ H]thymidine
APC	antigen presenting cell
BSA	bovine serum albumin
Con A	concanavalin A
CPE	cytopathic effect
CPMV	cowpea mosaic virus
CTL	cytotoxic lymphocyte
E64	trans-Epoxy succinyl-L-leucylamido-(4-guanidino) butane
EDTA	ethylenediaminetetraacetic acid
FCA	Freunds complete adjuvant
FCS	foetal calf serum
FIA	Freunds incomplete adjuvant
FMDV	foot-and-mouth disease virus
HA	haemagglutinin
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HIV	human immunodeficiency virus
HLA	human lymphocyte antigen
HPV	human papilloma virus
HRV	human rhinovirus
ICAM-1	intracellular adhesion molecule 1
kD	kiloDaltons

LPS	lipopolysaccharide
MEM	minimal essential medium
MHC	major histocompatibility complex
MS	mouse serum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIm	neutralising immunogen
NP	nucleoprotein
O.D.	optical density
OVA	ovalbumin
pIgG	purified IgG
PBMC	peripheral blood mononuclear cells
PMNL	polymorphonuclear leukocytes
PPD	purified protein derivative
SBMV	southern bean mosaic virus
SWM	sperm whale myoglobin
Th	helper T cell
TCR	T cell receptor
TMEV	Thieler's murine encephalomyelitis virus
TNP	trinitrophenyl

1.1. Human Rhinovirus (HRV)

Isolation and characterisation of human rhinovirus

Human rhinoviruses are the major causative agents of the common cold (Stott and Killington, 1972), and are probably the most common of acute infections in humans (Gwaltney, 1975). Long term studies of experimental common cold infections in volunteers, reviewed by Tyrrell (1968), lead to the isolation of virus which could be passaged in human embryonic lung tissue. This virus was demonstrated to be a rhinovirus (Andrewes, 1953). Two identical isolates of a virus named ECHO virus type 28 were identified from different sources (Pelon et al., 1957; Price, 1956) and later renamed human rhinovirus serotype 1A (HRV 1A). By reducing the incubation temperature of cells infected with isolated virus from 37°C to 33°C, Tyrrell and Parsons (1960) demonstrated that cytopathic effects occurred with a number of "common cold" virus isolates. Advances in tissue culture techniques and the development of human cell lines have facilitated the isolation and propagation of rhinoviruses (Hayflick and Moorehead, 1961; Conant and Hamparian, 1968) and enabled further characterisation of the viruses.

HRV was identified as a member of the picornavirus family (Tyrrell and Channock, 1963). Picornaviruses are small, non-enveloped RNA viruses which are ether stable. The picornavirus family also includes enteroviruses, e.g. poliovirus and

coxsackie viruses, aphthovirus (foot-and-mouth disease virus), cardiovirus e.g. mengovirus and encephalomyocarditis virus (EMC), and hepatitis A. All picornaviruses have been shown to have a similar morphology by electron microscopy. Rhinovirus is the largest group with over 100 serotypes of human rhinovirus and 2 serotypes of bovine rhinovirus. The picornavirus capsid is made up of multiple copies of four structural proteins and contains a single strand of positive sense RNA. The RNA codes for a large poly-protein (2178 amino acids in HRV 14) which is cleaved during translation by three virus encoded proteinases to eventually generate the structural proteins and proteins involved in virus replication and assembly. The structural proteins VP1, VP3, and VP0 are assembled to form the capsid at which time the RNA is packaged and the maturation cleavage of VP0 into VP2 and VP4 takes place.

The molecular structure of HRV

Picornavirus particles exhibit an icosahedral structure and display a 5:3:2 symmetry, having five-fold, three-fold and two-fold axes of symmetry brought about by the regular ordering of identical structural subunits (Crick and Watson, 1956). The HRV capsid is made up of 60 copies each of VP1 and VP3, 58 copies of VP2 and VP4, and 2 copies of VP0. The 20 sides of the capsid consist of one copy of each of the structural proteins with two faces containing VP0 instead of

VP2 and VP4. Viral proteins 1, 2, and 3 are exposed at the surface of the capsid whereas VP4 is located internally (Mendappa et al., 1971; Ruekert, 1976). Viral protein 4 of rhinoviruses is myristylated, as it is in other picornaviruses (Chow et al., 1987).

A major advance in the study of virus structure came with the first crystallisation of a complete animal virus, HRV 14 (Rossmann et al., 1985), to the resolution of single amino acids. Combining the X-ray diffraction patterns from the virus crystals with the protein sequence of the virus (Stanway et al., 1984) enabled a three dimensional model to be generated. The elucidation of this atomic structure was followed by the 3-dimensional structures of poliovirus (Hogle et al., 1985), FMDV (Acharya et al., 1989), mengovirus (Luo et al., 1987) and HRV 1A (Kim et al., 1990). The structure of the three external picornavirus proteins was found to be similar to that of icosahedral plant viruses such as southern bean mosaic virus (SBMV) (Abad-Zapatero et al., 1980), having an eight stranded anti-parallel beta barrel structure (Rossmann et al., 1987: figure 1.1.). The beta sheets forming the core structure of the proteins are joined by protein loops. Many of these loops protrude from the surface of the virion and are involved in formation of the antigenic determinants of the virus. Structural analysis of HRV 14 revealed a cleft or "canyon" running around the five fold axis of symmetry where five VP1 molecules join (Rossmann et al., 1985). This cleft was also present in another rhinovirus serotype, HRV 1A (Kim et al.,

1989). A comparison of the protein sequences of seven rhinovirus serotypes showed little variation within this site (Palmenberg, 1989) which has been proposed as the receptor attachment site for rhinoviruses (Rossmann and Palmenberg 1988). A series of antiviral compounds developed by Sterling Winthrop (WIN compounds) were found to inhibit viral uncoating and therefore replication. These drugs bind to a hydrophobic pocket which exists beneath the floor of the canyon (Smith et al., 1986) and stabilise the virion. Attachment of HRV 14 to cells was also inhibited by the compounds due to conformational changes in the canyon (Pevear et al., 1989) but binding of HRV 1A and HRV 2 remained unaffected.

Cellular receptors for HRV

Rhinoviruses are able to infect cells by attachment to specific receptors on the cell surface. Attachment of HRV to cellular receptors was found to be temperature dependant, and for certain serotypes, adsorption was enhanced by the inclusion of magnesium chloride in the incubation media (Stott and Killington, 1972). The rates of attachment of two rhinovirus serotypes, 2 and 14, varied considerably although the number of receptors per HeLa cell for the two serotypes was estimated to be similar, at 1×10^4 receptors per cell. Using competition assays to investigate the ability of different virus serotypes to inhibit virus attachment to HeLa cells, Lonberg-Holm and Korant (1972) also demonstrated that

HRV 2 and 1A utilised a different receptor to that used by HRV 14 and 51. These results were confirmed by Abraham and Colonna (1984) in a study using 24 HRV serotypes, 20 of which bound to one receptor, and 4 which bound to a different receptor. The first group of virus serotypes has been termed the major receptor group, and the second, smaller group, the minor receptor group. Further studies have characterised the receptor groups of 102 recognised HRV serotypes (Colonna et al., 1986) to give 91 serotypes belonging to the major receptor group and 10 serotypes in the minor receptor group. One serotype, HRV 87, did not bind to either of the HRV receptors but bound instead to sialic acid (Uncapher et al., 1991).

The major group receptor has been identified as the intracellular adhesion molecule ICAM-1 (CD54), a member of the immunoglobulin superfamily which has five immunoglobulin like domains (Staunton et al., 1988). Monoclonal antibodies (mabs) directed against HeLa cell surface components known to block the attachment of major group viruses were used to identify a 95kD cell surface glycoprotein which showed sequence homology with ICAM-1 (Greve et al., 1989). Staunton et al. (1989) used mabs against ICAM-1 to block the binding of major group virus HRV 14 to the molecule. Soluble ICAM-1 has also been shown to inhibit major virus group infection of cells (Marlin et al., 1990). The minor group receptor is as yet uncharacterised, but has been identified as a protein with an apparent molecular weight of 450kD (Mishak et al., 1988), made up of 120kD

subunits (Uncapher et al., 1991), giving the receptor a similar size to that of ICAM-1, the major human rhinovirus receptor.

Human rhinovirus exhibits a narrow host range, replicating only in human and higher primate cells. The existence of two receptors for HRV serotypes is reflected in the ability of viruses to attach to cells from different species. Cell binding studies showed that the major group virus HRV 15 bound only to human cell lines and chimpanzee liver cells (Colonno et al., 1986), agreeing with an earlier observation that virus replication occurred in chimpanzees (Dick, 1968). Major group viruses bind only to human ICAM-1 and not to mouse ICAM molecules (Staunton et al., 1989). Minor receptor group viruses are able to bind to cells from numerous species including mouse, hamster, insect, and human (Uncapher et al., 1991). Although minor receptor group viruses bind to mouse cells, replication does not normally occur (Yin and Lomax, 1983). However, an infectious variant of HRV 2 has been derived which is capable of replication in mice (Yin and Lomax 1986).

Antibody responses to HRV

Initial studies of virus infectivity, using nasal washings containing undefined viruses to infect human volunteers, revealed that volunteers infected with one sample were susceptible to infection by other samples but not to

reinfection with the original sample (Jackson et al., 1962). A role for antibody involvement in protection was demonstrated by Tyrrell et al. (1960) using pooled human immune globulin to neutralise virus present in nasal washes. The presence of specific HRV neutralising antibodies was shown to prevent infection in humans by that HRV serotype (Bynoe et al., 1961), as was a correlation between the antibody activity and resistance to infection. These findings lead to vaccination studies in which volunteers were immunised with various serotypes of HRV (Mufson et al., 1963; Doggett et al., 1963). Protection against infection induced by immunisation with one serotype was shown to be serotype specific (Doggett, 1965) and the antibodies produced to that virus were shown to be long lived.

HRV exhibits a large degree of antigenic variation, with over one hundred virus serotypes identified (Hamparian et al., 1987). Virus serotypes can be distinguished in virus neutralisation assays with serotype specific antibodies. The ability of virus specific antibodies to cross-neutralise other virus serotypes was studied using 90 serotypes of rhinovirus (Cooney et al., 1982). Serotypes were divided into groups on the basis of their neutralisation by heterotypic antisera. Most antisera exhibited little or no cross reactivity with heterologous virus, although three out of sixteen groups contained five or more viruses. Thus cross neutralisation by specific antibodies can occur but is not broadly cross reactive with the full range of HRV serotypes. Antibodies from

humans infected with two serotypes of HRV have been shown to react with both of the viruses (Mogabgab et al., 1975). Serological studies of virus neutralisation provide little information about the precise antigenic determinants of the virus to which the antibodies are directed. Monoclonal antibodies have been widely employed to investigate neutralisation epitopes in a number of picornaviruses by isolating virus escape mutants selected by specific antibodies and then analyzing the sequence changes in that region of the virus. These changes can also be transposed onto the structural models. Studies with poliovirus type 3 (Minor et al., 1983) revealed that the majority of antibodies were directed to a single site in the VP1 protein. Similar experiments with HRV 14 identified four major antigenic sites, referred to as neutralising immunogen (Nim) sites IA, IB, II, and III (Sherry and Ruekert, 1985; Sherry et al., 1986). Epitope mapping studies with HRV 2 revealed three antigenic sites. The positions of two of these corresponded to HRV 14 sites Nim IA and Nim IB, but the third showed no similarity to the other HRV 14 sites (Appleyard et al., 1990). The antibody binding sites identified for both viruses occur at hypervariable regions and at the most surface exposed areas of the structures. They were principally conformational determinants although one HRV 2 site, made up from VP1, VP2 and one VP3 residue, contained a stretch of linear amino acid sequence. A synthetic peptide representing the VP2 contiguous sequence has been shown to raise neutralising antibodies in

animals (Francis et al., 1987a), demonstrating its ability to act as a neutralisation epitope. The peptide also bound a significant proportion of antibodies in serum from immunised animals and infected humans (Hastings et al., 1990).

Cellular immune responses to HRV

Although the host response to HRV infection has been studied extensively at the level of antibody induced immunity, the role of other arms of the immune response has only recently begun to be investigated. In humans HRV causes a rapid lytic infection of the cells lining the nasal epithelium (Bynoe et al., 1961) and is often associated with loss of ciliated cells (Turner et al., 1982). Studies of the cellular immune response to HRV infection have revealed that it probably plays a role in producing the symptoms of the common cold. Turner (1988) isolated polymorphonuclear leucocytes (PMNL) from the nasal mucosa during the early stages of rhinovirus infection and these may contribute to the inflammatory responses seen at the onset of infection. A detailed study of the systemic cellular immune response to HRV infection demonstrated a role for both non-specific and specific immune activation (Hsia et al., 1990). Non-specific responses included the production of interleukin 2 (IL-2) and interferon (IFN) by peripheral blood mononuclear cells (PBMC) from infected individuals, and natural killer cell (NK) cytolytic activity. Low level production of interferon both in

vitro and in vivo has been demonstrated (Stoker et al., 1973; Fiala et al., 1972) was dependant upon the serotype of virus used. Specific activation of lymphocytes was observed in volunteers infected with human rhinovirus (Lewandowski, 1983) and was shown to correlate with clinical signs of HRV infection, such as mucus production and virus shedding (Hsia et al., 1990). During the course of HRV infection the number of circulating CD4 positive T-lymphocytes in the peripheral blood decreases due to migration of the cells into infected tissues, where they may provide in situ immune protection (Lewandowski et al., 1986). However, the exact mechanisms by which this cellular protection may occur are as yet unknown. To date there have been no reports of cytotoxic responses to human rhinovirus mounted by HRV specific cytotoxic T lymphocytes.

1.2. The role of T cells in the generation of immune responses to antigens

T cells play an important role in the generation of immune responses. Both CD4+ and CD8+ cells act in an antigen specific manner to modulate the immune response to both self and foreign antigens. CD4+ helper T cells (Th cells) are involved in the generation of both CD8+ cell mediated cytotoxic responses and in the generation of antigen specific T cell dependant antibody responses by B cells. Therefore Th cells are central to the generation of antigen specific immune

responses by both B and T cells. This role, together with the molecular basis of T cell recognition of antigens and the relationship between B and T cell epitopes in providing T cell help, particularly in the context of vaccine design, is discussed in this chapter.

T cell help

The generation of specific antibody responses to the majority of soluble antigens was shown to be thymus dependent (Gell and Benacerraff, 1959). When immunised with T cell dependant antigens, athymic mice failed to produce a significant antibody response. B cells therefore required help from T cells in order to proliferate and differentiate in response to protein antigens. T cell help was demonstrated experimentally by transferring spleen cells from mice immunised with haptened protein to recipient irradiated mice and measuring the secondary antibody responses towards the hapten. Antibody responses in the recipient mice were much higher when the carrier protein used for the secondary immunisation was the same as that used to prime the response to hapten in the donor mice, thus demonstrating that the helper T cell (Th) response was specific to the carrier molecule (Mitchison, 1971). Both T and B cells could be primed independently using carrier/hapten to prime B cells and a different protein to prime T cells, but a secondary immune response in recipient mice was seen only when the hapten was

covalently linked to the carrier to which the T cells had been primed. This finding suggested that the epitopes recognised by T and B cells needed to be linked for T cell help for specific antibody production to occur, although "bystander" B cell activation can be demonstrated by Th cells primed against a different protein antigen at high antigen concentrations.

T cells recognise processed protein fragments in conjunction with class II molecules on the surface of an antigen presenting cell (APC) (Schwartz, 1985). The molecular details of this interaction are discussed below but the requirement for processing and presentation presented difficulties for the simple "antigen-bridge" model of T/B interaction. However, the molecular basis of the phenomenon of T/B cell linkage is now understood in terms of B cells acting as antigen presenting cells capturing antigen via their surface immunoglobulin receptors (Chesnut and Grey, 1981). The interaction of Th cells with antigen presenting B cells was dissected by Lanzavecchia (1985) using tetanus toxoid (TT) specific B cell clones to present native and denatured tetanus toxoid to TT specific T cell clones. Presentation of TT by antigen specific B cells was shown to be highly efficient, with presentation of native antigen occurring at much lower antigen concentrations than were required for the denatured antigen, which did not bind antibody at the surface of the B cells. Enhanced uptake, processing and presentation of proteins by B cells therefore explains the concept of linked recognition of T and B cell epitopes within the same protein

antigen, with B cells recognising the native protein and T cells recognising epitopes resulting from processing of the protein by the B cell.

The close interaction of B and T cells appears to be involved in providing the necessary signal(s) for T and B cell activation. The proximity of the cells may promote the action of cytokines secreted by the activated T cell to provide localised activation of the B cells. Interaction of the T and B cells may also enable activation to occur through signalling interactions between the surface molecules of the cells (Noelle et al., 1989). Interactions between the cell surface molecules of T and B cells such as LFA/ICAM (Springer et al., 1987), CD4/class II (Doyle and Strominger, 1987) as well as recognition of processed antigen in conjunction with MHC class II by the T cell receptor (TCR), act both to bring the cells together, and to maintain this interaction. This interaction may then play a role in signal transduction in the responder B cell.

T and B cell epitopes

Protein antigens are recognised both by antibodies produced by B cells, and by T cells. However, different forms of the protein antigen are recognised by the different cells. The epitopes on native proteins recognised by antibodies are normally located on the surface of the molecule. The majority of these determinants are discontinuous, made up from amino

acids near to each other in the tertiary structure of the protein but not sequential in the primary protein structure (Benjamin et al., 1984). It is difficult to reproduce these B cell determinants synthetically using linear peptides. However there are antigenic determinants which are made up of, or contain, sequential amino acids and these can be synthesised as linear peptides and used to produce antibodies which recognise determinants on the native protein. Examples of such epitopes can be found in viral proteins from foot-and-mouth disease virus (FMDV) (Bittle et al., 1982), poliovirus (Heraud et al., 1987), hepatitis B (Neurath et al., 1984), influenza virus (Green et al., 1982), HRV (Francis et al., 1987) and many other proteins (reviewed by Millich, 1989). Antibodies raised towards peptides may recognise different epitopes to those recognised by antibodies elicited by the native protein (Sutcliffe et al., 1980, Lerner, 1982). A synthetic peptide B-cell epitope must be able to induce neutralising antibodies if it is to be effective as a vaccine.

In contrast, T cells were shown to recognise a denatured form of protein antigens using chemical or proteolytic cleavage fragments of protein antigens such as insulin (Barcinski and Rosenthal, 1978). The molecular basis for this difference between T and B cell epitopes lies in the nature of T cell recognition, which requires the interaction of T cell receptor (TCR) with antigen bound to MHC (see below). T cells elicited using native proteins respond equally well when challenged with denatured protein fragments as they do to the

native antigen. This has been demonstrated for a number of proteins including myoglobin (Berzofsky et al., 1979), lysozyme (Maizels et al., 1980), cytochrome c (Solinger et al., 1979), and ovalbumin (OVA) (Shimonkevitz et al., 1984). The OVA specific T-cells recognised a short (17 amino acid) tryptic peptide and synthetic peptides based upon this sequence were used to define the epitope involved in the T-cell recognition of antigen (Watts et al., 1985). Synthetic peptides have since been widely used to substitute for denatured protein fragments in the investigation of immune responses to a large number of proteins including cytochrome c (Schwartz et al., 1985), lysozyme (Allen et al., 1984), and myoglobin (Berkower et al., 1986) thus enabling the identification and characterisation of the major T-cell determinants present within these proteins (reviewed by Allen et al., 1987; Millich, 1989).

Algorithms to predict the site of T cell epitopes within protein molecules from the primary sequences were designed based on common features in the primary and secondary structures of the known T cell epitopes (DeLisi and Berzofsky, 1985; Rothbard, 1986). However, although predictive methods for T cell epitopes circumvented the need for multiple peptide syntheses and screening procedures, they did not necessarily identify all the relevant Th epitopes of a protein. They also provide no information as to whether the identified epitopes are functional, in the sense of being generated from and reactive with the native (viral) protein. To fully investigate

the Th cell immunogenicity of a protein the epitopes still have to be defined experimentally. Synthetic peptides have been used to identify the Th cell epitopes involved in responses to native virus and viral proteins for influenza nucleoprotein (NP) (Brett et al., 1991), HPV E7 protein (Cromerford et al., 1991) and the structural proteins of human rhinovirus (Hastings et al., 1992). Helper T cell epitopes from the proteins of other pathogens such as the 65kD protein of Mycobacterium tuberculosis (Lamb et al., 1987) and the malarial parasite Plasmodium falciparum (Good et al., 1987) have also been identified using synthetic peptides.

Structure of MHC antigens

T cells recognise peptide bound to MHC molecules and recent evidence has allowed the molecular basis of this interaction to be better understood (see below). The interaction of the T cell and the antigen presenting cell results from the formation of a trimolecular complex between the APCs MHC molecule, peptide, and the antigen specific T cell receptor.

The antigen specific T cell receptors (TCR) are heterodimers composed of two chains arranged in a similar manner to those of an immunoglobulin molecule. In adults the TCR on peripheral T lymphocytes is predominantly made up of alpha and beta chains, although different isoforms (the gamma/delta TCRs) are found during ontogeny and in a number of

specific anatomical locations in the adult. The α/β TCR is responsible for MHC restricted antigen recognition (Dembic et al., 1986). The α/β chains have sequence homology with immunoglobulin V, D, and J regions and the diversity of the receptors is generated by gene rearrangements (reviewed by Davis and Bjorkman 1988). The TCR is also non-covalently associated with molecules of the CD3 complex involved in cell signal transduction (Clevers et al., 1988).

The genes of the major histocompatibility complex code for the highly polymorphic MHC antigens, known as the HLA antigens in humans and H-2 antigens in mice (Flavell et al. 1986). Class I antigens are made up of a single glycosylated heavy chain of approximately 45kD which folds to give three globular domains α_1 , α_2 , and α_3 . The α_3 domain lies closest to the cell membrane and forms a non covalent association with β_2 microglobulin (Hood et al. 1983). Class II antigens are made up of two polypeptide chains, α and β , each forming two domains. Both class I and class II MHC antigens are members of the immunoglobulin superfamily (Williams and Barclay, 1988). Crystallographic studies on the human class I MHC antigen HLA-A2 enabled the three dimensional structure of the molecule to be elucidated (Bjorkman et al., 1987). The data showed the presence of a groove between the upper two domains with 8 anti-parallel B strands forming the floor of the cleft and two α -helices, one from each domain, forming the opening to the groove at the top of the molecule. This groove acts as the binding site for the peptides that result from processing of

protein antigens.

A key factor in understanding how T cells recognise antigen has been to understand how a limited set of MHC molecules can bind a wide enough variety of peptides to allow an immune response to most protein antigens. MHC molecules are highly polymorphic, and this polymorphism may allow different MHC molecules to bind different sets of peptides.

Structural characteristics of class I peptide binding

Direct evidence for the existence of a peptide binding site on class I molecules came from the finding that electron dense material, presumed to be peptides bound by the MHC molecules, was present in the groove of the crystallised HLA-A2 molecule (Bjorkman et al., 1987). Electron dense material was also seen in the crystal structure of HLA-Aw68 molecule (Garrett et al., 1989), but this differed from that found in the HLA-A2 molecules, although the precise nature of the bound material could not be resolved. A comparison between the structures of the two HLA molecules revealed that, although the molecules had a similar overall structure in the region forming the peptide binding site, HLA-Aw68 exhibited a negatively charged pocket extending from the peptide binding cleft which was thought to be involved in binding specific amino acid side chains of the peptide. The crystal structure of HLA-B27 gave a clearer pattern of electron density in the peptide binding groove which enabled the consensus structure

of the multiple bound nonomeric peptides to be determined (Madden et al., 1991). Peptide was shown to exhibit an extended conformation when bound in the groove with the side chains of residues at positions 2, 3, 7 and 9 bound by pockets at the base and sides of the binding groove. Amino acids at positions 4 and 8 pointed upwards from the MHC molecule and would therefore be able to interact with the T cell receptor. Residues at positions 5 and 6 spanned the width of the groove interacting with the α helixes, and could also be exposed for interaction with TCR, as could a suitable amino acid side chain at position 1. This pattern of peptide binding may be similar for other MHC class I molecules since they have been shown to exhibit a number of similar features, in particular the presence of binding pockets (Saper et al., 1991).

The binding of peptide by class I MHC molecules is essential for the stability of the complex (Townsend et al., 1990; Ljunggren et al., 1990). Isolated class I molecules have been shown to contain peptides derived from self antigens (Jardetsky et al., 1991) and, from cells infected with virus, virally derived peptides corresponding to CTL epitopes (Rotzschke et al., 1990). Peptides eluted from cells or from isolated MHC class I molecules were shown to be nonomers (Falk et al., 1991; Schumacher et al., 1991), in agreement with the structural analysis of bound peptides (Madden et al., 1991). A study of the amino acid sequences of naturally processed viral nonapeptides revealed that the epitopes bound by K^d MHC molecules all contained tyrosine as the second residue (Falk

et al., 1990) which contributed to a conserved binding motif for that MHC allele (Falk et al., 1991). Eleven peptides were isolated by elution from purified HLA-B27 molecules and the sequences of seven of the peptides were identified as being derived from intracellular proteins. Sequence alignment of these eleven peptides and viral peptides known to be recognised by HLA-B27 revealed a binding motif which showed that arginine was highly conserved at position 2, which could reflect the narrowness and depth of the binding pocket of HLA-B27 thought to bind this residue (Madden et al., 1991). Murine K^d class I molecules also recognised a conserved residue at this position, which suggests that the binding of peptides to mouse class I molecules occurs in a similar manner. The ability of the other binding pockets to accommodate amino acid changes at positions 3, 7, and 9, and for the other peptide residues to have variable amino acids, indicates that HLA-B27 would have the capacity to bind many different peptide sequences.

Structural characteristics of class II peptide binding

There is no crystallographic structural data available for MHC class II molecules but the strong sequence similarities between the upper $\alpha 1$ and $\beta 1$ domains of class II molecules and the class I $\alpha 1$ and $\alpha 2$ domains have allowed sequence alignments between the two types of molecule which indicate that class II molecules probably have similar

molecular structures (Brown et al. 1988).

Synthetic peptides can bind directly to isolated MHC class II molecules and are recognised by antigen specific T cells (Babbit et al., 1985). The structural characteristics necessary for peptides to act as antigens have been investigated in an attempt to identify the criteria required for MHC binding. Studies using truncated peptides showed that a core of 5-7 amino acids was required for binding to mouse (Sette et al., 1987) and human class II antigens (Rothbard et al., 1988), although longer peptides were often more potent in their ability to stimulate Th cells (Cease et al., 1986). Recently, peptides arising from intracellular processing of proteins have been eluted from class II molecules and these have been shown to be between 13 and 17 amino acids long (Rudensky et al., 1991).

Once peptide has bound to an MHC molecule, parts of it must be exposed to enable interaction with the T-cell receptor. Experiments using pigeon cytochrome c peptides and analogues showed that certain amino acids were involved in class II MHC interaction whilst others were involved solely in TCR recognition (Fox et al. 1987). A model for peptide binding was proposed in which peptide bound to the MHC in an extended conformation, as is seen in extended β strands, via key amino acids while leaving others free to interact with the TCR (Sette et al., 1987). Other experiments to determine the residues contacting MHC molecules indicated that peptide bound to MHC in a helical conformation (Allen et al., 1987). The

critical contact residues for six peptides derived from different protein sequences, three of which were known to interact with HLA-DR1Dw1 and three which bound other human MHC class II antigens were studied (Hill et al., 1991). Although the peptides adopted unique conformations, they were all bound by HLA-DR1Dw1 molecules. However, there was an element of similarity between the peptides in that they all contained at least one turn of an α helix, allowing residues at similar positions to be exposed for TCR recognition, and all had a bulky hydrophobic side chain residue near to the N terminus of the peptide. Binding studies with the influenza HA peptide 307-319, and analogues containing single amino acid substitutions, showed a requirement for a hydrophobic residue at position 309, and that when all residues of the original peptide, bar the tyrosine and a lysine, were substituted with alanine, the peptide was still able to bind (Jardetsky et al., 1990). On the basis of these observations and the ability of some peptides to bind to both class I and class II molecules (Perkins et al., 1989), Hill et al., (1991) suggested that the N terminus of class II binding peptides could interact with pockets in the peptide binding site of the class II MHC, in a similar manner to that employed by class I molecules.

The observation that class II molecules exhibit minimal structural constraints for peptide binding (Jardetsky et al., 1990) was also indicated by the lack of sequence similarities between naturally processed peptides eluted from class II molecules (Rudensky et al., 1991). These "natural" class II

peptides appeared to be distinct from class I peptides, since class I peptides were all 8 or 9 amino acids in length whereas the minimum length for class II peptides was 13 amino acids, implying that longer peptides were bound preferentially by class II MHC. The sequences of the eluted peptides revealed that only a small number of different peptides were bound and that these peptides were derived from intracellular proteins, exogenous proteins and a murine retrovirus, murine leukaemia virus (MuLV). Also, the different MHC molecules I-A^b and I-E^b, recognised distinct sets of peptides, binding different MuLV envelope derived peptides. Peptides varied in length only at their carboxyl termini, while the N terminus of a particular peptide was always the same. Therefore, the binding site of class II MHC could be a groove which is closed at one end, where the N terminus of the peptide binds, and open at the other end, allowing the C terminus of the bound peptide to be exposed and further cleaved by carboxypeptidases (Rudensky et al., 1991).

Antigen processing

Because of the constraints imposed by the MHC peptide binding groove, the majority of proteins require some form of denaturation (Allen, 1987), which can be either unfolding (Streicher et al., 1984) or proteolytic cleavage, to stimulate a T cell response. A small number of proteins, such as fibrinogen, require no denaturation in order to stimulate T

cell responses, since the linear T cell epitopes are readily processed from the native protein (Lee et al., 1988).

The majority of cells express class I molecules, although the level of expression varies, and therefore a wide range of cells are capable of processing and presenting class I restricted T cell epitopes. The cells which process and present peptides in conjunction with class II molecules are more specialised, B cells (Chesnut and Grey, 1981) and dendritic cells are the major populations of antigen presenting cells (APCs) although other cell types expressing class II molecules may also act as antigen presenting cells (Zeigler and Unanue, 1981). Protein antigens can either be synthesised endogenously in the cytoplasm of the processing cell as for example occurs in virally infected cells, or can be brought into the cell from the outer environment by endocytosis from the cell membrane. Antigens can enter cells by endocytosis after interaction between the antigen and cell surface molecules, such as binding to surface antibody on B cells, via cell surface Fc receptors binding pre-formed antigen/antibody complexes, or by interactions such as biotin/avidin binding or electrostatic interaction with the surface charge of the cell membrane (Lanzavecchia, 1990). Two different pathways of antigen processing have been proposed for endogenous and exogenous antigens whereby each pathway is principally associated with a separate class of MHC molecule (Townsend et al., 1986a; Morrison et al., 1986) Endogenous antigens are processed so as to be presented in conjunction

with class I MHC antigens, whereas exogenous antigens become associated with class II MHC (reviewed by Brodsky and Guagliardi, 1991; Levine and Chain, 1991). However, there are a number of examples where antigens which normally enter the cell via endocytosis can be introduced into the cytoplasm and be processed so as to associate with class I. Soluble ovalbumin, introduced into the cell via the fusion of liposomes containing the ovalbumin with the cell membrane, could associate with class I (Steartz et al., 1987; Lopes and Chain, 1992), and the class II restricted HIV glycoprotein gp 160 gave a cytotoxic lymphocyte response (CTL) when it was incorporated into immune stimulating compounds (ISCOMs) which introduce antigen into the cell cytoplasm (Takahashi et al., 1990). These experiments illustrate that processing of antigen by the different pathways can determine whether a peptide associates with class I or class II MHC.

Peptides are thought to associate with class I MHC molecules in the endoplasmic reticulum where the α -chain and β 2 microglobulin are assembled (Yewdell and Benninck, 1989). The association of peptide with class I molecules has also been shown to stabilise the molecule (Schumacher et al., 1990; Ljunggren et al., 1990). Proteins are thought to be processed in the cytoplasm and the resulting peptides transported to the endoplasmic reticulum (ER). Evidence for this process comes from the identification of specific genes associated with the MHC coding region which encode putative transporter proteins (Spies et al., 1992). The class II MHC region has also been

shown to encode genes for sub-units of large intracellular protein complexes, proteasomes (Martinez and Monaco, 1991; Kelly et al., 1991), which are involved in processing proteins prior to their transportation (Parham, 1990). Once peptide has bound, the class I molecules are transported through the Golgi, where they become glycosylated, to the plasma membrane.

The α and β chains of Class II molecules, and invariant chain (Ii), are also synthesised within the ER and pass through the Golgi, but unlike class I antigens they move from the Golgi to the trans Golgi network (TGN) where they remain for 2-3 h (Neefjes et al., 1990). Association of the invariant chain with class II molecules directs the association of exogenous peptides with class II, possibly by preventing binding of endogenous peptides in the ER and Golgi (Roche and Cresswell, 1990). For peptide to bind to class II invariant chain has to become dissociated from the molecule and there is evidence to suggest that it is degraded prior to dissociation (Blum and Cresswell, 1988). Therefore Ii has a regulatory role in peptide loading (Peterson and Miller, 1990), although other regulatory proteins may also exist. Peptide binding to class II molecules may also be involved in determining the stability of the class II molecules (Sadegh-Nasseri and Germain, 1991). Endocytosed antigens are processed into peptides during their passage through the endocytic pathway. The early endosomes contains many vesicular elements (Griffiths et al., 1989) and are involved in sorting the endocytosed material for further progress through the endocytic pathway or for recycling to the

cell surface. The late endosomes have a pH of 5-5.5 (lower than that of the early endosomes) and contain many of the intracellular proteinases associated with protein processing, such as cathepsins B, D, and E (Levine and Chain, 1991). The demonstration that late endosomes also contained MHC class II molecules (Guagliardi et al., 1990) and Ii (Cresswell, 1985) indicated that in these studies the late endosome was the site where peptide associates with class II. Naturally processed peptide fragments from Ii and MHC class II (Rudensky et al., 1991) have been eluted from class II molecules and this provided further evidence that these molecules were present in the same cellular compartment as that where MHC/peptide association occurred. If the late endosome is the cellular compartment in which class II and peptide associate then the α/β Ii complex has to be transported from the trans Golgi network to the endosome. Evidence for this comes from the finding that multivesicular bodies containing MHC class II were located near to the TGN (Harding et al., 1990), possibly providing a means of transfer of molecules between the TGN and the late endosomes. Recent studies have further demonstrated that peptide binding takes place during the course of intracellular transport of the MHC class II through an endosomal compartment (Germain and Hendrix, 1991; Neefjes and Ploegh, 1992).

Factors influencing the T cell response to protein antigens

The Th cell response to a multideterminant antigen can display different immunological characteristics being directed towards either immunodominant, subdominant, or cryptic determinants (Gammon et al., 1987). Immunodominant epitopes are the regions of the protein against which the majority of the Th cells respond, however other epitopes may also be recognised. These sub-dominant epitopes are recognised to a lesser extent by the population of Th cells responding to the protein. Cryptic epitopes are not recognised by Th cells in response to the protein antigen but are immunogenic when administered as peptides, raising Th cell responses against both peptide and protein (Scherer et al., 1990). The Th cell response to many proteins has been shown to be restricted to a small number of epitopes (Katz et al., 1982; Bixler et al., 1985), often to a single immunodominant epitope, and different epitopes were shown to be recognised by different strains of inbred mice (Roy et al., 1989).

There are a number of factors which influence the T cell recognition of proteins. A straightforward model for the role of MHC in determining the genetic variability in T cell responses to protein antigens is that MHC molecules from different genetic backgrounds express differences in their peptide binding sites and therefore bind different peptides. This was proposed by Rosenthal (1978) as the "determinant selection theory", to explain the action of the "immune

response genes". Experimental evidence that peptide binding to MHC molecules *in vitro* correlated well with their known ability to generate T cell responses *in vivo* were in agreement with the determinant selection theory (Babbitt et al., 1985). There is also evidence that different MHC isoforms from the same genetic background (I-A^b and I-E^b) bind different peptides from the same protein (Rudensky et al., 1991).

Differences in individual MHC/peptide binding may not be sufficient to explain all of the T cell repertoire selectivity, and more subtle effects may arise from different peptides competing with each other for the MHC binding site (Buus et al., 1987). Peptide competition has been demonstrated both *in vitro* and *in vivo* (Adorini et al., 1988). In one experiment, for example two covalently linked Th cell epitopes, known to be immunodominant in H-2^d mice, resulted in the generation of a "novel" junctional epitope, which was dominant over the other two epitopes (Perkins et al., 1991). The authors proposed that the immunodominance of this epitope was due to its ability to compete for MHC binding with the overlapping, covalently linked Th cell epitopes, and that immunodominance was a function of the MHC binding ability of a given epitope within the context of the whole protein. Foreign antigens, such as viral proteins and proteins from other pathogens, have to compete with self antigens for binding (Rosloniec et al., 1990). Since peptides derived from self and "foreign" peptides were eluted from class II molecules (Rudensky et al., 1991), both types of antigen are

able to bind.

The selective inhibition of protease activity, using leupeptin, was shown to enhance the presentation of certain T cell epitopes (Vidard et al., 1991), probably by preventing the destruction of known immunogenic epitopes by thiol and serine proteases. Antibodies may also play a role in antigen processing, either preventing epitopes from being processed, determinant protection (Ozaki and Berzofsky, 1987) or by protecting epitopes such that they are processed and become available for T cell recognition (Van Noort et al., 1991). This may be important in determining the T cell response to proteins which are processed and presented by B cells which have captured antigen via their surface IgG receptors.

The presence of T cells expressing receptors capable of recognising the peptide/MHC complex is also essential for the generation of a T cell response to antigen. The T cell repertoire has an enormous potential for diversification, generated by gene rearrangement, and therefore might be expected not to be a limiting factor in the recognition of T cell epitopes. However, a significant proportion of peptides from staphylococcal nuclease that were able to bind to MHC were shown not to be immunogenic (Schaeffer et al., 1989). This lack of recognition by T cell receptors could be explained by the presence of "holes" in the T cell repertoire. The loss of V β gene segments from mice has been shown to affect the T cell response to antigen (Nanda et al., 1991) and if particular gene segments were involved in the

formation of TCRs recognising dominant protein determinants, the absence of these V β genes would result in the holes in the T cell repertoire. T cell non-responsiveness may also be a result of tolerance towards the antigen resulting from the deletion of T cells responding to self antigens or microbial superantigens (Blackman et al., 1990).

Therefore the T cell response to protein antigens can be the result of a number of different processes which probably all act to determine which epitopes are recognised by T cells. The general conclusion is that the formation of the peptide/MHC complex is crucial in determining the overall specificity of the immune response mounted by an individual and that antigen processing plays a secondary role in determining the T cell response to a whole protein. There is less evidence for defects in the T cell response because T cell gene deletions would still allow a large amount of diversity to be generated in outbred populations.

1.3. Synthetic peptide vaccines

Approaches to the design of synthetic viral vaccines

Vaccination is a means of inducing an immune response which is directed towards an infectious agent and is protective against reinfection by that agent. There are a number of diseases for which effective vaccines exist, but there are still a large number of diseases against which there

is no available vaccine. For instance there are effective vaccines for only a small number of viral diseases in humans including smallpox, polio, yellow fever, measles, influenza and hepatitis B (Warren, 1986). Conventional viral vaccines have been produced by either inactivating the virus or selecting for an attenuated strain of virus (reviewed by Budowsky, 1991). There are a number of disadvantages associated with both these conventional approaches. Inactivated vaccines require careful handling prior to inactivation and the inactivation process must be 100% efficient to eliminate the possibility of the presence of infective virus in the vaccine. Viruses which are attenuated for use in vaccines, such as poliovirus, may revert to an infectious form (Evans et al., 1985). Also impurities such as toxins or chemicals introduced by treatment of the infectious agent may cause adverse reactions in the immunised individual, and co-contamination of cell lines used to propagate viruses with other infectious species, e.g. the infective agent of bovine spongiform encephalitis (BSE) in culture media or human immunodeficiency virus (HIV) contamination of blood products. Vaccines consisting of proteins derived from the infectious agents, but produced in the absence of infectious material would not have many of the problems normally associated with conventional vaccines.

The development of novel vaccines provides the possibility of altering the natural immune response of the host to an infectious agent, for example encouraging either

cellular or humoral responses which may be more advantageous to the host. For example antibodies specific for one serotype of dengue virus can result in the development of haemorrhagic fever in an individual exposed to another serotype (Brandt, 1990), therefore vaccination against dengue virus must be effective against all four of the virus serotypes. Immune responses could also be directed towards different protein determinants by using a synthetic vaccine rather than inactivated antigen. In this way antibody responses to epitopes which may be cross-reactive between different serotypes or strains of viruses could be generated, rather than the serotypic responses (McCray and Werner, 1987). Antibodies (or CTLs) could also be directed towards different protein sequences of different proteins of the pathogen, again conferring an advantage to the host.

There is also the possibility that natural immunity could be improved by using highly defined vaccines to elicit immune responses. Therefore attempts have been made to identify and isolate the individual proteins of an infective agent, or specific regions of those proteins which are involved in the generation of immune responses. Approaches to the development of "new generation" vaccines include the use of anti-idiotypic antibodies to present an internal image of the antigen (Utydettaag and Osterhaus, 1985), expression of defined antigenic epitopes by recombinant proteins (Clarke et al., 1987), the generation of chimeric viruses containing the immunogenic determinants of two or more virus serotypes or

different viruses (Burke et al., 1988), and the chemical synthesis of the antigenic determinants of the virus as peptides (reviewed by Millich, 1989; Vjada et al., 1990). Although recombinant proteins expressing defined B cell and T cell determinants can be used to elicit effective immune responses (Moss and Flexner, 1987), chemically synthesised antigens have certain advantages over recombinant vaccines (Francis and Clarke, 1990). Synthetic peptide vaccines would be defined vaccines containing only the epitopes required for the generation of specific immune responses, unlike genetically engineered proteins which also contain epitopes from the native protein which would be capable of stimulating B and T cell responses towards that protein as well as, or possibly instead of, responses towards the defined "foreign" epitopes. Technically the production of peptide vaccines has the advantages that peptides are stable, allowing them to be handled and stored easily, and can be synthesised by controlled processes leading to less variability between different batches of vaccine. Such vaccines would be produced in the absence of infectious or potentially infectious materials.

Synthetic peptides were initially used as experimental antigens to investigate the various parameters controlling antigenicity (Sela, 1966; 1969). Anderer (1963), showed that a peptide from tobacco mosaic virus (TMV) raised antibodies to the virus and thus demonstrated a role for protein fragments and synthetic peptides in the generation of immune responses

to native proteins. The availability of protein sequences, derived either by direct sequencing or from the nucleic acid sequence of the gene encoding a protein, enabled the synthesis of defined epitopes. Advances in solid phase peptide synthesis techniques (Merrifield, 1963) allowed peptides to be synthesised efficiently and in large numbers (Houghten, 1985), enabling their wide use in immunological research. This approach was developed further by Lerner (1982) with a view to using synthetic peptides in the design of novel vaccines.

Factors influencing the identification of helper T cell epitopes

Vaccines aimed at generating antibody responses to foreign antigens must include epitopes representing the protein region recognised by neutralising antibodies and be capable of generating a Th cell response. Whether these two functions can be mediated by the same peptide is discussed below. Other vaccines may be required to generate T cell responses only, inducing cytotoxic cellular responses towards the antigen. However, for both types of vaccine the generation of effective T cell responses which can be stimulated by reinfection with the native antigen is crucial (Bottomly, 1988).

An important prerequisite to the development of any peptide based vaccine is the identification of suitable peptide immunogens. Immunisation with peptides may elicit

peptide specific Th cells which do not recognise that epitope on the native protein, due to a number of factors arising from the cellular processing of protein and the presentation of the processed fragments in conjunction with MHC (Berzofsky et al., 1987). Either the protein fragment represented by the peptide fails to be generated by cellular processing, or the processed fragment does not bind to the MHC molecule in the same manner as the peptide (Brett et al., 1988). The route of immunisation can also affect the protein epitopes which become the dominant T cell epitopes. For example, whether an individual encounters virus by immunisation or by natural infection can alter the Th cell repertoire. Influenza A virus provides a good model system to study such differences in the T cell response to virus as mice can be experimentally infected with the virus.

Th cell clones generated from mice infected with influenza virus recognised a number of determinants on the hemagglutinin (HA) molecule (Mills et al., 1986) whereas immunised mice displayed a more restricted Th cell response (Hurwitz et al., 1984). The ability of influenza A virus to infect mice also allows the responses of mouse and human T cells to be compared. Human CD4+ T cell clones were shown to recognise a single immunodominant determinant of HA which was different to those epitopes recognised by murine cells (Lamb et al., 1982), indicating that different Th epitopes may be recognised by different species. Significant Th responses are produced towards another influenza virus protein, nucleoprotein, and an epitope immunodominant for HLA-DR2

individuals (Brett et al., 1991) was also recognised by murine cells, although immunised mice recognised other Th cell epitopes (Gao et al., 1989).

Infection with influenza A virus also generates strong cytotoxic T cell (CTL) responses (Townsend and McMichael, 1985). A single site on the HA molecule has been defined using mouse CTL cells (Braciale et al., 1987), but the CTL response to influenza virus was shown to be directed predominantly towards the internal viral protein, nucleoprotein (NP) (Townsend et al., 1984). Human CTL responses have also been demonstrated against a defined region of the matrix protein of influenza A (Gotch et al., 1987). Using synthetic peptides, the CTL response to NP in the mouse was also shown to be limited to a small number of discrete sites on the protein (Townsend et al., 1986b). These Tc sites were also distinct from the Th epitopes identified for NP (Gao et al., 1989). The recognition of the CTL epitopes was demonstrated to be controlled by the class I molecule phenotype of the responding individuals in both mice (Taylor et al., 1987) and humans (McMichael et al., 1986), although different human class I molecules have been demonstrated to bind a wide range of peptides (Frelinger et al., 1990). This suggested that there is a level of permissiveness in the class I MHC binding of peptides.

The many factors controlling the immune response to an antigen therefore influence the selection of T cell epitopes within the individual, and binding of the processed protein

fragments to the MHC molecules does not necessarily result in the generation of antigen specific T cell responses. However, there are examples of Th epitopes which bind to a number of class II molecules, and generate T cells which recognise both peptide and protein. Such "promiscuous" Th epitopes have been identified in a number of proteins, including HPV E7 protein (Tindle et al., 1991), Tetanus toxin (Pania-Bordignon et al., 1989), measles fusion virus protein (Partidos and Steward, 1990), and a protein from the malarial parasite Plasmodium falciparum (Sinigaglia et al., 1988).

Enhancement of antibody responses to peptides by the addition of Th cell epitopes

The immunogenicity of peptides, in terms of stimulation of specific antibody, can be enhanced by coupling to a large immunogenic carrier protein which provides T-cell help for antipeptide antibody production (Mitchison, 1971). However, this approach is not practical for use in peptide vaccines since carrier proteins could induce hypersensitivity and carrier linked suppression (Jacob et al., 1985). The use of defined T cell epitopes would overcome the problems associated with carrier proteins and provide specific T cell help for T and B cell responses (Francis and Clarke, 1990). Peptides representing B cell epitopes capable of inducing antibody responses in the absence of additional T cell help often exhibit MHC restriction. A FMDV peptide, VP1 141-160, raised

peptide and virus reactive antibodies only in H-2^k mice (Francis et al., 1987b). This restriction could be overcome by adding defined Th sites from other proteins, one from ovalbumin (OVA) and two from SWM. Using chimeric peptides consisting of FMDV 141-160 synthesised N terminal to the Th epitopes, antibodies were elicited in H-2^d mice (Francis et al., 1987b; Francis et al., 1988). Although all three constructs gave anti-peptide antibodies, one of the Th epitopes failed to induce virus neutralising antibodies, indicating that the choice of the Th determinant can influence the specificity of the antibodies produced to the B-cell component. Antibodies to a B cell epitope of bovine rotavirus, not normally immunogenic unless coupled to a carrier, were produced in H-2^d mice when an influenza H-2^d restricted Th epitope was added (Borras-Cuesta et al., 1987). Moreover the activity of the antibodies produced was comparable to that obtained with the epitope conjugated to bovine serum albumin. Some peptides may be less immunogenic when coupled to carrier than when administered as free peptides (Francis et al., 1990a). These findings indicate that defined Th epitopes can be as good as, if not better than carrier molecules in inducing antibody responses directed towards B cell peptides. Although demonstrating that genetic restriction could be overcome, responses to the Th epitopes used in the experiments remained restricted to a small number of haplotypes. Th epitopes that were recognised by a number of MHC class II molecules would prove to be better vaccine components since

one Th epitope could provide help for antibody production in an outbred population. Combinations of such broadly specific T cell epitopes could be used in synthetic vaccines to provide T cell help in a number of, if not all, genetic backgrounds.

The positioning of the Th epitope(s) with respect to the B cell epitope may influence the final antibody response. A study by Cox et al. (1988) showed that a Th epitope from the Mycobacterium tuberculosis 65kD protein (65-85), cosynthesised with B cell epitope from the same protein (422-436), could induce antibodies to the B cell determinant only when positioned C-terminal to the B cell epitope (B-T) and not in the other orientation (T-B), probably due to the loss of the alpha helix structure in the T-cell peptide. Other studies have shown that the converse can occur and that it is the configuration T-B which provides help for B epitope specific antibody. Partidos et al. (1991) cosynthesised the "promiscuous" Th determinant from measles virus fusion protein both C- and N-terminal to a B cell determinant of measles HA known to be non immunogenic in H-2^k mice. The construct B-T peptide produced antibodies on secondary immunisation only towards the T cell determinant, whereas construct T-B gave antibodies recognising the whole construct and the B cell epitope. A similar finding was reported for respiratory syncytial virus epitopes synthesised as T-B peptides which when chemically cross linked to produce the B-T orientation gave a strong antibody response to the T cell component but no response towards the B cell determinant (Leveley et al.,

1990). Therefore the position of a Th epitope relative to the B cell determinant can drive antibody production towards a particular region of the chimeric peptide. This linear synthesis of epitopes can also create novel junctional Th epitopes within the peptide which may or may not detract from the activity of the intended Th epitope. The inclusion of multiple Th epitopes in a linear peptide may also result in hierarchy effects with certain epitopes becoming dominant over others (Ria et al., 1990).

Most of the chimeric peptides in these examples have been synthesised either in a linear sequence or with spacer residues such as gly-pro-gly between them. Peptide constructs can also be produced using multimeric peptides covalently coupled to a branched lysine carrier to produce a multiple antigen peptide, MAP (Tam, 1988). MAPs constructed using either multiple copies (either 4 or 8) of a single FMDV peptide exhibiting both T and B cell activity enhanced antibody responses to the virus (Francis et al., 1991). Joined octameric MAPs, composed of eight T cell peptides and eight B cell peptides from hepatitis B virus surface antigen also enhanced the immune response to virus (Tam and Lu, 1989).

Towards a rhinovirus vaccine

Antibodies to one rhinovirus serotype are generally specific and not cross reactive with other serotypes. Therefore intact virus is unlikely to be an effective vaccine,

eliciting broad cross protection against infection. The four major antigenic sites on the virus surface (Sherry et al., 1986) are highly variable and show little sequence homology between serotypes (Palmenberg, 1989). Nevertheless, some experiments aimed at studying antibody responses to these regions using peptide antigens have been carried out. Since some of the amino acids contributing to these antigenic sites are sequential within the primary protein structure, portions of the antigenic determinants can be represented by linear peptides or combinations of peptides. Peptides were selected for HRV serotype 2 using the amino acid sequence (Skern et al., 1985) and the three dimensional structural model of HRV 14 (Rossmann et al., 1985) to locate the four NIm sites and other surface accessible regions (Francis et al., 1987a). Peptides were conjugated to keyhole limpet haemocyanin (KLH) and used to raise antibodies. All the peptides tested raised antibodies which recognised peptide and denatured virus. However, only antibodies to the NIm II peptide VP2 156-170 recognised intact virus and gave a low degree of neutralisation. This peptide corresponded with the protein sequence recognised by a neutralising monoclonal antibody produced against intact HRV 2 (Skern et al., 1987). Antibodies from human sera with anti-HRV 2 neutralising activity also recognised this peptide, and these peptide cross-reactive antibodies were capable of neutralising virus infectivity (Hastings et al., 1990).

Once a suitable B-cell epitope for HRV is identified, its

immunogenicity could be enhanced by the addition of HRV specific Th epitopes, enabling HRV specific memory Th and B cell responses to be generated. Five potential Th epitopes were predicted from the HRV 2 sequence and synthesised C-terminal to the VP2 sequence 156-170. These chimeric peptides induced virus reactive antibodies in a number of mouse haplotypes, thus demonstrating the principle that T cell help could be provided by HRV sequences (Francis et al., 1989).

These preliminary studies indicated that HRV would be a good model antigen for the study of the Th cell epitopes involved in generating Th cell responses towards the virus. Once the "natural" helper T cell epitopes of HRV have been identified peptides representing these epitopes would be able to elicit Th cell responses in vivo and provide T cell help for the generation of antibody responses towards the B cell epitopes of HRV.

Aims of the project

The aim of this thesis was to investigate the role of helper T cells in the cellular immune responses to human rhinovirus. More specifically, to determine the serotype specificity of Th cell responses, to identify the regions of the virus structural proteins involved in generating Th cell response using synthetic peptides, and to demonstrate that these peptides could represent the functional Th cell epitopes of HRV and generate T cell help for antibody production by B

cells. Such epitopes would be potentially useful for inclusion in synthetic vaccines against HRV and possibly other viruses. Studying the Th cell response in mice to one HRV serotype, HRV 1A, provides a model for study of the parameters governing the immune response to a complex antigen, such as a picornavirus. The amino acid sequences of the four structural proteins of HRV 1A (figure 1.2.) and the crystallographic structure of the virus are known (Kim et al., 1989), enabling the positions of the defined Th cell epitopes to be interpreted in the context of the virus structure. The location of the Th epitopes could also be related to the major antigenic sites of HRV which have been defined for two serotypes.

Thus, by combining the defined Th cell epitopes and the B cell determinants for HRV, an immunogenic map of the virus can be made which could provide essential information on the relationship between T and B cell epitopes within the complex structure of a virus particle, and how these epitopes are able to generate antigen specific immune responses.

Figure 1.1. Diagrammatic representation of the protein folding of rhinovirus VP1, VP2, and VP3

The structure of the three large structural proteins of HRV 14 produced by Rossmann et al. (1987) are shown. The shaded areas indicate the positions of the four HRV 14 NIm regions IA, IB, II, and III. It should be noted that HRV 14 contains a number of small insertions and deletions from the HRV 1A sequence (and from other sequenced HRV serotypes) and therefore the amino acid numbers do not directly correlate with the HRV 1A sequence.

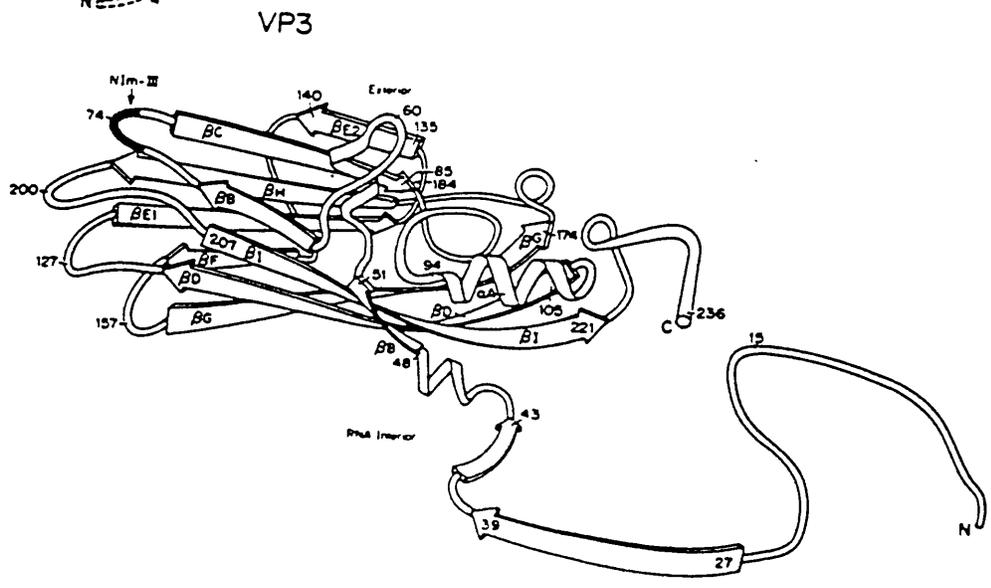
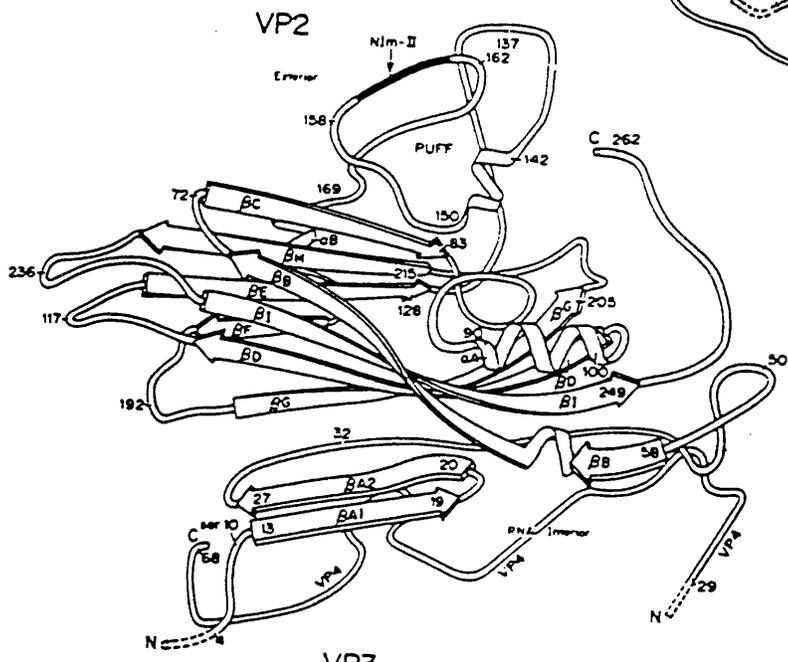
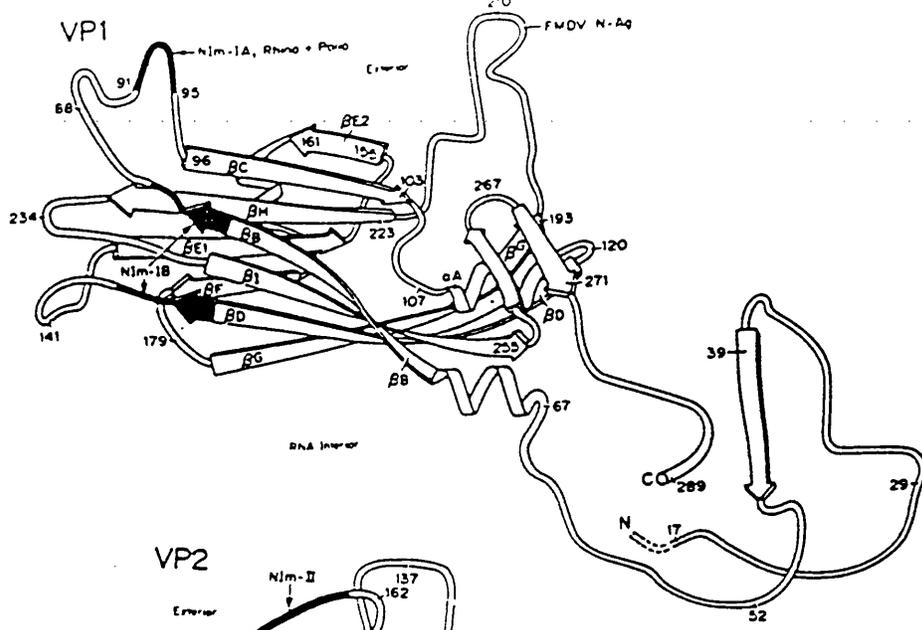


Figure 1.2. Amino acid sequence of the four structural proteins of HRV 1A

The amino acid sequence of HRV 1A is that reported by Palmenberg (1989) and used by Kim et al. (1989) in the elucidation of the three dimensional structure of the virus. The single letter amino acid code is used.

A	alanine	M	methionine
C	cysteine	N	asparagine
D	aspartic acid	P	proline
E	glutamic acid	Q	glutamine
F	phenylalanine	R	arginine
G	glycine	S	serine
H	histidine	T	threonine
I	isoleucine	V	valine
K	lysine	W	tryptophan
L	leucine	Y	tyrosine

HRV 1A VP1

1 20 40
NPVENYIDEVLNEVLVVPNIKESHHTTSNSAPLLDAAETG
HTSNVQPEDAIETRYVITSQTRDEMSIESFLGRSGCVHIS
RIKVDYTDYNGQDINF TKWKITLQEMAQIRRKFELFTYVR
FDSEITLVPCIAGRGDGIGHIVMQYMYVPPGAPIPSKRND
FSWQSGTNMSIFWQHGOFFPRFSIPFLSIASAYYMFYDGY
DGDNTSSKYGSVVTNDMGTICSRIVTEKQKLSVVITTHIY
HKAKHTKAWCPRPPRAVPYTHSHVTNYMPETGDVTTAIVR
RNTITTA

HRV 1A VP2

1 20 40
SPSVEACGYSDRIMQITRGDSTITSQDVANAVVGYGVWPH
YLTPQDATAIAKPTQPD TSSNRFYTLESKHWNGSSKGWWW
KLPDALKDMGIFGENMYYHFLGRSGYTVHVQCNASKFHQG
TLLVAMIPEHQLASAKHGSVTAGYKLTHPGEAGRDVSQER
DASLRQPSDDSWLNF DGTLLGNLLIFPHQFINLRSNNSAT
LIVPYVNAVPMDSMLRHNNWCLVIIPIPLRSETTSSNIV
PITVSI SP MCAEFSGARAKNIQ

HRV 1A VP3

1 20 40
GLPVYITPGSGQFM TDDMQSPCALPWYHPTKEISIPGEV
KNLIEMCQVDTLIPVNNVGNVGNVSMYTVQLGNQTGAMQ
KVFSIKVDITSTPLATTLIGEIASYYTHWTGSLRFSFMFC
GTANTTLKLLLAYTPPGIDEPTTRKDAMLGTHVVWDVGLQ
STISLVVPWVSASHFRLTADNKYSMAGYITCWYQTNLVVP
PSTPQTADMLCFVSACKDFCLRMARDTDLHIQSGPIEQ

HRV 1A VP4

1 20 40
GAQVSRQNVGTHSTQNSVSN GSSLNYFNINYFKDAASSGA
SRLDFSQDPSKFTDPVKDVLEKGIPTLQ

CHAPTER 2. Materials and Methods

2.1. Preparation of antigens

Peptide synthesis

Peptides were synthesised on an Applied Biosystems 430A Automated Peptide Synthesiser. Briefly, peptides were synthesised using the t-Boc strategy of Merrifield (1963) on a mesh p-methylbenzhydrylamine, 1% crosslinked divinylbenzene resin. Syntheses were carried out on the peptide synthesiser using modified 0.1mM small scale rapid cycles. Peptides were cleaved off the resin with 90% hydrogen fluoride 10% anisole, triturated with ether and vacuum dried before lyophilisation from 15% acetic acid.

HRV serotypes

a) Origins of HRV serotypes

HRVs 3, 14, 15, and 49 were received from W. Barclay, MRC common cold unit, Salisbury, U.K., HRVs 7 and 29 from V.V. Hamparian, Childrens hospital, Columbus, Ohio, USA., HRV 1A from D.C. Pevear, Stirling Winthrop, USA., HRV 1B from The Wellcome Foundation, Beckenham, U.K., and HRV 2 was received from D.Blass, Vienna, Austria. The viruses were plaque purified three times and their identities confirmed in neutralisation assays using virus serotype specific antisera.

b) Propagation of virus stocks

Confluent cell sheets of OH-5 cells, a cloned line from O-HeLa cells which is highly susceptible to rhinovirus infection, were inoculated with 50 μ l of plaque purified virus. After one hour at room temperature 20ml of Eagles MEM containing 2% foetal calf serum (FCS) and 30mM magnesium chloride (MgCl₂) were added and incubated at 34°C until a cytopathic effect (CPE) was observed, generally 36 to 48 h post infection depending on the virus serotype. Virus was released by freeze thawing twice at -20°C, cells were removed by centrifugation at 3000 rpm in a bench centrifuge, and 0.5ml aliquots of virus were stored at -70°C. The once passaged (x1) stocks were passaged similarly (using 0.5 ml to infect the cells) to produce twice passaged virus, 5ml of which were used to infect roller cultures of approximately 10⁹ confluent cells. Eagles MEM containing 2% FCS (100ml) was added, the cells were incubated at 34°C until complete CPE had occurred and the cells were then frozen at -20°C.

c) Purification of virus

Virus was harvested from 10 to 20 roller cultures of confluent Hela cells grown at 37°C, inoculated with virus (see above) by freeze thawing twice at -20°C, cells were removed by centrifugation and the virus was precipitated by the addition of an equal volume of saturated ammonium sulphate in 0.04M phosphate pH 7.0. After 1 hour at 4°C the precipitate was spun down at 3000 rpm for 40 min and then resuspended in

120ml of ice cold Eagle's MEM made 1% sarkosyl and 5mM EDTA. Virus was pelleted at 33 000 rpm for 90 min using a Beckman T35 rotor, resuspended in 1ml of 10mM tris 1mM EDTA 0.1% 2ME buffer at pH7.5, and fractionated on a linear 15%/45% (w/v in PBS) sucrose gradient for 2 h at 40 000rpm in a SW40 rotor. Pooled 0.5ml fractions were taken from two gradients and the virus peak was detected by measuring the A_{260} in a spectrophotometer. Fractions comprising the peak were pooled, sterile filtered through a 0.22 μ m filter, and stored at -70°C.

The protein concentration of purified virus was measured using a microplate assay. Samples were diluted, in duplicate, 1/2 in 100 μ l of distilled water down eight wells of a microplate, as was a protein standard solution of bovine serum albumin (BSA) at 100 μ g/ml. 100 μ l of Biorad protein assay reagent diluted 1 in 5 in distilled water were added and the absorbance read at 600nm using an automated plate reader. The protein concentrations were calculated using linear regression analysis of the standard curve.

d) Preparation of radiolabelled virus

A 150cm² flask of confluent O-H5 cells was infected with 0.5 ml of virus (x1 passaged). Virus was allowed to attach for 1 h at room temperature. Eagles MEM (20ml) supplemented with 2% FCS and 30mM MgCl₂ magnesium chloride were added and the cells incubated at 34°C until CPE occurred. Virus was released by freeze thawing, cell debris was removed by centrifugation, and virus was used to infect another confluent

monolayer. Virus was allowed to attach for 1 h at room temperature, tipped away, and 20ml of MEM 2% FCS added and incubated at 34°C for 3 h. The media was discarded and the monolayer washed twice with methionine free MEM (with no serum added). Cells were incubated at 34°C for 1 h in the methionine free medium. ³⁵S methionine (200μCi) were added for 2 h at 34°C and a further 200μCi were added and incubated at 34°C overnight. Virus was purified and its position on the sucrose density centrifugation was determined. A 10μl aliquot from each fraction was added to vials containing 90μl of 10% BSA and 2ml of liquid scintillant were added. Samples were counted on an automatic scintillation counter.

e) Infectivity assay

Healthy, confluent monolayers of OH-5 HeLa cells were grown at 37°C in 60mm petri dishes. The media was discarded to leave a confluent cell sheet. A series of ten fold dilutions of the purified virus were made in MEM, and 100μl of each dilution were allowed to attach to the monolayers for 1 h at room temperature. The cell sheets were overlaid with 6ml of 0.6% agarose in MEM (supplemented with 2% FCS, 15mM MgCl₂, and DEAE-D at 100μg/ml) at a temperature of 34°C, and the dishes were incubated at 34°C until virus plaques were visible (2-3 days for HRV 1A). The agarose was then removed and the plates stained with crystal violet (0.02% crystal violet, 2% formalin and 5% methanol in saline). Plaques on duplicate plates were counted and used to calculate the infectivity of the virus in

plaque forming units per millilitre (pfu/ml).

Preparation of haptenated antigens

A 10 mg/ml solution of trinitrobenzyl sulphonic acid (TNBS) was made in 0.1 M bicarbonate buffer, pH 8.2, and 1 ml was added to an equal volume of a solution of peptide at 5mg/ml, or ovalbumin at 2mg/ml in the same buffer. TNBS was used at a twice molar excess to the peptide or protein. The reactants were stirred in the dark for 1 h at room temperature and a bright yellow colour indicated that the reaction had occurred. The solution was passed down a disposable 5ml sephadex G-10 column to separate the components and the fractions containing the bright yellow component were collected and pooled. The protein concentration was determined by measuring the A_{280} using a spectrophotometer.

2.2. Immunisation schedules

Immunisation for Th cell responses

a) Immunisation with HRVs

Purified virus was diluted in sterile PBS and emulsified 1:1 with Freund's complete adjuvant (FCA, Difco), to give a final virus concentration of $2\mu\text{g/ml}$. Female 8-10 week old mice, either H-2^d (Balb/c), H-2^k (C3H.He or CBA), or H-2^b (C57/Bl10) were obtained from Charles River, U.K., or from the

ICRF, U.K. Mice were immunised sub-cutaneously at the base of tail with 100 μ l of vaccine, each mouse receiving 0.2 μ g of virus. Different virus doses in FCA, or vaccines made up of Freund's incomplete adjuvant (FIA) were prepared in a similar manner.

Virus administered in aluminium hydroxide was prepared by diluting the purified virus in PBS containing 20% aluminium hydroxide (obtained from the Wellcome Foundation, U.K.) and allowing the vaccine to stand overnight at 4°C. Mice were immunised as described above. Virus was also administered in the tail base diluted in PBS and care was taken not to allow leakage of the preparation from the immunising site.

b) Immunisation with synthetic peptides

Peptides were diluted in sterile PBS and emulsified 1:1 with FCA to give a final concentration of 500 μ g/ml. Mice were immunised with 100 μ l, each mouse receiving 50 μ g of peptide. For both peptide and virus immunisation groups of between 4-12 mice were generally used for a single experiment.

Immunisation for antibody responses

a) Antibody responses to HRV 1A and CPMV

HRV 1A and cowpea mosaic virus (CPMV) were diluted in sterile PBS to give a range of virus concentrations from 4-0.04 μ g/ml for HRV 1A and 40-0.04 μ g/ml for CPMV. Equal volumes were emulsified 1:1 with FIA and groups of six Balb/c mice

were immunised intramuscularly in the left hind flank with 100 μ l of each preparation. Both viruses were also administered in PBS alone and in 20% v/v aluminium hydroxide such that each animal received 0.2 μ g of virus. The mice were bled from the tail 14, 28 and 56 days post immunisation and were boosted with 100 μ l of the same preparations on day 56. Further bleeds were taken on days 63, 70 and 84 post primary immunisation. Serum was prepared by allowing the blood to clot overnight and then removing red cells by centrifugation. Serum was stored at -20°C until required.

b) Peptide priming for enhanced antibody responses towards HRV1A

Groups of five CBA mice were immunised sub-cutaneously at the tail base with 100 μ l of peptide emulsified 1:1 in FCA such that each animal received 50 μ g of peptide. 21 days post immunisation the mice were bled from the tail and immunised intraperitoneally (i.p.) with 100 μ l of HRV 1A emulsified 1:1 with FIA, at a concentration of 0.02 μ g/ml, each mouse receiving 0.002 μ g of purified virus. Mice were bled 28 and 42 days after the primary immunisation.

c) Antibody response to haptens peptides

Groups of five CBA (H-2^k) mice were immunised sub-cutaneously at the tail base with 100 μ l of HRV 1A diluted to a concentration of 2 μ g/ml in PBS containing 20% aluminium hydroxide. Fourteen days later the mice were bled and

immunised i.p. with 100 μ g TNP-peptide, also in aluminium hydroxide. Mice were bled 21, 28, 42, and 70 days after the primary immunisation.

2.3. Cell preparations

Single cell suspension from normal mouse spleens

Spleens were taken from normal mice and placed in cold RPMI medium. A single cell suspension was prepared by passing the spleens through a stainless steel mesh using a sterile 2ml syringe plunger to mash the tissue through. The cells were washed through the sieve with cold RPMI (or MEM) and left to stand for a few minutes to allow tissue debris to settle out. The cells were washed twice by centrifugation and resuspended in media supplemented with 5% heat inactivated FCS.

Spleen cell preparation enriched for low density cells

An enriched preparation of dendritic cells was made from a single cell suspension of 10-20 normal mouse spleens in MEM (no serum) following the method described by Ellis et al., (1991). After washing with MEM the cells were resuspended in 2ml MEM were centrifuged through a stepwise percol gradient at 2000 rpm for 15 min. Low density cells from the 50/40% and the 40/30% interfaces were removed, washed three times in MEM, resuspended in 10ml of MEM 5% FCS and left for 2h at 37°C to

allow the cells to adhere to the flask. Non-adherent cells were washed off with warm medium and the adherent cells were incubated overnight at 37°C, after which time the cells rounded off and were harvested.

Antigen stimulated spleen cells

Activated spleen cells were obtained by adding Concanavalin A (Con A, Sigma) at 1µg/ml and lipopolysaccharide (LPS, Sigma) at 10µg/ml, to 5ml aliquots of spleen cells at 5×10^6 cells/ml in RPMI 5% FCS. The cells were cultured for 3 days at 37°C to give predominantly T-cells in the Con A stimulated preparation and B-cells when stimulated with LPS.

Single cell suspension from lymph nodes

Seven to ten days after immunisation, the draining inguinal and para-aortic lymph nodes were dissected out and placed in ice cold RPMI-1640 supplemented with penicillin (100U/ml), streptomycin (100µg/ml), and 20mM glutamine (RPMI). The lymph nodes were passed through a stainless steel mesh to produce a single cell suspension and the cells were washed through with 20 ml of cold RPMI. Cells were washed twice with RPMI, viable cells were counted in trypan blue and cells were resuspended on ice at 5×10^6 cells/ml in RPMI supplemented with 1% normal mouse serum and 2-mercaptoethanol (1×10^{-4} M).

2.4. Cellular proliferation assays

In vitro cellular proliferation

Purified HRV serotypes were diluted in RPMI to give a range of working concentrations from $20\mu\text{g/ml}$ to $2 \times 10^{-5}\mu\text{g/ml}$. Dilutions were always made on the day of assay from the original aliquot as HRV preparations appeared to be "sticky" with certain plastics. Peptides were used over a range of working concentrations of $100\mu\text{g/ml}$ to $0.1\mu\text{g/ml}$. Control antigen Con A, at $500\mu\text{g/ml}$, was used to demonstrate non-specific cellular proliferation and purified protein derivative, PPD (1000U/ml, Evans) was included as a further control of antigen specific proliferation when mice had been immunised with FCA.

Antigen ($100\mu\text{l}$) was added in triplicate to the wells of flat bottomed 96 well plates containing $100\mu\text{l}$ cells at 5×10^6 cells/ml. Control wells, containing cells and media alone (no antigen) were also set up in triplicate. When cells were available, more control wells were set up to provide a better indication of the background cellular proliferation. Plates were incubated at 37°C in a 5% CO_2 humidified atmosphere for 4 days. The cultures were pulsed with $1\ \mu\text{Ci}$ [^3H]thymidine (^3HT , obtained from Amersham) by the addition of $25\mu\text{l}$ of ^3HT diluted in RPMI, for 16 hours prior to harvesting onto filter mats using an automated cell harvester (Skatron) and measuring the amount of radiolabel incorporated into the cells by

liquid scintillation counting using a Pharmacia Betaplate automated counter. In some assays the level of incorporated radiolabel was measured by harvesting the wells onto filters and then placing the discs in separate scintillation vials for counting using a scintillation counter.

Antibody specific complement depletion of lymph node cells

Tissue culture supernatants containing monoclonal antibodies (Mabs) against CD4 (GK1.5), CD8 (3.168), and Thyl (H.0139), were used in conjunction with guinea pig complement (Seralab) to deplete different populations of primed lymph node cells. Cells (1ml at 2×10^7 /ml) were incubated with 1ml of Mab at 4°C for 45 min, washed twice with ice cold RPMI, and then incubated at 37°C with complement at a final dilution of 1/20 for 30 min. Cells not treated with antibody were also included as a control. After washing twice viable cells were counted and the preparations resuspended at 5×10^6 cells/ml and assayed in triplicate for proliferation with control and specific as described above. Cells were also cultured with antigens in the presence of TIB 120, which binds MHC class II, at a final concentration of 20µg/ml.

Inhibition of proliferation by virus specific antibodies

a. Preparation of reagents

Virus serotype specific antibodies towards HRV 1A and 15

raised in rabbits were available in the laboratory. The serum was used to prepare purified IgG (pIgG) by affinity chromatography on a protein A column using FPLC (Pharmacia). The fractions containing pIgG were desalted against PBS (pH 7.5) using a pharmacia fast desalting column containing sephadex G-25, sterile filtered, and stored at -20°C . Fab fragments were generated from the purified IgG using papain digestion. The pIgG was concentrated 5 fold to 5-10mg/ml by centrifugation using centricon 30 tubes spun at 2000g for 20 min, and dialysed against 20mM phosphate, 10mM EDTA pH 7.0 overnight at 4°C . Digestion buffer (0.5 ml of 20mM NaH_2PO_4 , 20mM cysteine-HCl, 10mM EDTA pH 7.0) and 0.5 ml of immobilised papain (Pierce), also in digestion buffer, were added to 1ml of dialysed rabbit pIgG in a glass tube and mixed gently overnight at 37°C using an end over end shaker. The pH was adjusted by the addition of 1.5ml 10mM Tris-HCl pH 7.5 and the immobilised papain removed by centrifugation. Fab fragments were separated from Fc and undigested IgG using a protein A column and FPLC as described above. The purity of the Fab separation was determined by PAGE.

b. Cellular proliferation assay

Rabbit antiserum, purified IgG and Fab fragments were diluted in RPMI in siliconised tubes to give 200 μl of dilutions x2 greater than required. These were mixed with an equal volume of purified virus at concentrations of 0.4, 0.04, and 0.004 $\mu\text{g}/\text{ml}$, and incubated overnight at 4°C . Three 100 μl

aliquots were used as antigen in proliferation assays with cells from mice primed against HRV 1A. Cellular proliferation was determined as described previously and the results expressed as a percentage of the proliferation observed with cells incubated with virus in the absence of antibody.

Inhibition of cellular proliferation by protease inhibitors

Protease inhibitors pepstatin (Sigma) and E64 (Sigma) were diluted in RPMI to give a range of concentrations from 200-10 μ g/ml. 50 μ l of each dilution were added in triplicate to lymph node cells from antigen (either HRV 1A or peptide) primed mice. Antigen (50 μ l) was also added to give concentrations of 0.02 and 0.002 μ g/ml for HRV 1A and 10-1 μ g/ml for peptide. The proliferation assay was performed following the previously described protocol and the results expressed as a percentage of the proliferation of cells in the presence of antigen alone.

2.5. Cell binding studies

Radioimmunoassay

a. Direct binding

Cells used in the radioimmune assays (RIAs) included, normal spleen and lymph node cells, Con A and LPS stimulated spleen cells, O-H5 cells, a murine B-cell hybridoma cell line

(A20) and a murine T cell hybridoma (IE5). Cells were resuspended at either 2.5×10^7 or 5×10^7 cells/ml in RPMI containing 2% FCS and 30mM magnesium chloride and 200 μ l were pipetted into 1.5 ml eppendorf tubes. An equal volume of ^{35}S labelled virus, diluted to give approximately 2000 counts per tube, were added and the tubes were incubated at 34°C for up to 60 min. Cells were spun at 1200 rpm for 8 min and washed with 400 μ l RPMI. Cells were resuspended in 400 μ l medium, spun down, and the supernate removed into a scintillation vial. The pellets were lysed with 400 μ l 1% SDS in H₂O and transferred to scintillation vials. Assays were performed in duplicate and the level of ^{35}S in both the supernate and the pellet were measured by liquid scintillation counting.

b. Inhibition assay

Prior to incubation with radiolabelled virus, cells at 5×10^7 /ml were first incubated with unlabelled virus. Virus was added at concentrations of between 10 and 0.1 μ g/ml and incubated for 60 min at 34°C in 400 μ l of RPMI (2% FCS, 30mM MgCl₂). Cells were washed once, resuspended in 200 μ l of supplemented RPMI and ^{35}S radiolabelled virus was added to give approximately 2000 counts per tube and the assay was performed as described previously.

Fluorescence antibody cell staining

Two hundred microlitres of OH-5 cells or low density cells

at 1×10^6 cells/ml in RPMI (containing 2% FCS, 30mM $MgCl_2$) were mixed with an equal volume of purified HRV at 20 μ g/ml. Cells were incubated at 34°C for 40 min, washed and resuspended in 400 μ l of RPMI. Cells incubated in the absence of virus were included as controls. A single drop of cell suspension from a 1ml pipette tip was added to the wells of microscope slides pre-coated with polylysine. The cells were allowed to adsorb for 20 min and then fixed for 30 min by the addition of a drop of 3% formaldehyde. Wells were washed twice with PBS by adding and removing drops using a pipette and blocked for 30 min with 50% horse serum (HS) in PBS. Anti-virus or non-virus specific purified rabbit IgG diluted 1/100 in 10% HS was added for 30 min. After washing, FITC labelled anti-rabbit IgG (Sigma) diluted 1/100 in 10% HS was added and incubated for 30 min. Mouse cell preparations were also stained for CD3 and surface IgG using biotinylated anti-mouse IgG (Amersham) and biotinylated anti-mouse CD3 (obtained from Dr. J. Marvel, ICRF Tumor Immunology Group). Texas red labelled streptavidin (Amersham) was used to bind to the biotin labelled cells. Finally a drop of Citifluor was added to each of the wells and the slides covered with a glass slip. Fluorescence was observed using a fluorescence microscope and cells were counted and photographed under phase contrast or fluorescent illumination.

2.6. Enzyme linked immunosorbent assay (ELISA)

Measurement of mouse anti-virus antibodies

Microtitre plates were coated overnight at 4°C with 50µl of purified HRV 1A diluted in carbonate buffer (0.1 M, pH 9.6) to give a final concentration of 0.5µg/ml. The plates were washed three times with PBS containing 0.5% Tween 20 (Sigma) and the mouse antisera were diluted 1/10 in 100µl 1% BSA in PBS, added to the first row of the plate and diluted 1/2 down the plate in 50µl of 1% BSA. The plates were incubated for 1 h at 37°C. After washing with PBS/Tween, 50µl of peroxidase labelled goat anti-mouse antibody (Sigma) diluted 1/1000 in 1% BSA/PBS were added and incubated at 37°C for 1 h. The plates were washed and 50µl of the enzyme substrate (0.04% o-phenylenediamine plus 0.004% hydrogen peroxide in phosphate/citrate buffer, pH 5.5) were added. The orange/brown colour reaction was stopped after 10-15 min by the addition of 12.5µl of 12.5% sulphuric acid and the absorbance at 490nm was measured in an automated plate reader. The A_{490} values were plotted against the reciprocal dilution of the antiserum and the endpoint titres were calculated with reference to the A_{490} value given by a negative standard (1/10 dilution of negative MS). The mean end point titre and the standard error from the mean (S.E.M.) was calculated for each group of mice.

Measurement of mouse anti-TNP antibodies

Plates were coated overnight at 4°C with 50µl of TNP-ovalbumin at 100µg/ml diluted in coating buffer. The ELISA was then performed as described above and the antibody titres were calculated as described above. The mean end point titre and the S.E.M. was calculated for each group of mice.

CHAPTER 3. Th cell responses to human rhinovirus

3.1. Introduction

Infection with human rhinovirus, HRV, results in the production of virus specific neutralising antibodies which confer long-term protection against reinfection with the homologous HRV serotype (Dogget 1965). The generation of antibody responses to other picornaviruses such as poliovirus (UytdeHaag et al., 1985) and FMDV (Collen et al., 1989) has been shown to require T cells. Virus specific Th cell responses have been demonstrated against poliovirus in humans vaccinated with the virus and also in cattle, a host species for FMDV, vaccinated against FMDV (Collen and Doel, 1990). It was likely that the response to HRV would also be T cell dependant. Experiments using HRV to infect human volunteers have also provided evidence for HRV specific Th cell responses. During the course of a HRV infection the number of circulating T cells in the peripheral blood decreased, possibly due to cellular migration into infected tissues (Levandowski et al., 1986). HRV specific and non-specific cellular proliferation and cytotoxicity can be demonstrated in peripheral blood mononucleocytes taken from infected individuals (Hsia et al., 1990), indicating that a systemic cellular immune response is generated by HRV infection.

Studies of the immune responses of mice immunised with FMDV (Collen et al., 1989) and poliovirus (Katrack et al.,

1991) have shown that strong virus specific Th cell responses were induced. Therefore, as a prelude to more difficult studies in humans, and also to set up a model system to study the parameters governing immunogenicity to a complex antigen such as a virus, we set out to study the Th cell response to HRV in mice. The primary objectives of the study were to investigate the serotype specificity of the Th cell response, to identify the specific Th cell epitopes involved in the generation of the Th cell response, and to analyse the main features which determine the immunogenicity of HRV.

The results reported below demonstrate that a HRV specific Th cell response was generated in each of the three different mouse strains used in this study and that the proliferating cells from HRV immunised mice were mostly CD4+ Th cells. Th cells primed against HRV 1A were also shown to proliferate in response to a number of different HRV serotypes. This heterotypic response was also seen in all three mouse strains tested. Therefore the Th cell response to HRV, compared to cellular immune responses generated towards other picornaviruses, will be discussed in this chapter.

3.2. Optimisation of the assay

The usual assay for measuring cellular proliferation in response to antigen depends upon the observation that dividing cells incorporate radiolabelled thymidine into their DNA (Corradin et al., 1977). A mitochondrial stain, 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) has also been used as an indicator of cellular proliferation because of the close relationship between proliferation and the level of mitochondrial activity in the lymphocytes. It should be noted, however that neither incorporation of radiolabelled thymidine, nor MTT are a direct measure of cellular proliferation. Both DNA synthesis and mitochondrial activation can occur in the absence of cell division. A further problem in the interpretation of proliferation assays is that, although the difference in response in the presence and absence of antigen is generally described as "antigen specific", much of the response observed probably arises from recruitment of non-specific "bystander" T cells, activated indirectly by cytokines secreted in the cultures. Despite these limitations, conventional assays provide a straightforward and informative assay of many T cell responses. Antigen specific cellular proliferation can be determined using purified populations of T cells (Rosenwater and Rosenthal, 1978) but the purification procedure is labour intensive and the proliferation assay then requires the addition of antigen presenting cells. It was therefore decided to employ the simplest method to measure cellular proliferation using a single cell suspension made from the lymph nodes of immunised mice, and to allow the antigen presenting cells (including B cells) contained within the mixed cell preparation to present antigen to the primed Th cells. As a preliminary to this study, the experimental

conditions for measuring HRV specific responses were optimised. For this purpose a number of factors which could affect the cellular proliferation assay were varied. These factors included the number of cells used in the assay, the length of time cells remain in culture, the type and concentration of serum used in the assay, the immunising dose of virus and the length of time mice were immunised.

3.2.a. The effect of altering the concentration of cells used in the antigen specific proliferation assay

Lymph node cells from Balb/c mice immunised with $2\mu\text{g}$ of HRV 1A were used at concentrations from 3×10^6 - 6×10^6 cells/ml and the proliferation to HRV 1A determined over a concentration range of 0.001 - $10\mu\text{g/ml}$. The T cell mitogen Con A was included as a control for overall T cell responsiveness antigen and cells cultured in medium alone provided a base line against which to compare antigen induced responses. Assays were carried out initially using 10% heat inactivated FCS in the culture medium. There was little difference between the virus dose response profiles obtained using 5×10^6 cells/ml and 6×10^6 cells/ml (figure 3.1.). However, when the cell number was reduced to 4×10^6 a dramatic decrease in cellular proliferation was observed, and no cellular proliferation was seen at the lower virus concentrations. Virtually no antigen specific cellular proliferation occurred when cells were used at 3×10^6 cells/ml. The proliferative response to Con A was similar at all four cell concentrations (data not shown).

Therefore cells were used in subsequent assays at a concentration of 5×10^6 cells/ml.

3.2.b. The effect of altering the length of time cells are cultured in vitro on antigen specific proliferation

To investigate the effect of varying the length of time for which cells were cultured with antigen, three 96 well tissue culture plates were set up with lymph node cells from Balb/c mice immunised with $2 \mu\text{g}$ of HRV 1A in FCA, and the cultures were incubated at 37°C for 3, 4, and 5 days. At the end of each time interval one plate was pulsed with $1 \mu\text{C}$ ^3HT for 16 hours and then harvested and the uptake of radiolabelled thymidine was measured. Cellular proliferation was determined over a range of virus concentrations. Cells cultured for 4 and 5 days gave higher levels of proliferation than cells cultured for 3 days (figure 3.2.). Cells cultured for four days also gave higher levels of proliferation at lower in vitro virus concentrations than did the 3 and 5 day cultures. Although the concentration of virus giving the optimal proliferative response was the same, $0.02 \mu\text{g}/\text{ml}$, for all three time points, both the level of proliferation and the range of virus concentrations at which the cells responded were higher when cells were cultured with virus for four days. In subsequent assays cells were therefore routinely incubated at 37°C with antigen for four days prior to the addition of ^3HT .

3.2.c. The effect of altering the type of serum used in the antigen specific proliferation assay

The use of 10% FCS in the cell culture medium gave high levels of background ^3HT incorporation and this background proliferation was variable between assays. This made direct comparisons of results from different experiments difficult. Therefore mouse serum (MS), which supports the growth of mouse cells at much lower concentrations than FCS (Chain et al., 1987), was used in HRV 1A specific assays. Antigen specific cellular proliferation could be measured at concentrations of MS from 0.5-2%. Mouse serum was routinely used at 0.5%. Results from a number of experiments in which the HRV 1A specific proliferation had been assayed either using FCS or MS are presented in table 3.1. The results demonstrate that FCS often gave high levels of background proliferation and variable results between experiments, whereas culturing cells with MS reduced the background proliferation and gave results which were more reproducible. The majority of experiments described in this thesis were performed using MS in the culture medium.

3.2.d. The effect on the antigen specific proliferation of using different virus concentrations to prime mice

Mice were immunised with different concentrations of purified HRV 1A to determine the lowest amount of virus required to give reproducible antigen specific cellular responses. Although a low dose of virus, $2\mu\text{g}$ per animal, had

been used in initial experiments, it was not known whether this was the optimal amount. Mice were immunised with 0.04, 0.2, and 1 μ g of HRV 1A per animal and the proliferative responses were measured over a range of virus concentrations. Cells from mice immunised with 1 μ g gave similar response profiles (figure 3.3.) to those of cells primed with 2 μ g of virus (previous experimental observations). Mice primed with lower amounts of virus (0.04 μ g and 0.2 μ g per mouse) also responded to HRV 1A in vitro. Mice primed with the lowest dose of virus gave lower levels of ³HT incorporation at all virus dilutions but exhibited an optimal level of proliferation at the same in vitro virus concentration as the other two groups (0.02 μ g/ml). On the basis of these results, and other experiments in which immunisation with 0.2 μ g of HRV 1A was shown to give reproducible proliferative responses, 0.2 μ g of HRV 1A was selected as the standard amount of virus for immunisation.

3.2.e. The effect of altering the time interval between immunisation and measurement of the cellular proliferation

Antigen specific proliferation assays are routinely carried out seven to ten days between immunisation. This length of time was also optimal for HRV 1A specific cellular responses (figure 3.4.a.), but it was interesting to note that virus specific Th cell responses were still observed over a range of virus concentrations after 14, 21 and 28 days, although the level of ³HT incorporation was lower. A similar

experiment using a HRV 2 peptide to immunise mice demonstrated that the Th cell response to peptide was not so long lived, with peptide specific cellular proliferation dropping considerably after 14 days (3.4.b.).

3.3. HRV 1A primed lymph node cells from H-2^d mice proliferate in response to HRV 1A and heterologous HRV serotypes

The proliferative response of HRV 1A primed cells to 8 other rhinovirus serotypes was investigated over a range of antigen concentrations from 2×10^{-5} - $2 \mu\text{g/ml}$. Cellular proliferation was observed in response to HRV 1A and with seven of the other serotypes tested. Only one, HRV 15, gave a low level of proliferation (figure 3.5.). The responses to the different serotypes varied. Cells responded to HRV 2 and HRV 29 to a similar extent as HRV 1A with high levels of ^3HT incorporation at low concentrations of virus. HRV 1B and HRV 49 also stimulated good incorporation, but at higher virus concentrations. HRV 3, 7, and 14 proliferated to a lesser extent and only at higher in vitro antigen concentrations.

3.4. HRV 1A primes cells which respond to HRV 1A and other HRV serotypes in mice of different haplotypes

To determine whether the response to heterologous HRV serotypes and the pattern of the cross reactivity seen with Balb/c (H-2^d) mice was unique to that haplotype, C57.BL (H-2^b)

and C3H.He (H-2^k) mice were immunised with HRV 1A and assayed for proliferation with the same panel of viruses (figure 3.6). H-2^b mice (figure 3.6.a.) responded well to the immunising virus, HRV 1A and the heterologous HRV 29 as did H-2^k mice (figure 3.6.b.). Cells from both haplotypes also proliferated with 1B, 2 and 49 but gave lower levels of ³HT incorporation at higher antigen concentrations. The responses to HRV 3, 7, and 14 were lower still. Cells from both mouse strains failed to proliferate in response to HRV 15.

3.5. HRV 15 primed cells exhibit different cellular proliferative responses to HRV 1A primed cells

Since little or no proliferative response to HRV 15 was observed in HRV 1A primed animals, Balb/c mice were immunised with HRV 15 and tested for responses to the panel of rhinoviruses (figure 3.7.). The level of the response to homologous virus was much lower than that of HRV1A immunised mice, and occurred only at the two highest concentrations of virus. Cells proliferated with HRV 3 to a similar degree but only responded to the other major receptor group viruses HRV 7 and 14 at the highest antigen concentration, 2 μ g/ml. Little or no response was seen with the minor group viruses HRV 1A, 1B, 2, 29, and 49.

3.6. Cellular proliferation is HRV specific

To demonstrate that HRV preparations did not act as non-specific T cell mitogens in vitro, cells from mice primed only with FCA were cultured with the two highest in vitro concentrations of each of the serotypes (0.2 and 2 μ g/ml). Table 3.2. shows that these cells gave very little proliferation in response to the HRV serotypes. To further demonstrate that the response of virus primed cells to HRV in vitro was not due to non-viral contaminants found in the viral preparations, proliferation of HRV 1A primed lymph node cells was measured using an extract of HeLa cells and dilutions of sucrose in PBS. Table 3.2. shows that the ³HT incorporation of HRV 1A primed cells in response to these preparations was negligible, demonstrating that the cellular proliferative response was indeed due to the presence of HRV.

3.7. The proliferating lymph node cells are CD4+ Th cells

The phenotype of the proliferating cells was determined by depleting lymph node cell preparations of various subpopulations using monoclonal antibodies against specific cell surface markers and complement. Viable cell counts obtained after treatment were used as an indication that depletion had occurred. Treatment with anti-Thy 1 antibody (H.0139) eliminated ³HT incorporation indicating that the cells proliferating in response to HRV 1A were T cells (table 3.3).

After treatment with anti-CD4+ (GK 1.5) proliferation was greatly reduced, whereas anti-CD8+ antibody (3.168) had no effect. In addition to the cellular depletion assays using complement and specific antibodies, un-treated lymph node cells were cultured with antigen in the presence of TIB 120, a monoclonal antibody which binds MHC I-A/I-E molecules and prevents antigen specific cellular proliferation. The cells did not respond to HRV 1A, demonstrating their I-A/I-E restriction. All cells responded to control antigens Con A and PPD, with the PPD responses affected by antibody treatment in a similar manner to those observed with virus (data not shown).

3.8. U.V. inactivated and heat inactivated HRV 1A prime for Th cell responses to both inactivated and live virus

Although minor group HRV 2 has been shown not to replicate in mouse cells (Yin and Lomax 1983), it could be argued that a small degree of replication of HRV 1A in the immunised mice could contribute to the high immunogenicity of HRV observed. Viral replication can be eliminated by treating the virus with ultraviolet light. Treatment of HRV with low pH (4-5) or heat (for 1h at 60°C) also reduces virus infectivity possibly by inducing conformational changes in the capsid structure which alters the ability of the virus to bind to cells. Therefore HRV 1A exposed to U.V. or heated at 60°C for 1 hour, was compared to live virus for its ability to

immunise for a T cell response. The infectivity of live and inactivated virus preparations was assayed on Hela cell monolayers (chapter 2.). The live HRV 1A preparation had a titre of 2.9×10^9 , virus treated with U.V. light for 3 min had a titre of 1×10^1 (essentially 100% inactivated), and heated virus, a titre of 3.9×10^6 making it 99.9% inactivated.

Figure 3.8.a. shows that cells primed with live virus respond to both U.V. and heat-inactivated HRV 1A; the response to U.V. inactivated virus is similar to that seen with live virus. Immunisation with U.V. inactivated HRV 1A generated T cells which responded to all three virus preparations (figure 3.8.b.) and the optimum responses occurred over a similar range of virus concentrations as did those of cells primed with native virus. Therefore the ability of low concentrations of HRV 1A to stimulate Th cell responses in vivo or in vitro was not related to the ability of virus to replicate. It was interesting to note that the heated HRV 1A primed for lower levels of proliferation against HRV 1A and U.V. inactivated HRV 1A, and that the proliferative response of these cells to heated HRV 1A was even lower (figure 3.8.c.). The in vitro responses of cells primed against HRV 1A and U.V. inactivated HRV 1A were also slightly lower using in vitro heated virus as antigen, compared to the response against live virus. This suggested that heat-inactivated HRV 1A was less efficient at generating T cell responses both in vivo and in vitro.

3.9. Discussion

In the course of experiments designed to determine the optimal conditions for measuring Th cell response to HRV 1A, a number of interesting observations were made which suggest that HRV 1A is unusually immunogenic in mice. The amount of virus required to prime for a proliferative CD4+ Th cell response was much lower than the amounts of protein used to prime for similar responses to many experimental protein antigens such as ovalbumin, which is normally used in amounts of 10-200 μ g per animal. In contrast, amounts of HRV 1A as low as 0.2 μ g were shown to be sufficient to generate effective Th cell responses. The in vitro concentration of HRV 1A required to give optimum proliferation was 10-100 fold lower than those reported for virus specific Th cell responses in mice immunised with another picornavirus, poliovirus (Wang et al., 1989). The observation that the HRV 1A specific Th cell response remained relatively strong 28 days after priming was also an indication of the high degree of immunogenicity of minor group HRVs.

The results obtained using U.V. inactivated or heat inactivated virus confirmed that the high level of immunogenicity at low HRV 1A virus concentrations was not a product of virus replication in the immunised animals. However, heat inactivated HRV 1A gave lower levels of cellular proliferation, whether used in vivo or in vitro. Treatment of minor group HRVs at low pH (pH 4-5), with 2M urea or at high

temperatures (60°C) alters the conformation of the virus particles, such that antibodies directed against the native virus fail to recognise the altered forms of virus (Lonberg-Holm and Yin, 1973). Acid treated virus particles were shown to have a reduced ability to attach to cells, leading to a loss in infectivity (Noble and Lonberg-Holm, 1973). Thus the reduction in the Th cell immunogenicity of heated HRV 1A may be a function of the altered conformation of the virus capsid.

The Th cell response to HRV was shown to be highly cross-reactive, with Th cells primed using HRV 1A responding well to eight of the nine HRV serotypes tested in three haplotypes. In contrast to these results, responses to HRV 15 when used as an immunogen and in vitro were much lower. Heterotypic Th cell responses have been reported for the three serotypes of poliovirus (Wang et al., 1989; Katrack et al.1991) but the response to poliovirus has also been described as serotype specific (Leclerc et al., 1991).

The ability of cells primed towards one HRV serotype to cross-react with other HRV serotypes was encouraging for the development of peptide vaccines based upon defined HRV Th cell epitopes. The panel of HRV serotypes used in this study represented approximately one twelfth of the total number of HRV serotypes identified to date. There is also a high level of sequence homology between certain regions of the viral proteins of serotypes (Palmenberg 1989) not tested in this assay and HRV 1A, suggesting that HRV 1A primed Th cells may cross-react with a number of other virus serotypes.

There is also an indication that the Th cell response to HRV in humans is cross-reactive with other HRV serotypes. Lymphocytes from human volunteers infected experimentally with HRV were shown to proliferate in vitro, even though the absence of an antibody response to the experimental virus indicated that they had not been exposed to the HRV serotype prior to the experiment (Hsia et al., 1990). This finding suggested that cells previously primed by infection with another HRV serotype, or serotypes, were capable of recognising similar determinants on the experimental HRV serotype. Cross-serotype Th cell responses to poliovirus have also been demonstrated using cloned human Th cells and similar cross-serotype responses have been seen with bovine T lymphocytes in response to FMDV. It therefore appears that there may be similarities in the Th cell response to a variety of picornaviruses and the results obtained from investigating the Th cell response to HRV may contribute to the understanding of immune responses to other picornaviruses.

All three mouse strains used in this study mounted similar heterotypic responses to HRVs when immunised with serotype 1A, demonstrating that T cell recognition of HRV 1A and the other serotypes could occur in the context of a variety of MHC haplotypes. Differences in the T cell proliferative responses of inbred mouse strains have been reported for two picornaviruses, poliovirus (Wang et al, 1989) and TMEV (Clatch et al, 1987). Again our results are encouraging for the development of an HRV based Th cell

vaccine, but there was no indication that the same epitopes were recognised in each of the different haplotypes.

Human rhinoviruses can be divided into two groups, major and minor, depending on the cellular receptor to which they bind (Abraham and Colonno, 1984). HRV 1A primed cells from all three mouse strains showed a similar pattern in their responses to HRV serotypes from the two different receptor binding groups, responding preferentially to other minor receptor group serotypes. Minor group serotypes 1A, 1B, 2, 29 and 49 gave high incorporation of radiolabel at low concentrations of in vitro antigen, whereas major group viruses required high concentrations of virus to produce similar or lower levels of proliferation. No proliferation was observed with HRV 15 in Balb/c mice at the concentrations used whereas when mice were immunised with HRV 15 the cells proliferated to the homologous virus but to a much lower extent than did HRV 1A primed cells with HRV 1A. HRV 15 primed cells also proliferated with HRV 3, 7, and 14 but not with the minor group viruses. The results suggested that there was a fundamental difference in the ability of the two receptor groups of virus to stimulate a T cell response. The possible molecular basis for this phenomenon is studied in more detail in chapter 4.

Figure 3.1. Effect of cell number on HRV 1A specific proliferation of lymph node cells

Lymph node cells taken from Balb/c mice immunised with 0.2 μ g HRV 1A were cultured with virus in triplicate at different cell numbers per well. The dose response curves show the mean ^3HT incorporation at each virus concentration minus the ^3HT incorporation of cells cultured in medium alone (background ^3HT incorporation).

- 6x10⁶ cells/ml
- 5x10⁶ cells/ml
- 4x10⁶ cells/ml
- 3x10⁶ cells/ml

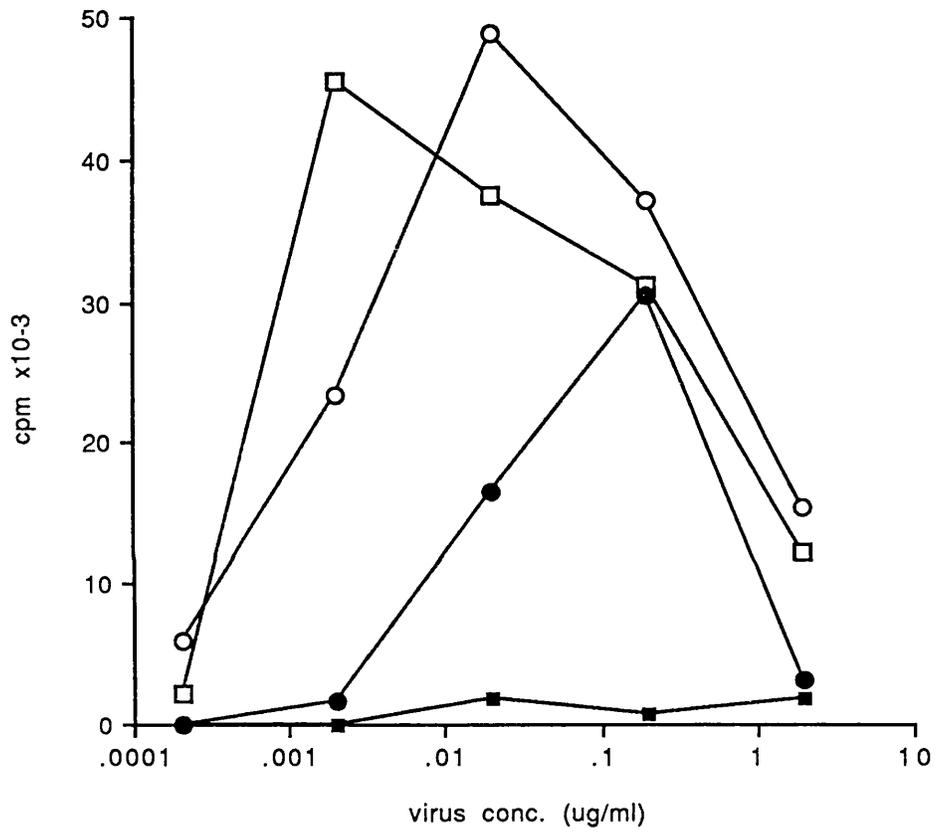


Figure 3.2. Cellular proliferation of HRV 1A specific lymph node cells after 3, 4 and 5 days in culture

HRV 1A specific lymph node cells were cultured with a range of virus concentrations for different time periods. ^3HT incorporation was measured 16 hours after pulsing, and the results show the mean ^3HT incorporation of triplicate culture wells minus the background cellular proliferation.

○ 3 days

□ 4 days

△ 5 days

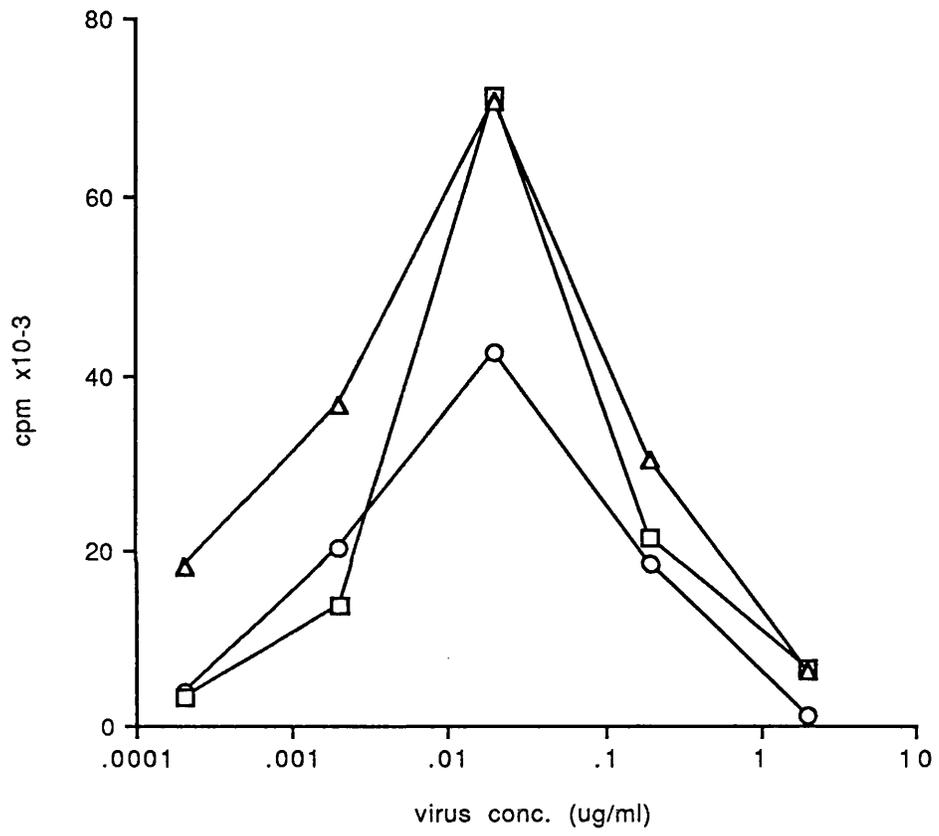


Figure 3.3. Cellular proliferation of lymph node cells primed using different amounts of HRV 1A

Cells from Balb/c mice immunised with 1, 0.2 and 0.04 μ g of HRV 1A were cultured in triplicate with a range of in vitro concentrations of virus. ^3HT incorporation was measured and the results show the mean ^3HT incorporation of triplicate culture wells minus the background cellular proliferation.

- 1.0 μ g HRV 1A
- 0.2 μ g HRV 1A
- Δ 0.04 μ g HRV 1A

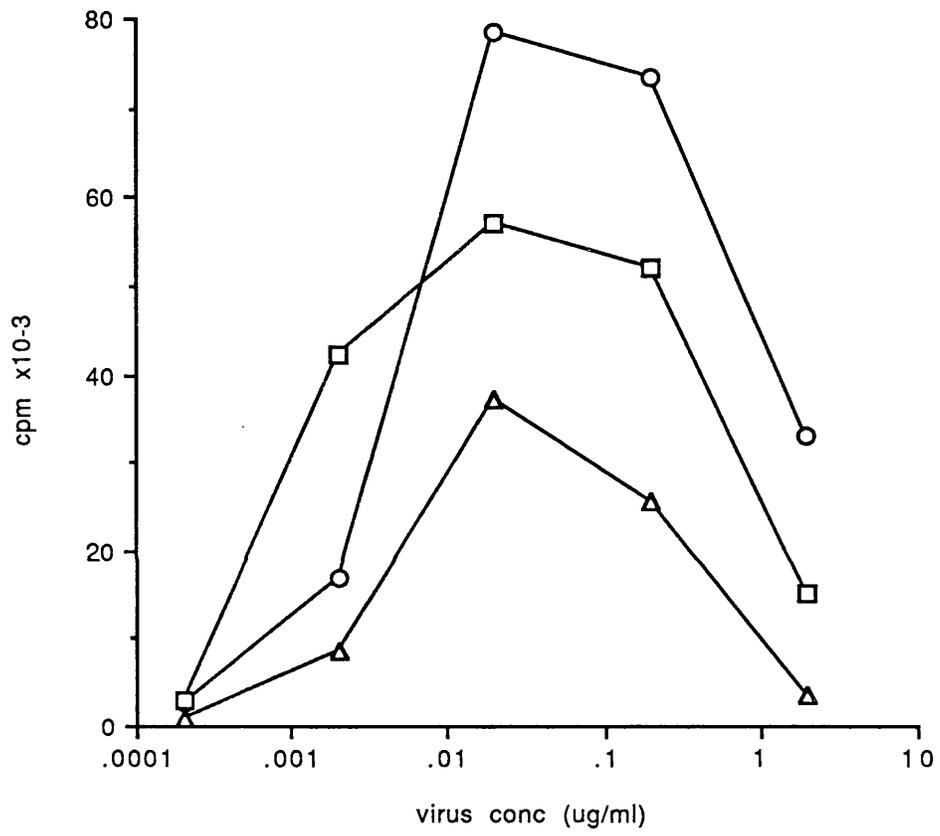


Figure 3.4. Antigen specific proliferative response of cells taken from animals at different time intervals post immunisation

Four groups of Balb/c mice were immunised with 0.2 μ g HRV 1A and the virus specific cellular proliferative response was determined at 7, 14, 21 and 28 days post immunisation (figure 3.4.a.). A similar experiment was performed using mice immunised with 50 μ g of a peptide containing a B cell epitope cosynthesised with a 10 amino acid T cell epitope from the HRV 2 sequence (VP2 156-170, VP1 251-260) and tested in vitro with peptide over a concentration range of 100-1 μ g/ml (figure 3.4.b.) 3 HT incorporation was measured and the results show the mean 3 HT incorporation of triplicate wells minus the background cellular proliferation.

- 7 days
- 14 days
- 21 days
- 28 days

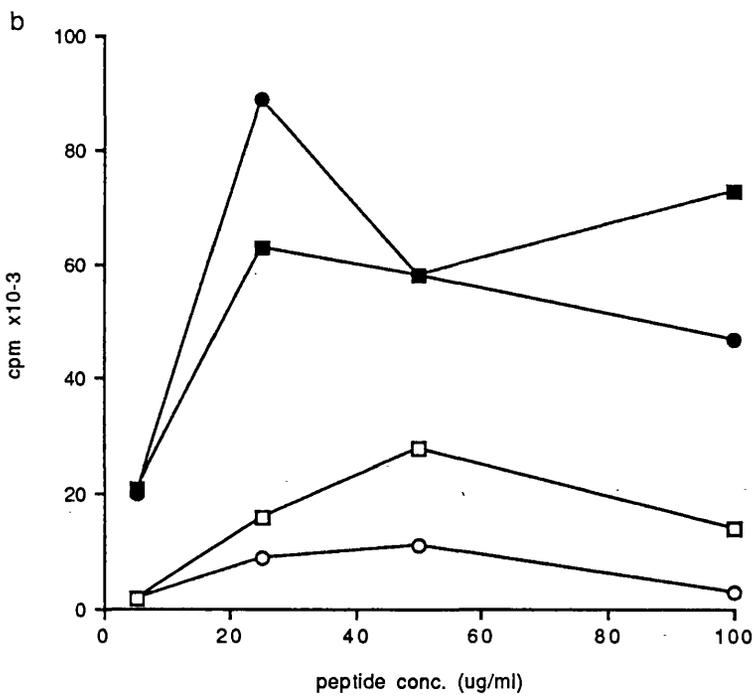
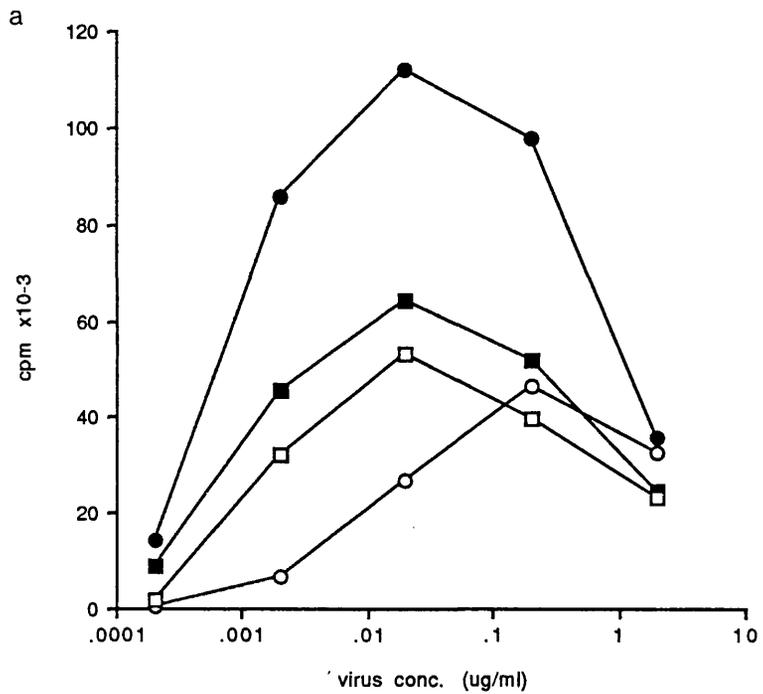


Figure 3.5. Cellular proliferation of HRV 1A primed H-2^d cells to nine virus serotypes

Balb/c mice were immunised with 0.2 μ g HRV 1A and the cellular immune response to HRV 1A and eight other HRV serotypes was determined in triplicate over a range of virus concentrations. The results are expressed as the mean ³HT incorporation of triplicate wells minus the background ³HT incorporation. The results represent the mean of three experiments and the error bars the S.E.M.

HRVs 1A, 1B, 2, 29, and 49 belong to the minor group;

HRVs 3, 7, 14, and 15 belong to the major group.

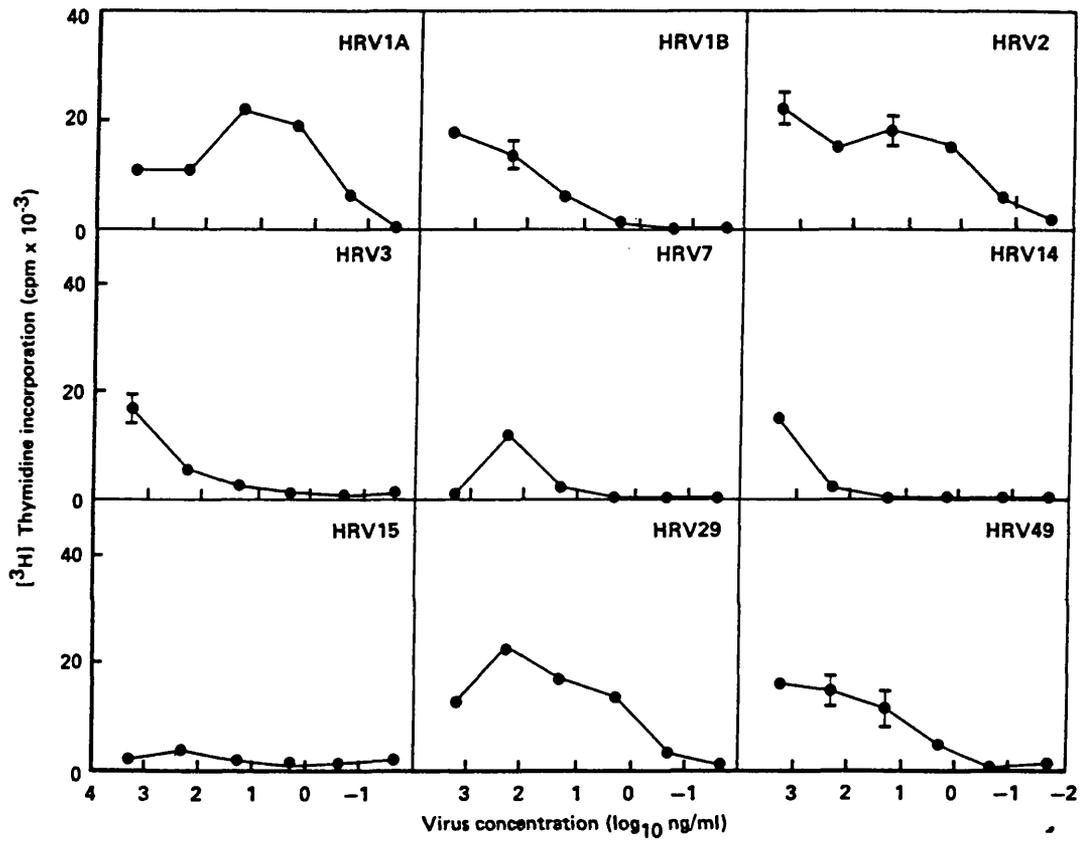


Figure 3.6. Cellular proliferation of HRV 1A primed cells from other mouse haplotypes to the nine virus serotypes

C57/BL10 (H-2^b) and C3H.He (H-2^k) mice were immunised with 0.2 μ g HRV 1A and the cellular immune response to HRV 1A and eight other HRV serotypes was determined over a range of virus concentrations. Figure 3.6.a. shows the proliferative responses of H-2^b mice and figure 3.6.b. shows the responses of H-2^k mice. The results are expressed as the mean ³HT incorporation of triplicate culture wells minus the background ³HT incorporation. The results for both figures 3.6.a. & b. represent the mean of two experiments and the error bars the S.E.M.

HRVs 1A, 1B, 2, 29, and 49 belong to the minor group;

HRVs 3, 7, 14, and 15 belong to the major group.

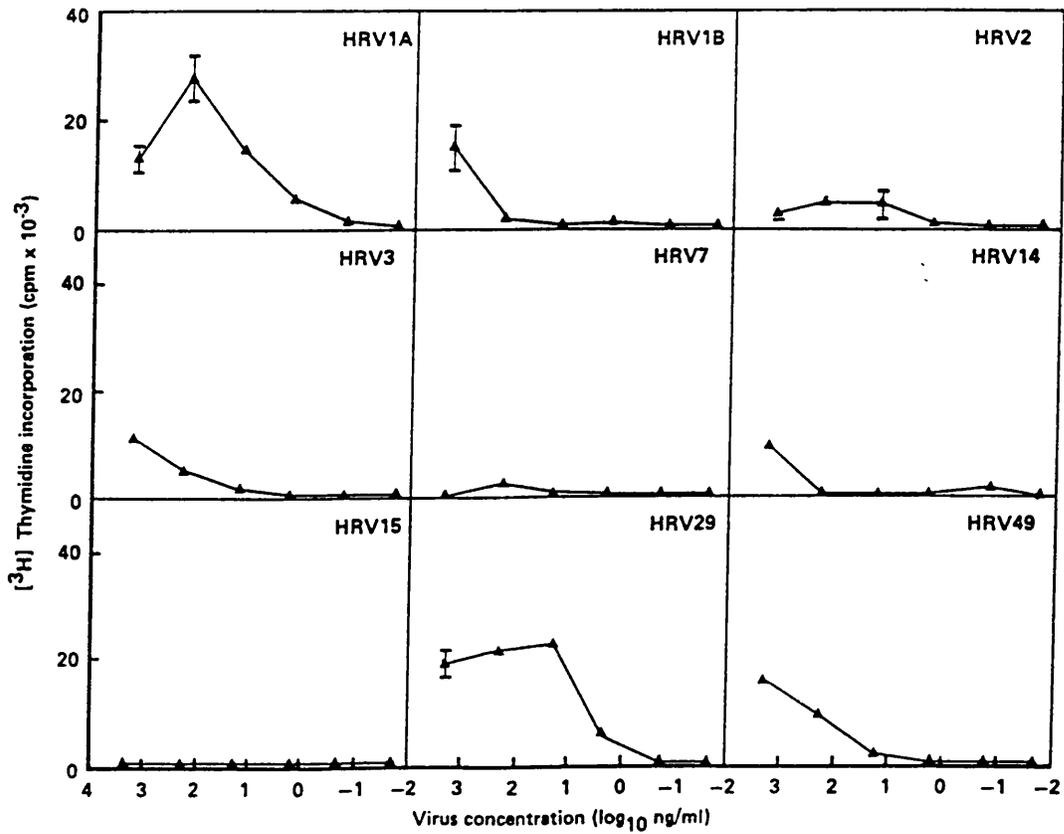
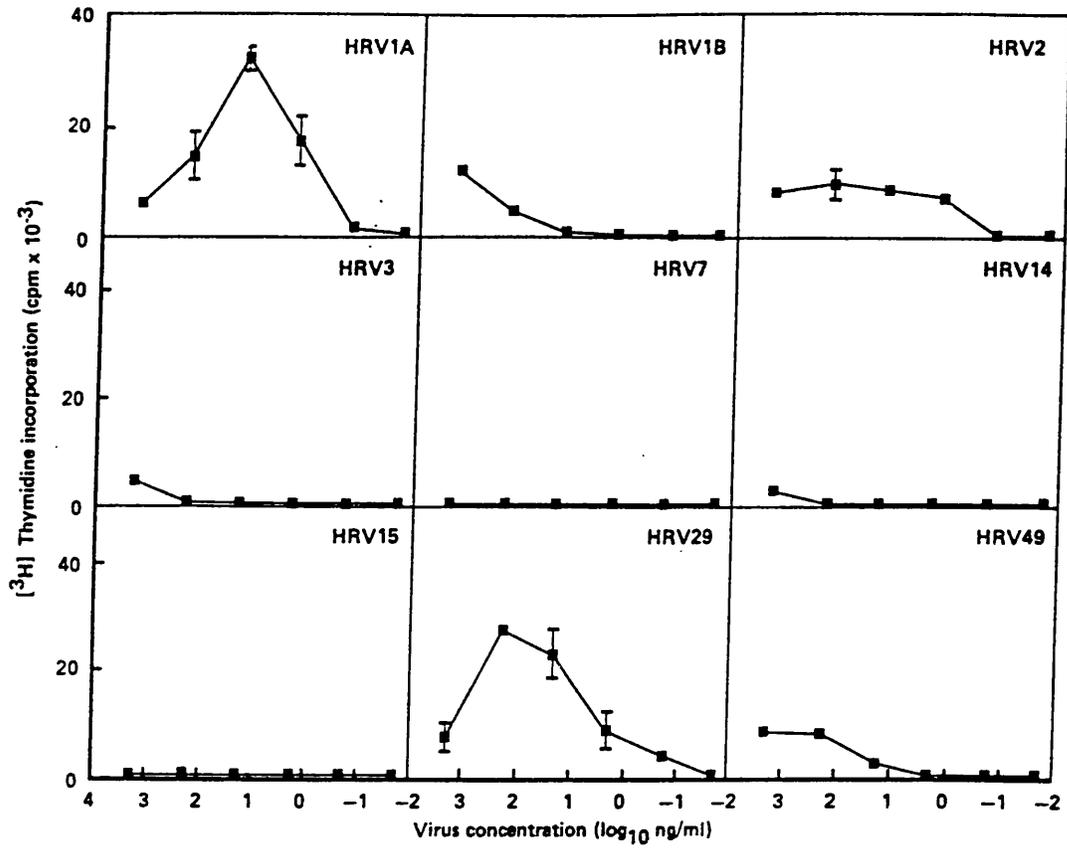


Figure 3.7. Cellular proliferation of HRV 15 primed H-2^d cells to the nine virus serotypes

Balb/c mice were immunised with 0.2 μ g HRV 15 and the cellular immune response to HRV 1A and eight other HRV serotypes was determined over a range of virus concentrations. The results are expressed as the mean ³HT incorporation of triplicate culture wells minus the background ³HT incorporation. The results represent the mean of three experiments and the error bars the S.E.M.

HRVs 1A, 1B, 2, 29, and 49 belong to the minor group;

HRVs 3, 7, 14, and 15 belong to the major group.

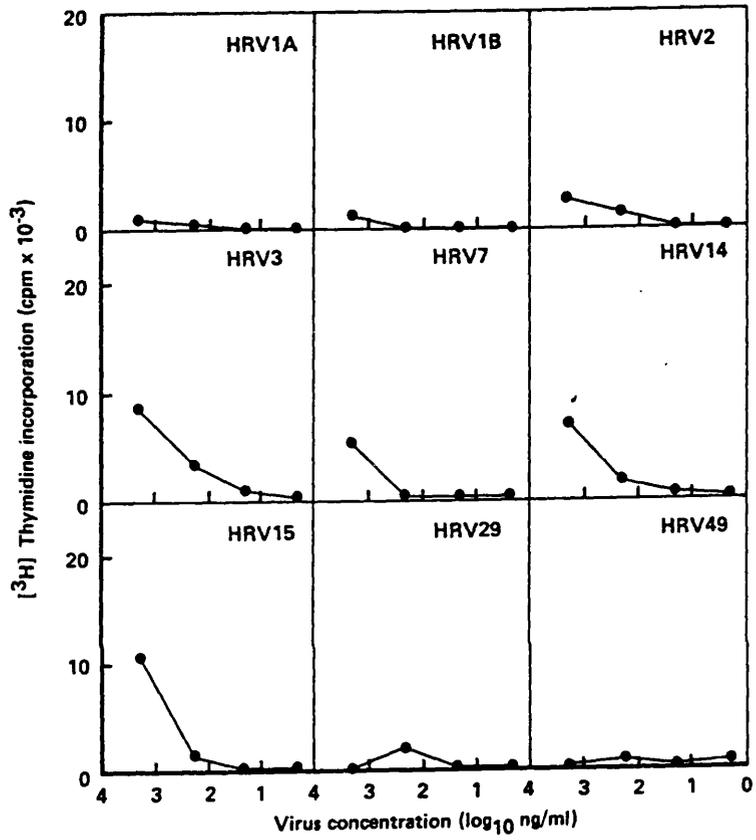


Figure 3.8. Proliferative responses of cells primed with native, U.V. inactivated and heat inactivated preparations of HRV 1A

Three groups of eight Balb/c mice were immunised with 0.2 μ g of either HRV 1A, U.V. inactivated HRV 1A or HRV 1A heated at 60°C for 1h. Ten days post immunisation the cellular proliferative responses towards each of the three virus preparations were measured. Figure 3.8.a. shows the response of HRV 1A primed cells towards the three virus preparations, figure 3.8.b. shows the response of mice immunised with U.V. inactivated HRV 1A , and figure 3.8.c. shows the cellular proliferative responses of mice immunised with heated virus. The results are expressed as the mean ³HT incorporation of triplicate culture wells minus the background ³HT incorporation.

- live HRV 1A
- U.V. inactivated HRV 1A
- △ heat-inactivated HRV 1A

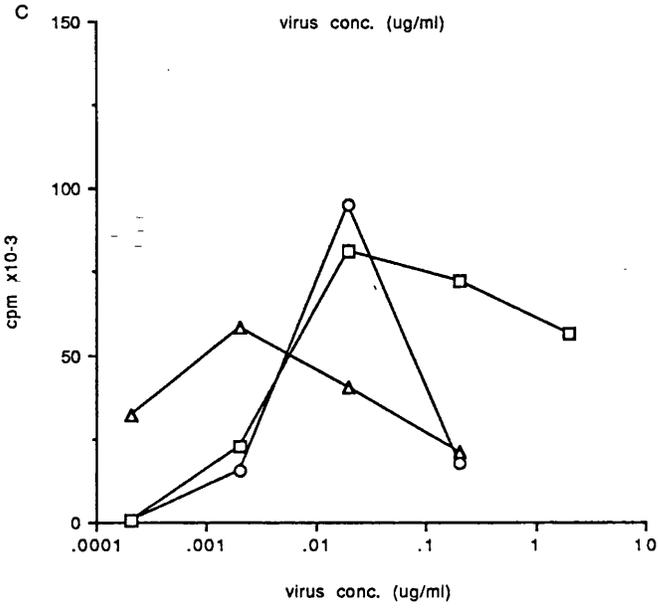
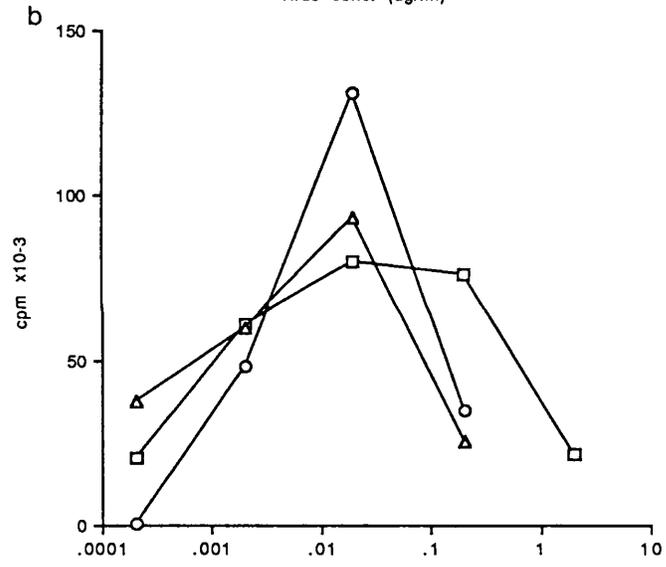
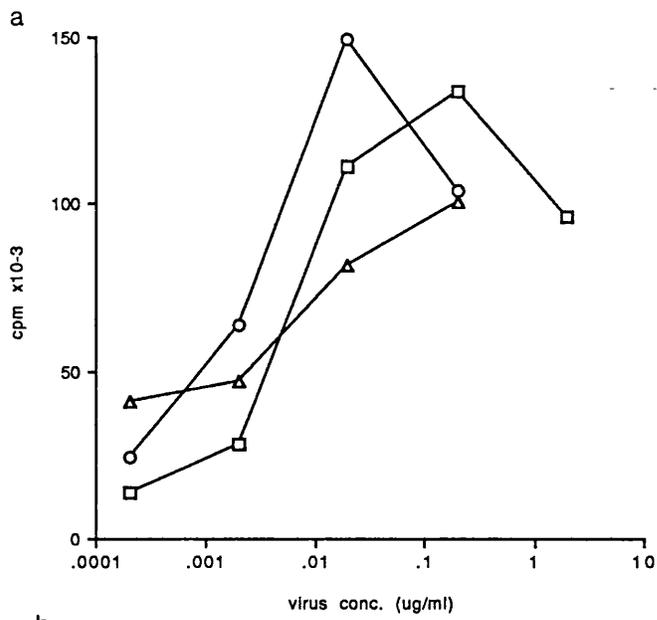


Table 3.1. In vitro cellular proliferation assays show better correlation when cells are cultured with MS

The results shown are taken from randomly selected assays performed during 1989. All assays were carried out with cells taken from Balb/c mice immunised with 0.2 μ g HRV 1A and cells were cultured in media containing either 10% FCS or 0.5% MS. Cellular proliferation was measured over a range of antigen concentrations and the results were expressed as the mean ³HT incorporation of three culture wells. The mean ³HT incorporation of three wells containing medium alone is also shown.

virus conc (ug/ml)	serum					
	10% FCS	10% FCS	10% FCS	10% FCS	0.5% NMS	0.5% NMS
0.2	84 869	35 410	47 743	21 620	11 327	26 446
0.02	86 667	21 727	20 768	31 529	23 897	23 959
0.002	131 853	13 228	6 245	36 207	31 572	21 896
cell background	37 990	13 533	4 811	1 173	1 359	1 275

Table 3.2. The cellular proliferative response to HRV serotypes is virus specific

Balb/c mice were immunised with PBS emulsified 1:1 with FCA and the lymph node cells were assayed with each of nine HRV serotypes at concentrations of 2.0 and 0.2 μ g/ml. The results are expressed as the mean 3 HT incorporation of triplicate wells minus the background 3 HT incorporation. Cells were also cultured in triplicate with Hela cell extract at 2.0 and 0.2 μ g/ml protein and 10 and 1% solutions of sucrose in PBS.

virus conc. (ug/ml)	virus serotype										Hela extract (ug/ml)	cpm	sucrose cpm (%)
	1A	1B	2	3	7	14	15	29	49	73			
2	1027	76	3012	702	45	26	65	34	73	2	79	10	0
0.2	546	0	2126	0	0	0	0	898	126	0.2	46	1	0

Table 3.3. Antibody and complement depletion of HRV 1A primed lymph node cells

The proliferative response of cells treated with specific antibody and complement to HRV 1A at 0.02 and 0.002 μ g/ml was determined and the results are expressed as the mean 3 HT incorporation of triplicate culture wells minus the background 3 HT incorporation.

antibody used to treat cells					
HRV1A	none	CD4	CD8	Thy1	TIB 120
(ug/ml)					
0.02	21 555	N.D.	21 339	N.D.	112
0.002	22 781	1 961	27 248	6	0

CHAPTER 4. Immunogenicity of human rhinovirus and the role of virus/cell interaction

4.1. Introduction

As detailed in the introduction, human rhinovirus serotypes can be divided into two groups by virtue of their ability to bind to different cellular receptors (Abraham and Colonno 1984). The major group of viruses have been shown to bind to the cell surface molecule ICAM-1 (CD54) (Greve et al., 1989; Staunton et al., 1989) and the minor group of viruses bind to cells via an as yet unidentified receptor. The interaction between HRV and CD54 is species-specific, and does not occur in rodents. However, minor type HRVs do bind specifically to murine as well as human cells, although this binding does not lead to productive infection of the target cell (Yin and Lomax, 1983).

The observation (chapter 3; Hastings et al., 1991) that Th cells from mice immunised with minor group viruses proliferated preferentially with HRVs of the same receptor group, and that the overall level of response was higher towards minor group virus suggested that there were differences in the abilities of the two groups of HRV to prime Th cell responses in vivo and/or stimulate in vitro cellular responses. This different species specificity of major and minor group HRV binding was used to explore the possible role of this binding in modulating T cell immunological responses.

Thus the Th cell responses to HRV serotypes belonging to different receptor groups were studied in more detail, as was their ability to bind to cells from the murine immune system.

The overall conclusion drawn from the experimental findings was that the binding of HRV 1A to cells of the murine immune system, particularly antigen presenting cells, contributed to its high level of immunogenicity in mice. The demonstration that inhibition of the specific binding of minor group HRVs to murine cells reduced the levels of antigen specific proliferation, suggested that the enhanced immunogenicity of minor receptor group viruses was occurring via a receptor mediated, facilitated uptake of the virus by cells of the immune system.

4.2. HRV 1A is more effective than HRV 15 in stimulating in vitro T cell responses

Balb/c mice (H-2^d) were immunised with different concentrations of HRV 1A and HRV 15 in FCA and virus specific cellular proliferation was measured over a range of antigen concentrations (figure 4.1). HRV 1A primed mice gave high in vitro levels of cellular proliferation over a wide range of virus concentrations (0.2-0.002 μ g/ml) and the level of proliferation remained high even at the lowest immunising concentration, 0.1 μ g per mouse. HRV 15 required a 1000 fold higher concentration of virus in vitro to give similar levels of proliferation to those seen with HRV 1A. Also, the maximum

proliferative responses to HRV 15 were only 50% of the maximum response seen with HRV 1A. The in vivo and in vitro doses required to give HRV 15 specific cellular proliferation are similar to those reported for mice immunised with poliovirus (Wang et al., 1989), which does not infect murine cells and binds only to a small degree (Selinka et al., 1992).

4.3. HRV 1A has intrinsic adjuvant activity in its ability to induce Th cell responses

Groups of five H-2^d mice were immunised with 0.2 μ g per mouse of HRV 1A and 15 in adjuvants FCA, FIA, aluminium hydroxide, and in PBS alone. The dose response curves obtained with HRV 1A (figure 4.2.a) showed that a strong Th cell response was generated by each of the three adjuvants, and also in the absence of adjuvant. A high level of proliferation was seen with the group immunised with aluminium hydroxide, a similar finding to that reported for poliovirus administered in alum (Katrack et al., 1991), but an elevated proliferative response was not seen with HRV 15 immunised mice (figure 4.2.b). Cells from mice immunised with HRV 15 in PBS alone did not respond to virus. Repeat experiments with HRV 1A showed that the level of the proliferative response of mice given virus in aluminium hydroxide was at least as high as the response of FCA immunised mice. The experiment performed with HRV 1A gave lower overall levels of ³HT incorporation than was typical. Additional experiments using 0.2 μ g HRV 1A in PBS to

immunise mice generated variable proliferative responses, suggesting that HRV 1A was at a limiting concentration. Therefore mice were immunised with $1\mu\text{g}$ of HRV 1A or HRV 15. Proliferative responses were seen with cells from HRV 1A primed mice but not with those from HRV 15 (figure 4.3). Indeed, priming with $1\mu\text{g}$ of HRV 1A in PBS raised a similar level of response to that generated by immunisation with $0.2\mu\text{g}$ HRV 1A in FCA.

4.4. HRV 1A has intrinsic adjuvant activity in its ability to raise virus specific antibody responses

In an investigation into the in vivo immunogenicity of HRV 1A, groups of eight Balb/c mice were immunised i.m. with different concentrations of HRV 1A emulsified 1:1 in Span-Marcol, an oil adjuvant licensed for use in animals which acts in a similar manner to FIA. Mice were also immunised with $0.2\mu\text{g}$ of HRV 1A in aluminium hydroxide and PBS. The antibody responses to virus were monitored over a period of ten weeks and compared to the responses of mice immunised with different doses of a "picornavirus-like" plant virus, cowpea mosaic virus (CPMV), also in Span-Marcol, aluminium hydroxide and PBS. The purpose of this study was to compare the immunological response to a non-mammalian virus to which the mice would almost certainly be immunologically naive. The antibody titration curves (figures 4.4 a & b) show that lower concentrations of HRV 1A are required to generate higher virus

specific antibody titres than those observed with CPMV. CPMV was not immunogenic at the lowest dose in Span-Marcol (0.002 μ g) or in aluminium hydroxide, and when given in PBS, raised only low antibody titres after secondary immunisation with virus. However, HRV 1A was highly immunogenic in both aluminium hydroxide and PBS and at the lowest dose in Span. It would have been interesting to repeat this experiment using different doses of HRV 15 in the different adjuvants, but time did not allow these experiments to be performed. However, it can be concluded that HRV 1A is highly immunogenic and induces strong antibody and Th cell responses.

4.5. Minor group HRVs bind specifically to cells from the murine immune system

Previous binding studies used L cells, a mouse fibroblast cell line, to demonstrate that only minor group HRV serotypes bound to mouse cells. It proved difficult to obtain a preparation of radiolabelled HRV 1A which has been used throughout this present study, so radiolabelled HRV 2, a closely related virus serotype belonging to the minor receptor group, was prepared and used in the assays described below. Figure 4.5 shows the binding of radiolabelled HRV 2, a minor group virus, and HRV 15, a major group virus, to mouse B and T cell lines, and splenic B and T lymphocytes. The human cell line OH-5 HeLa was also included as a positive control. Over 28% of the total counts of HRV 2 added bound to the murine T

cell line IE5 and to activated splenic T cells, and a significant amount (over 5%) of HRV 2 bound to the B cell line A 20 and activated splenic B cells. However, binding was not demonstrated to normal, resting spleen cells. In agreement with previous studies (Abraham and Collono 1984) no binding occurred at 4°C. In contrast, HRV 15 did not bind significantly to either of the murine cell lines but did bind to HeLa cells, as did HRV 2.

The binding of HRV 2 to murine cells was inhibited by the addition of excess unlabelled HRV 2 (5µg/ml), and by HRV 1A at the same concentration, but was not inhibited by type 15 virus, confirming that the binding of the minor group viruses to the murine cells was saturable and receptor group specific (figure 4.6).

4.6. Minor group HRVs bind to cells of the mouse immune system, including antigen presenting cells

The large number of cells required to perform radioimmune assays excluded the possibility of assaying direct binding to murine antigen presenting cells, which could not be obtained in sufficient numbers by this method. Instead an immunofluorescence assay was developed using virus specific purified rabbit IgG to detect virus bound to cells. The assay was developed using OH-5 cells and both HRV 1A and HRV 15 were used to demonstrate virus binding (figure 4.7). It was interesting to note the different appearance of the cell

labelling. HRV 15 labelled evenly over the cell surface, whereas cells with bound HRV 1A were labelled in patches, often a single cluster, possibly indicating capping of the cell membrane molecules.

Binding of HRV 1A was detected on a preparation of normal spleen cells enriched for lymphoid dendritic cells (figure 4.8 and Table 4.1). Contaminating B cells, T cells and macrophages were excluded by labelling with anti-mouse Ig and anti-mouse CD3, using two colour immunofluorescence to visualise the markers and the virus simultaneously. Approximately 60% of cells were lymphoid dendritic cells and HRV 1A was shown to bind to this population, and to a lesser degree to murine B and T cells and macrophages (Table 4.1). HRV 1A had bound to 17% of the total number of cells counted, 12.9% were dendritic cells (cells not labelled with the T and B cell specific staining) and 4.1% were B cells, T cells, or macrophages. Assuming that all cells not labelled as contaminating cells were dendritic cells, 18% of this population had HRV 1A bound to their surface. Control experiments demonstrated that the antibodies were serotype specific and did not bind directly to murine cells, and also that HRV 15 did not bind to the cell preparation.

4.7. Cellular proliferation to virus is blocked by virus specific antibodies

To test the importance of virus/cell interaction in

generating an immune response, both major and minor group viruses were pre-treated with virus specific neutralising antibodies prior to their use as an in vitro antigen in cell proliferation assays. HRV 1A specific antibodies, but not control antibodies, inhibited the proliferative response to HRV 1A in a dose-dependent way (Table 4.2). Fab fragments generated from the HRV 1A specific antibodies also inhibited proliferation of HRV 1A specific cells, indicating that the antibodies were blocking in a manner which was not dependent on the Fc portion of the molecule. In contrast, neutralising antibodies to HRV 15 failed to inhibit HRV 15 specific proliferation. Therefore, formation of complexes between antigen and antibody per se did not block the processing and presentation of virus and in fact an enhancement was seen with HRV 15, probably due to immune complexes binding Fc receptors. In control experiments with both HRV 1A and 15 a non-virus specific purified IgG (pIgG) did not block or enhance cellular proliferation in response to antigen.

4.8. Discussion

The observation that HRV 1A, and other minor receptor group viruses, exhibited an enhanced immunogenicity at the level of the Th cell response to virus compared to major group viruses lead to the hypothesis that binding of minor receptor group viruses to murine cells was involved in this process. The demonstration that minor receptor group HRVs bound to

cells from the murine immune system was in agreement with this hypothesis. Virus was shown to bind to those cells involved in antigen processing and presentation, and also to activated murine T and B cells from mitogen stimulated spleen cells and hybridoma cell lines. In both cases T cells bound more virus than B cells, possibly due to different levels of expression of receptor on these cell types. Virus bound to different cell types could play different roles in the generation of enhanced immune responses. Virus bound to B cells could enhance their ability to process and present antigen (Chesnut and Grey, 1981), whereas by binding to T cells virus could act as an antigen bridge, bringing cells into closer proximity and helping to maintain this interaction possibly allowing secreted cytokines to be more effective.

Binding of HRV 1A to "professional" antigen processing cells indicated a role for these cells in the enhanced immunogenicity of minor group HRVs. Rhinoviruses are thought to enter cells by a process of receptor mediated endocytosis and become internalised into endosomal compartments (Neubauer et al., 1987). Recovered virus particles displayed similar antigenic features to virus which had been treated with acid (Korant et al., 1975) indicating that virus had undergone conformational change which resulted in the degradation of VP2 and cleavage of VP1. Therefore the route of entry of minor group HRVs into murine cells is similar to that of the exogenous antigen pathway in APCs where external antigen is endocytosed, processed, and presented (Lanzavecchia, 1990;

Levine and Chain, 1991). It is therefore likely that cells which trap antigen, such as minor group HRV, via surface receptors are capable of processing and presenting that antigen to a greater extent.

The effect of targeting antigen onto B-cells, using antibodies against surface molecules conjugated to myoglobin specific antibodies, was to decrease the amount of antigen required for antigen specific T cell proliferation 1000 fold (Ozaki and Berzofsky 1987). Using an antibody conjugated to PPD to target antigen to presenting cells, Montgomery et al. (1992) also showed that lower concentrations of antigen are required for Th responses. A difference of up to 1000 fold was observed in the concentration of virus required to give optimal proliferation of T cells between HRV 1A, which binds to mouse cells, and HRV 15, which does not. This finding suggests that binding of minor group HRVs to virus specific receptors on the surface of murine cells was responsible for the differences in immunogenicity between the two virus groups. Receptor mediated uptake of antigen has been demonstrated using a number of experimental systems in which presentation of antigen was shown to be enhanced (Lanzavecchia 1990).

The receptor mediated enhancement of virus presentation is reflected in the in vivo immunogenicity of minor group rhinoviruses, in their ability to generate Th cell responses (Hastings et al., 1991) and virus specific antibodies. Low doses of HRV 1A were sufficient to prime Th cells in vivo

which responded optimally in vitro to even lower concentrations of virus. Antigen concentrations required for optimal proliferation of HRV 15 primed cells were similar to those reported for poliovirus primed mouse T cells (Wang et al., 1989; Katrack et al., 1991). Another example of the high degree of immunogenicity of minor group HRVs is the ability of HRV 1A to prime cellular immune responses in the complete absence of adjuvant, indicating that it has an intrinsic adjuvant activity, which may be due to its cell binding properties. Therefore binding of minor group HRVs to cells from the murine immune system could account for the differences observed in the immunogenicity of different virus serotypes. Since human cells express receptors for all HRV serotypes, human cells may exhibit a facilitated uptake of all rhinoviruses serotypes which could contribute to the overall immunogenicity of HRVs in their natural host.

Figure 4.1. Virus specific Th cell responses to different in vivo doses of HRV 1A and HRV 15

Groups of five Balb/c mice were immunised with different amounts of HRV 1A or HRV 15. The cellular proliferation was measured over a range of virus concentrations and expressed as the mean ^3HT incorporation of Three culture wells minus the mean c.p.m. of cells cultured in medium alone. The error bars represent the S.E.M.

- 1 μg HRV 1A
- 0.2 μg HRV 1A
- △ 0.1 μg HRV 1A
- 1 μg HRV 15
- 0.2 μg HRV 15
- ▲ 0.1 μg HRV 15

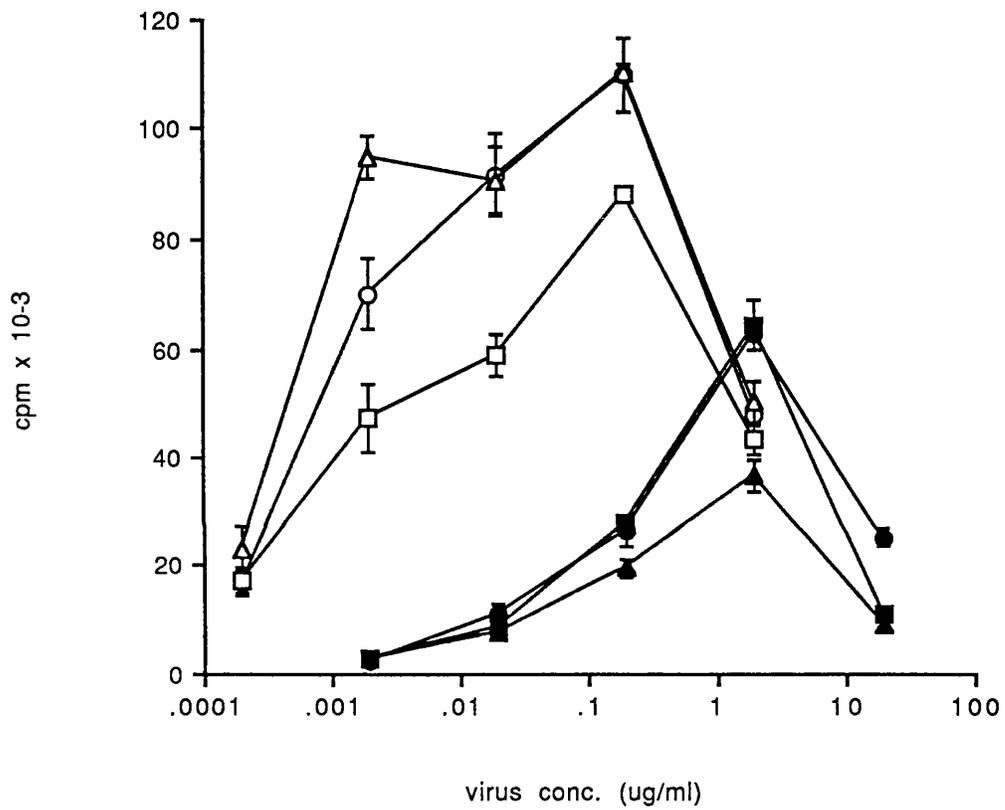


Figure 4.2. Ability of HRV 1A and 15 administered in different adjuvants to prime for Th cell responses

The dose response curves show the proliferation of lymph node cells from Balb/c mice primed with a single dose (0.2 μ g) of a. HRV 1A and b. HRV 15 given in different adjuvants. The cellular proliferation was measured over a range of virus concentrations and expressed as the mean ³HT incorporation of three culture wells minus the mean c.p.m. of cells cultured in medium alone. The error bars shown in figure 4.2.a. represent the S.E.M.

- virus in FCA
- virus in FIA
- virus in aluminium hydroxide
- virus in PBS

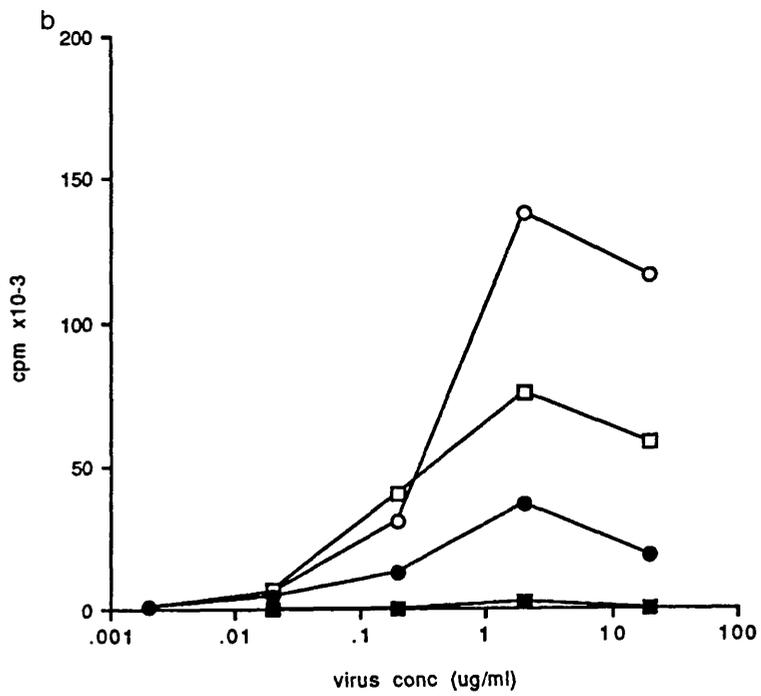
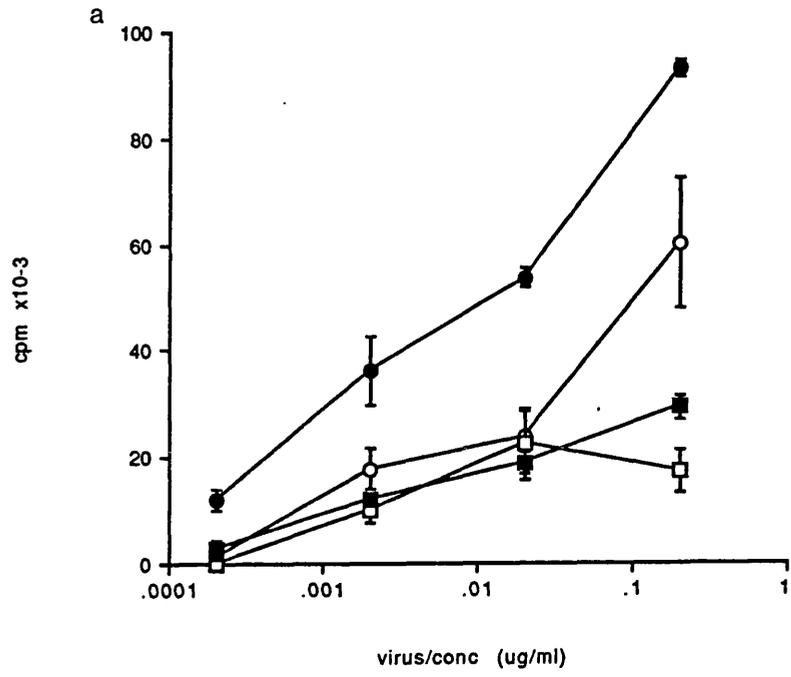


Figure 4.3. HRV 1A is an effective immunogen in the absence of adjuvant

Dose response curves of lymph node cells from H-2^d mice primed with different amounts of HRV 1A and HRV 15 in PBS. Cellular proliferation is expressed as the mean ³HT incorporation of triplicate cultures of cells with each of the virus concentrations minus the mean ³HT incorporation of cells cultured with medium alone.

- cells primed with 1 μg HRV 1A,
- cells primed with 0.1μg HRV 1A,
- cells primed with 1 μg HRV 15,
- cells primed with 0.1μg HRV 15.

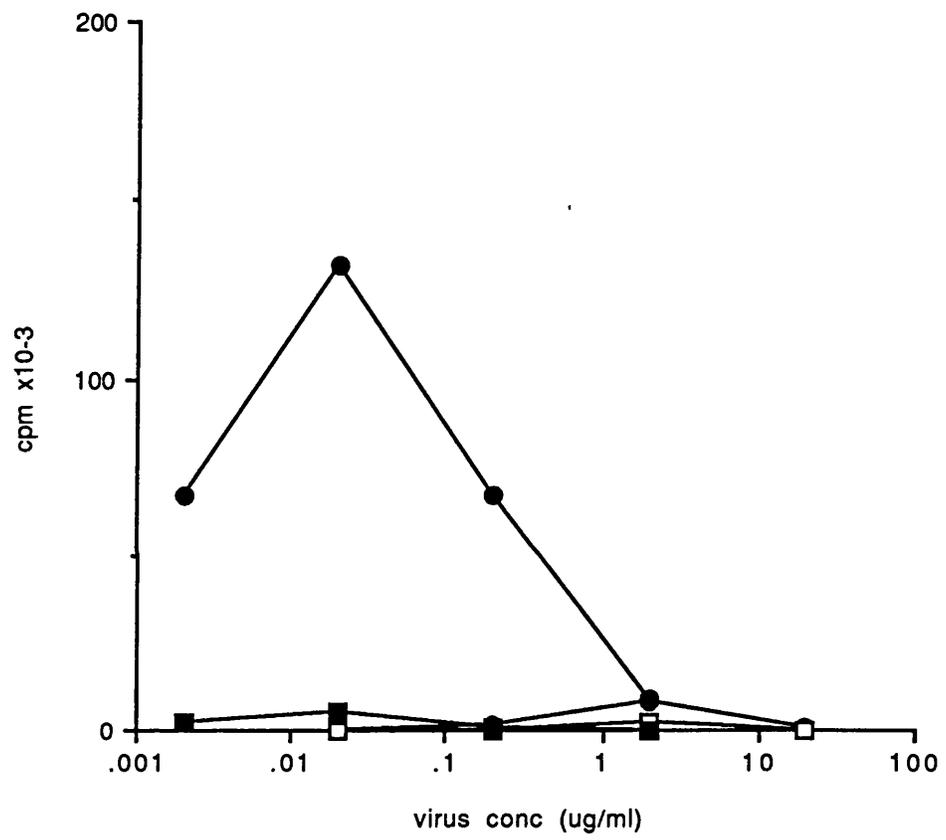


Figure 4.4. Virus specific antibody responses to HRV 1A administered in different adjuvants

The curves show the mean end point titre (expressed as the reciprocal dilution) of sera taken from groups of six Balb/c mice at the time intervals indicated. Mice were immunised on day 0 and received a secondary immunisation 6 weeks later. The virus specific antibody titres were determined by ELISA. Figure 4.4.a. shows the antibody responses of mice immunised with HRV 1A and figure 4.4.b. shows the responses of mice immunised with CPMV.

- 2 μ g virus in Span
- 0.2 μ g virus in Span
- 0.02 μ g virus in Span
- △— 0.002 μ g virus in Span
- 0.2 μ g virus in aluminium hydroxide
- 0.2 μ g virus in PBS

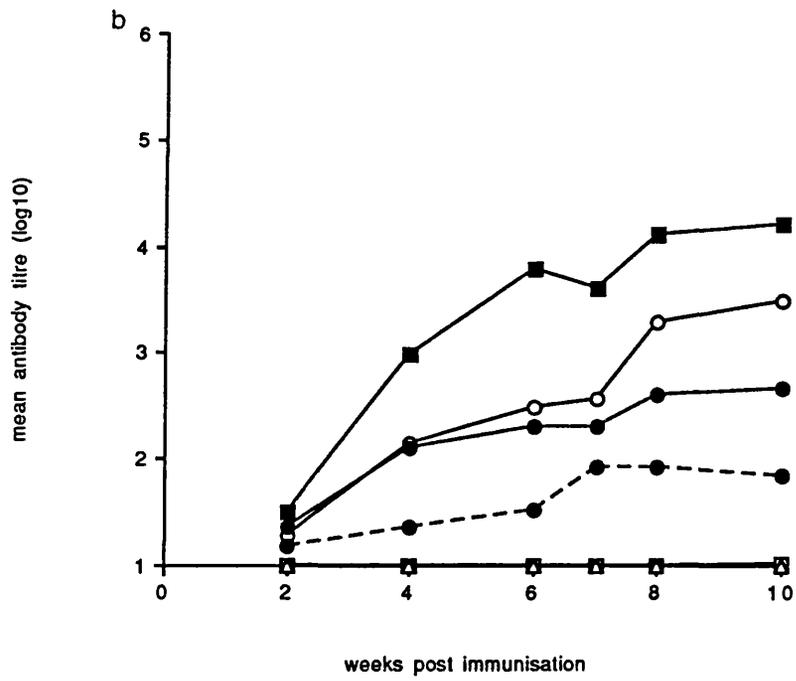
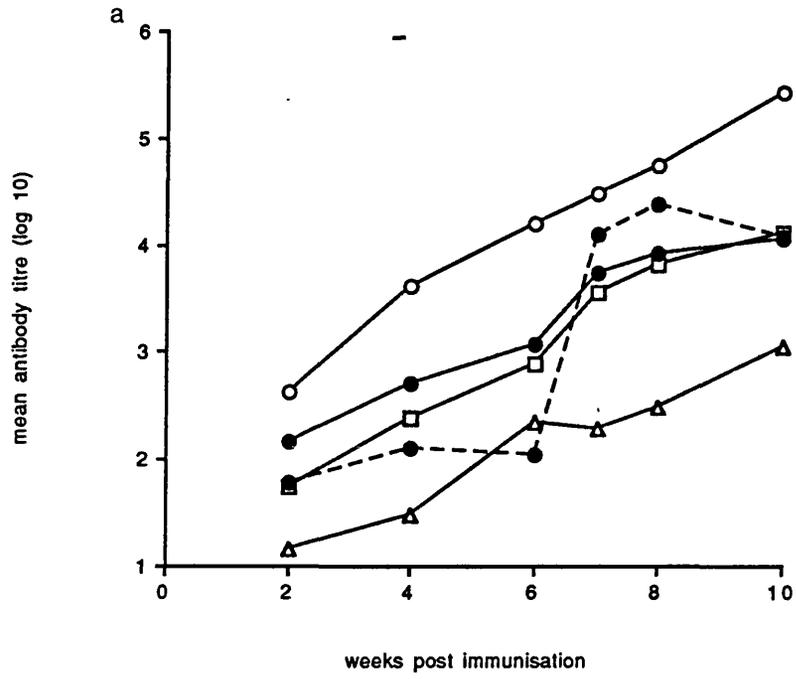


Figure 4.5. Binding of radiolabelled HRV 2 to cells from the murine immune system

The cell preparations were incubated with approximately 2000 counts of radiolabelled virus, either HRV 2 or HRV 15 as indicated. Each bar represents the mean of duplicate determinations of virus bound.

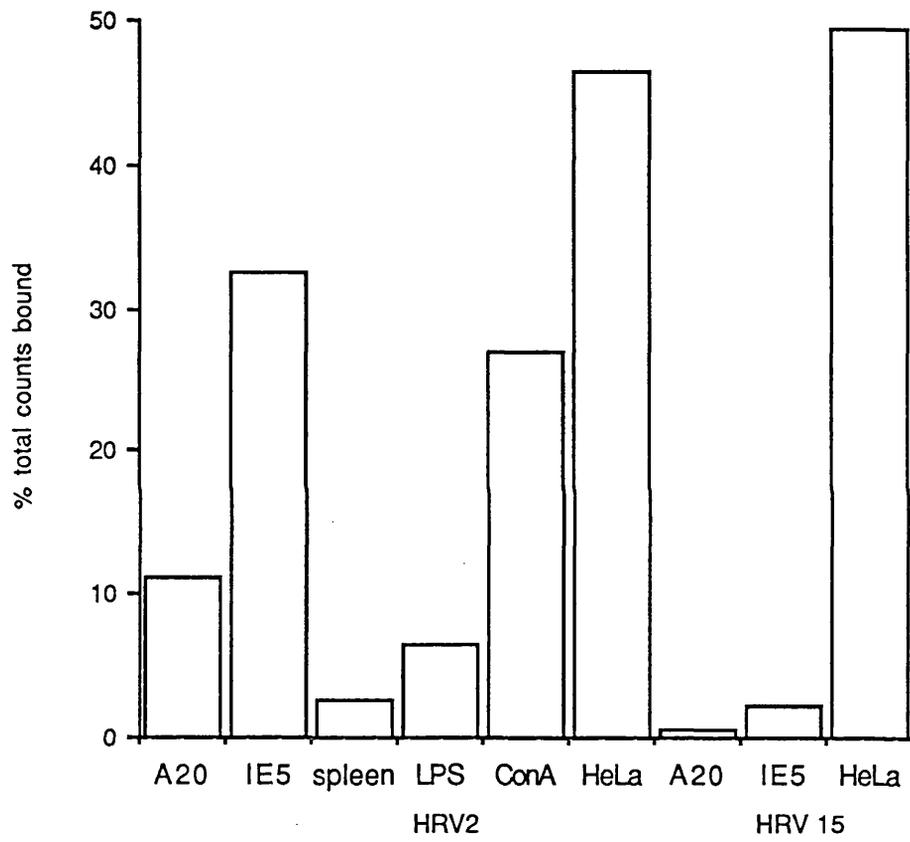


Figure 4.6. Minor receptor group HRVs bind specifically to murine cells.

Preparations of murine cells were incubated with unlabelled HRVs at a final concentration of $5\mu\text{g/ml}$ prior to the addition of approximately 2000 counts of ^{35}S labelled HRV 2. Assays were performed in duplicate and the percentage inhibition of binding compared to that of cells pre-incubated with medium alone (100% binding) was calculated.

■ HRV2, □ HRV 1A, ☒ HRV 15.

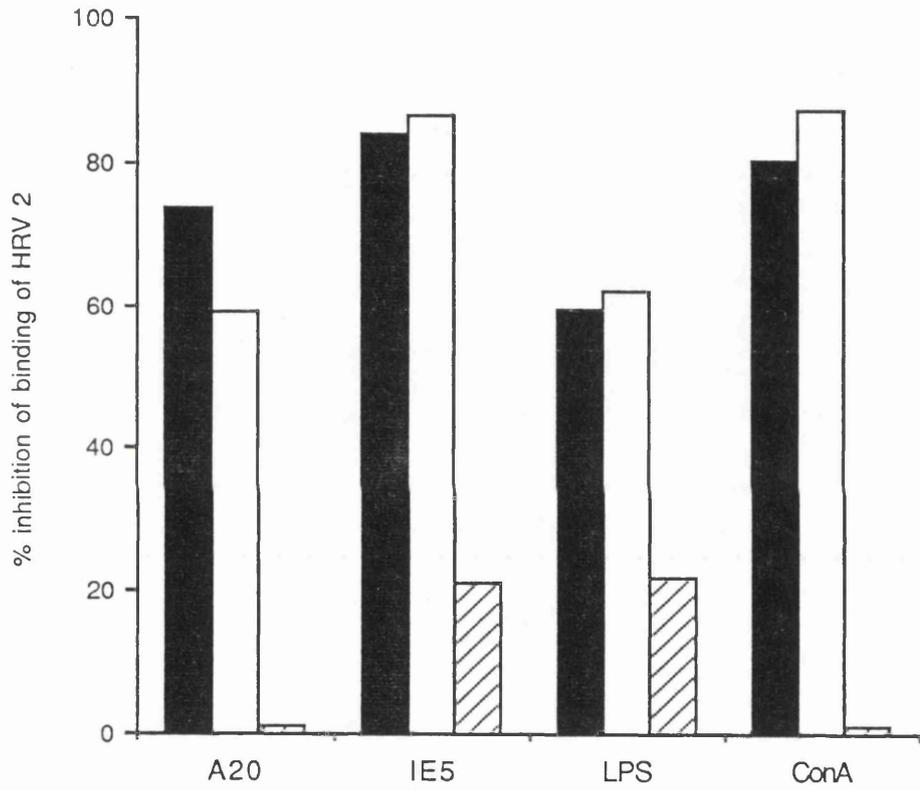
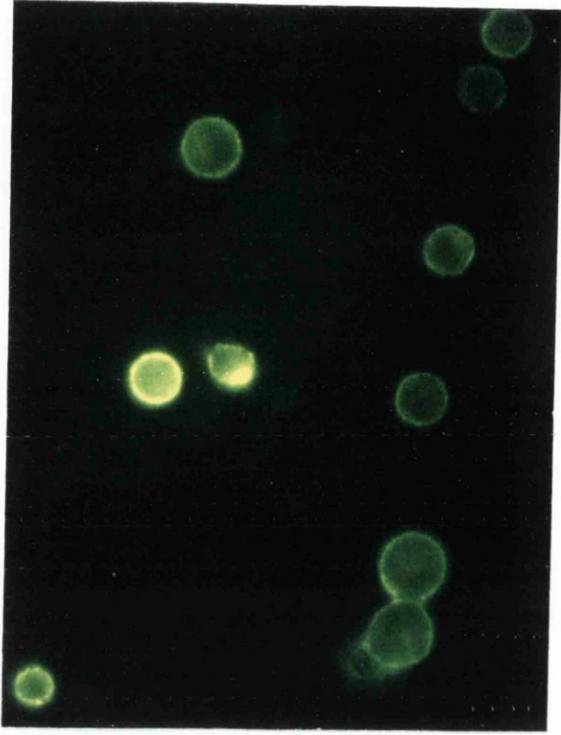
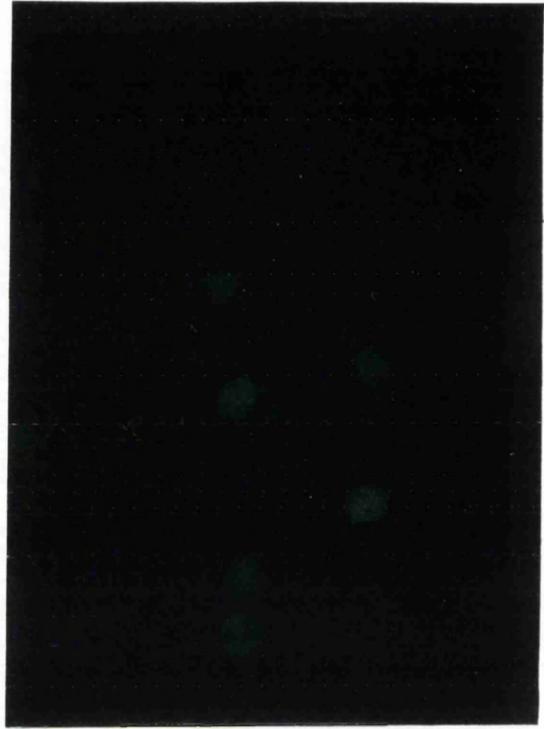


Figure 4.7. Fluorescence labelling of HeLa cells with HRV 1A and 15

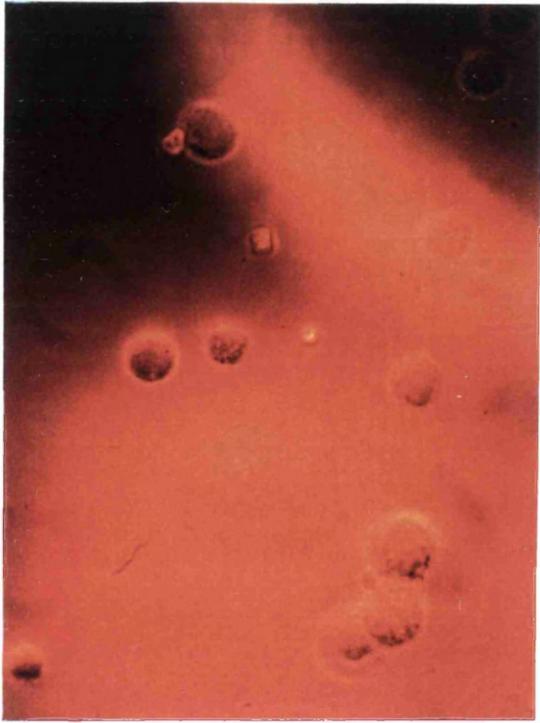
Virus was allowed to bind to OH-5 HeLa cells in solution prior to fixing the cells and labelling with virus specific antibodies. a) cells under phase contrast, b) the same field of view as shown in figure 4.7.a. in which cells were labelled with HRV 15 specific fluorescence antibody, c) HeLa cells with HRV 1A specific fluorescence antibody labelling, and d) HeLa cells incubated without virus but with HRV 1A specific antibody and fluorescent labelled antibody. Cells in figures a and b are shown at a magnification of x60 and cells in figures c and d are shown at a magnification of x40.



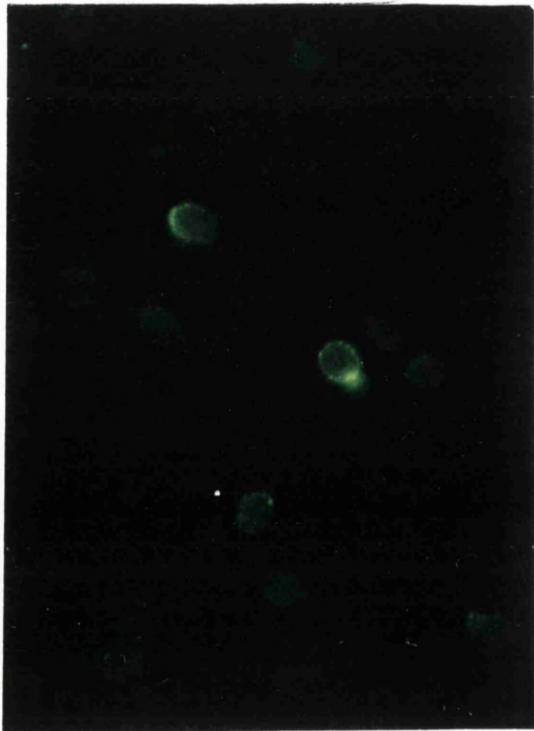
b



d



a



c

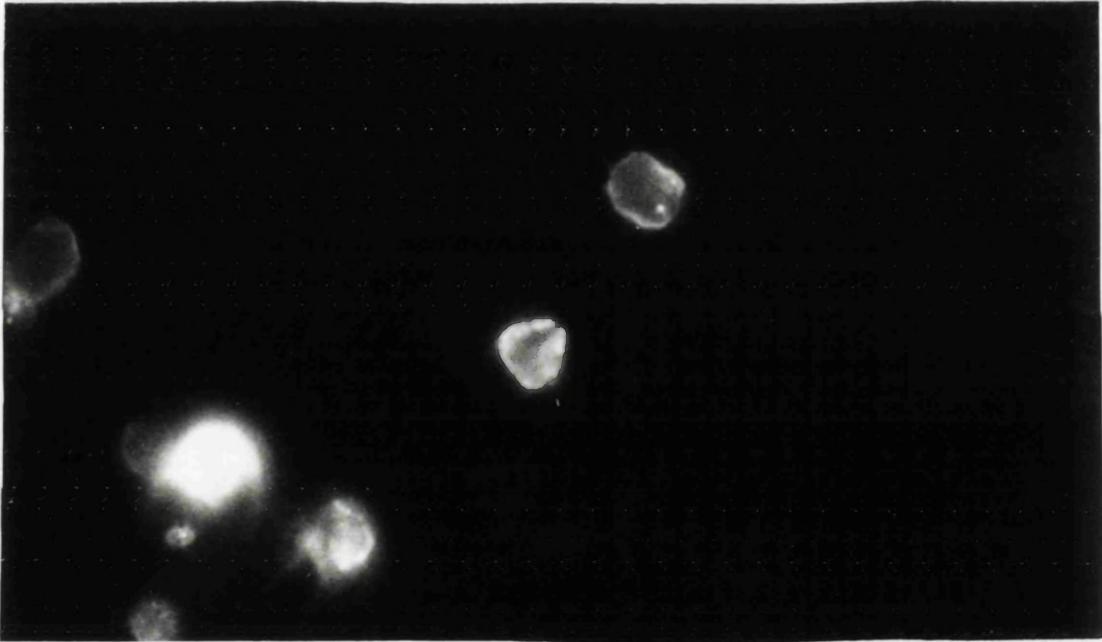
Figure 4.8. HRV 1A binds to normal mouse spleen cells enriched for dendritic cells

HRV 1A was allowed to bind to the low density cell preparation in solution prior to fixing and labelling with HRV 1A specific reagents (chapter 2). Figure 4.8.a. shows cells under phase contrast, 4.8.b. shows the same field of view in which cells were labelled with texas red for B and T cell blasts, and 4.8.c. shows the same cells labelled with FITC for the presence of bound HRV 1A.

a



b



c

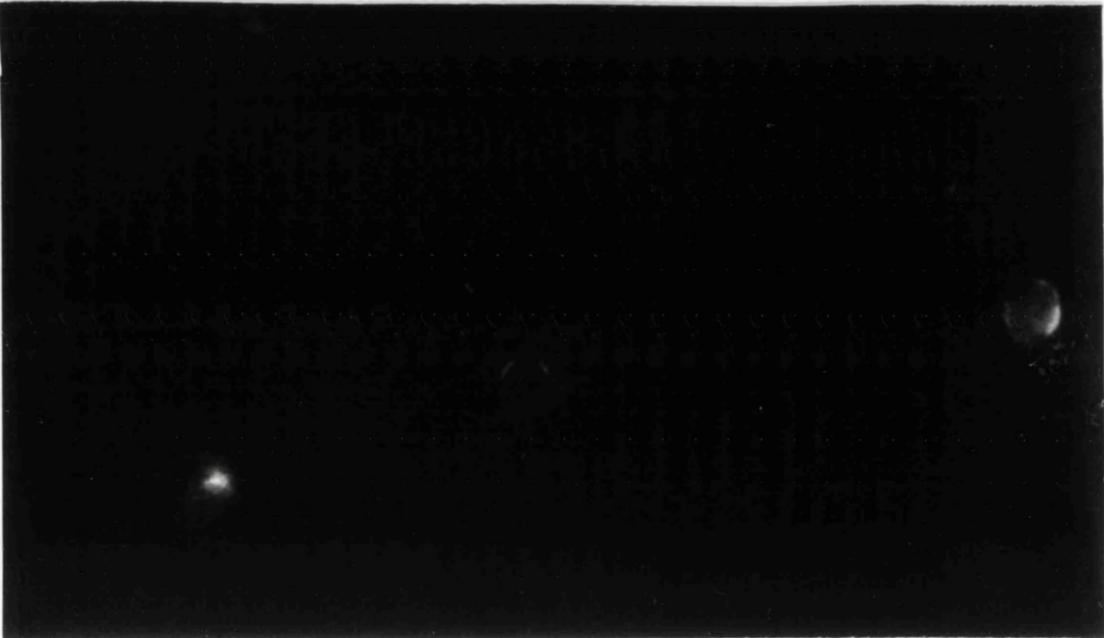


Table 4.1. HRV 1A binding to normal mouse spleen cells enriched for dendritic cells

The total number of cells were counted under phase contrast in ten fields of view (at a magnification of x40). Cells labelled with texas red (B and T cells blasts) and/or FITC (HRV 1A specific cell staining) in these fields of view were also counted.

cell staining	number of cells labelled	percentage of cells labelled
HRV 1A	51	12.9
T and B cell markers	102	25.8
Virus and T and B markers	16	4.1
unlabelled cells	226	57.2

Table 4.2. Inhibition of cellular proliferation by virus specific antibodies

Lymph node cells taken from Balb/c mice immunised with HRV 1A or HRV 15 were cultured with different concentrations of virus and antibody. The mean ^3HT incorporation for three individual wells was taken and used to calculate the cellular proliferation expressed as a percentage of the total number of counts obtained when cells were cultured with the given concentration of virus in the absence of antibody.

virus	antibody	antibody conc. (ug/ml)	percentage proliferation
HRV 1A (0.02ug/ml)	plgG anti HRV 1A	10	2.9
		5	18.5
		1	58.1
	Fab anti HRV 1A	5	5.0
		1	124.6
	non-specific plgG	1	154.4
HRV 15 (0.02ug/ml)	plgG anti HRV 15	10	141.0
		5	188.3
		1	100.6
	non-specific plgG	1	99.8

Chapter 5. Identification of Th epitopes from the structural proteins of HRV 1A

5.1 Introduction

HRV 1A has been shown to be a powerful immunogen, raising strong virus specific antibody and CD4+ T cell proliferative responses in mice. Moreover, the levels of proliferative responses of HRV 1A primed lymph node cells from three inbred mouse strains towards HRV 1A and other HRV serotypes were similar (Hastings et al., 1991). In order to identify the specific protein regions of the virus involved in generating these strong Th cell responses, peptides representing sequences from the four structural proteins of HRV 1A were synthesised. Four sets of peptides were made, one for each of the four virus structural proteins. Peptides were synthesised so as to cover the entire sequence of the four HRV 1A structural proteins (figure 1.2.) in lengths of 20 amino acids. Each peptide overlapped the previous peptide by 10 amino acids, allowing potential Th cell epitopes occurring across the junction of two consecutive 20 amino acid peptides to be represented by the overlapping peptide. Although peptides as short as 5-7 amino acids in length have been shown to stimulate Th cell responses (Sette et al., 1987), a length of 20 amino acids was chosen to allow for effective class II MHC binding. Recently the optimal length for a class II restricted peptide has been shown to be 13-17 amino acids

(Rudenski et al., 1991) and, therefore a length of twenty amino acids may be more representative of the peptides arising from antigen processing in vivo than the short synthetic peptides containing the minimal epitopes recognised by the T cell. Residues other than those involved in direct binding to MHC may also influence the ability of peptides to bind to class II MHC (Brett et al., 1988), possibly by inducing an appropriate conformation in the peptide and/or stabilising the interaction of the peptide/MHC complex.

The structural proteins of the virus, rather than the non-structural proteins, were selected for this study. Helper T cell responses are normally mounted against exogenous antigens, whilst proteins synthesised endogenously within the infected cell normally elicit cytotoxic T cell responses (Yewdell et al., 1988). Although minor group viruses bind to mouse cells they do not replicate (Yin and Lomax, 1983), therefore the Th cell response against virus in immunised mice would be directed predominantly towards the capsid proteins. Th cell determinants have also been shown to be located on the capsid proteins of a number of other picornaviruses such as poliovirus (Leclerc et al., 1991) FMDV (Francis et al., 1987) and coxackievirus (Beck and Tracy, 1989) in both mice and the natural host.

Th cell epitopes which have been selected from the sequences of a number of proteins (reviewed by Millich 1989), either by the use of predictive algorithms (Partidos and Steward, 1990; Cromerford et al., 1991) or by identification

using synthetic peptides (Millich et al., 1988), have been successfully demonstrated to provide T cell help for antibody production against known B cell epitopes. In the design of synthetic vaccines against viruses there is an advantage to using both T and B cell epitopes from the same virus as this would generate protective, lasting immunity against the virus. The epitopes included in such vaccines could be derived from the same viral protein, possibly sequential to each other (Francis et al., 1985; Leclerc et al., 1991), or may be taken from different proteins of the same virus, (Millich, 1988 ; Partidos et al., 1991). Therefore determining the precise protein sequences involved in generating the Th cell response to HRV would enable the incorporation of these sequences in composite T and B cell vaccines for HRV (Francis et al., 1989) using an identified linear B cell epitope for HRV (Francis et al., 1987a; Hastings et al., 1990).

The Th cell response to HRV has been shown to be heterotypic, with three different mouse strains responding to a number of HRV serotypes. Using peptides as antigens in cellular proliferation assays with HRV 1A primed Th cells allows the regions of the four viral structural proteins containing the Th cell epitopes to be identified, and thus addresses the question of whether similar epitopes were recognised by Th cells primed against HRV serotypes other than HRV 1A, and therefore whether these epitopes were involved in the heterotypic responses. Measuring the cellular proliferation in response to peptides of HRV 1A primed Th

cells from the three mouse haplotypes also enabled the genetic restriction of the Th cell response to HRV 1A to be determined. Therefore peptides were used to delineate the precise protein sequences involved in the generation of the Th cell response to HRV and allowed further investigations into the immunological factors, such as MHC restriction (Buus et al., 1987), influencing the Th cell response.

5.2. Proliferation of HRV 1A primed lymph node cells with HRV 1A peptides

Initially, cells from Balb/c (H-2^d) mice primed with the optimal dose of HRV 1A (0.2 μ g) in FCA were assayed for proliferation with each of the 28 VP1 peptides at a concentration of 100 μ g/ml (4x10⁻⁵M). A single peptide was identified as giving a positive response, i.e. a level of ³HT incorporation which was greater than twice the background incorporation of radiolabel observed with cells cultured in media alone. This peptide was used to determine the optimum concentration for screening virus primed cells with the peptides. Peptide concentrations higher than 500 μ g/ml (2x10⁻⁴M) appeared to have a toxic effect on the cells in culture, and concentrations lower than 10 μ g/ml were unable to induce in vitro proliferation. Therefore 100 μ g/ml was selected as the concentration used in subsequent screening assays. Later experiments showed that screening with peptides at the lower concentration of 10 μ g/ml selected the same positive peptides

as were selected using 100 μ g/ml, although the level of ^3HT incorporation was generally lower.

Virus primed cells from each of the three inbred mouse strains, C57/BL10 (H-2^b), Balb/c (H-2^d), and CBA (H-2^k) were assayed with each set of peptides in two or more separate experiments and each time the same peptides were identified as positive (having an stimulation index (S.I.) greater than 2.0). The results shown in figures 5.1- 5.4 are those obtained in a single representative assay. Cells from H-2^d mice proliferated with peptides from VP1, VP2 and VP3, H-2^k mice recognised predominantly VP1 and VP2 peptides and H-2^b mice responded to peptides from VP2 and VP3. The response of H-2^b mice to VP2 peptides was mostly directed towards two peptides, 31-50 and 201-220. However, the levels of response varied between assays, with 31-50 giving a dominant response in one experiment and 201-220 the dominant peptide in the second assay. A third assay was performed in which the proliferative response was directed towards VP2 peptide 201-220. This observation suggests that competition may occur between these two VP2 epitopes either at the level of MHC binding and/or at the generation of the epitopes by antigen processing (Gammon et al., 1990). None of the mouse strains demonstrated a significant response to any peptide from VP4. A Th cell epitope from VP4 of HRV 2 has been previously shown to be capable of providing help for antibody production (Francis et al., 1989) and this epitope was relatively well conserved between HRV serotypes, including HRV 1A. However this study

used peptide only as an immunogen and did not test whether this epitope was generated by the in vivo processing and presentation of HRV 1A (see below). Furthermore, there is evidence to suggest that when virus binds to cell receptors it undergoes conformational changes (Neubauer et al., 1987) which could result in the loss of VP 4 and therefore explain the lack of Th cell response in all strains of mice we tested. Finally, because of its small size (68 amino acids), VP 4 may be degraded by proteolytic enzymes before it can form a stable association with class II antigens.

Cells from H-2^k mice gave consistently higher proliferative responses, both in the presence and absence of antigens, than did cells from the other two mouse strains. Therefore absolute c.p.m. values were not directly compared between mouse strains. Instead the stimulation indices of the proliferative response to peptides were compared (Table 5.1) (Hastings et al., 1992). Seven peptides giving strong proliferative responses, two peptides that gave a weak but dominant proliferative response for a particular protein, and 10 other peptides generating a weak response in vitro were identified. Out of a total of 83 peptides covering the four proteins, 19 were recognised by virus specific Th cells; H-2^b mice responded to 6 peptides, H-2^d recognised only 4 peptides and H-2^k showed the widest response profile, responding to 10 peptides. Only one peptide, the VP2 peptide 101-120, was recognised by more than one haplotype.

5.3. Peptides were not inhibitory or stimulatory for non-virus specific Th cells

To investigate the possibility that responses to peptides were either negative due to a potential toxicity or inhibitory activity, or positive due to a non-specific stimulation, peptides were assayed with cells from H-2^d mice primed with FCA alone. A standard concentration of Con A was added to triplicate wells containing peptide and the percentage proliferation compared to that of the Con A stimulated proliferation was calculated (figure 5.5). No peptide inhibited by more than 50% and peptides did not exhibit non-specific stimulatory activities.

5.4. Antigen processing is required for virus induced proliferation

Inhibitors of proteolytic enzymes were used in in vitro proliferation assays with both HRV 1A and peptide (VP1 131-150) primed cells from H-2^k mice. Earlier experiments had indicated that it was difficult to demonstrate inhibition of virus specific proliferation of HRV 1A primed cells, possibly due to the strong and multi-component proliferative response seen even at low concentrations of virus. A peptide previously identified as inducing HRV 1A responses in H-2^k mice was used as an immunogen (see below) in order to study a more restricted HRV 1A response. Two proteinase inhibitors,

peptstatin, a reversible microbial inhibitor of aspartic proteinases, and trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), an irreversible synthetic inhibitor of cysteine proteinases were used over a range of concentrations. Initial experiments demonstrated that the levels of HRV 1A and peptide specific cellular proliferation could be reduced by the addition of proteinase inhibitors, but that high concentrations of both inhibitors were toxic to the cells, probably due to the high concentration of organic solvent used to solubilise the inhibitors. The results shown in tables 5.2.a. & b. demonstrate that inhibitors of enzymes previously implicated in the cellular processing of protein antigens also inhibit HRV 1A specific cellular proliferation. The inhibition of HRV 1A specific proliferation demonstrates that HRV 1A requires processing in order to generate the Th cell epitopes necessary for cellular proliferative responses in vitro. This effect is more pronounced with virus and peptide specific cells inhibited with pepstatin than with inhibitor E64. Both aspartic and cysteine proteinases may be involved in the processing of a protein antigen. Particular protein epitopes may be more susceptible to the action of one or other type of proteinase (Eisenlohr et al., 1988), or both proteinases may act together to generate a given epitope. The two types of proteinase could act sequentially to generate a T cell epitope from the protein (Van-Noort et al., 1991), or one proteinase may cause fragmentation of the protein whilst the other enzyme acts in an independent step in the processing pathway, such as

in bringing about dissociation of the invariant chain from the MHC molecules (Diment, 1990). Peptide specific proliferation, either with peptide or virus primed cells was not inhibited by pepstatin indicating that the peptide did not require processing by aspartic proteinases in order to induce peptide specific cellular proliferation in vitro. It was interesting to note that E64 had an inhibitory effect on both virus and peptide specific proliferation. This suggests that the peptide may require some degree of processing by cysteine proteinases in order to function optimally, or that E64 has a non-specific inhibitory effect on cellular proliferation.

5.5. H-2^d mice primed with different adjuvants show the same response profile with VP1 peptides

Th cells from Balb/c mice immunised with HRV 1A in FCA displayed a restricted response to VP1 peptides, responding only to peptide 211-230. The response of cells primed against virus using FCA, FIA and aluminium hydroxide as adjuvants and the response of cells primed with HRV 1A in PBS (no adjuvant) was assayed to determine whether the restricted response to VP1 was influenced by the type of adjuvant used. All four groups responded predominantly to the same peptide, 211-230 (Figure 5.6). In this assay, however, cells primed with FCA also responded weakly towards peptide 11-30. Mice primed with FIA and aluminium hydroxide recognised only 211-230, but mice primed with virus in the absence of adjuvant also exhibited

weak responses to peptides 11-30, 161-180, 171-190, and 221-240. Immunisation with virus in PBS gave lower levels of ^3HT incorporation. For aluminium hydroxide, although the level of response towards peptide was much lower than in the FCA immunised group, the stimulation index was considerably higher. Immunisation with virus in aluminium hydroxide also generated a very strong virus specific response. It is interesting to note that the level of response to peptide 211-230 does not reflect these differences in the response to intact virus. It is also clear that, although peptide 211-230 is the major immunogenic determinant for VP1, other epitopes may be generated by differential antigen processing, either due to variation between groups of experimental animals or an effect of the adjuvant in which the antigen is administered.

5.6. Cells primed with HRV serotypes 1B and 2 respond to the same VP1 peptide as HRV 1A primed cells

H-2^d mice were immunised with two HRV serotypes, 1B and 2, which belong to the same receptor group as HRV 1A and exhibit a high degree of homology between their amino acid sequences (Palmenberg, 1988). Cells primed towards HRV 1B responded to the panel of peptides representing the sequence of VP1 of HRV serotype 1A in an almost identical fashion to HRV 1A primed cells (figure 5.7.a.). HRV 2 primed cells also responded to peptide 211-230 (figure 5.7.b.), but only weakly (FCS was used in the culture medium, accounting for the high

background in this experiment and this may be masking some specific cellular proliferation). The protein sequence represented by HRV 1A VP1 peptide 211-230 has two amino acid changes in the HRV 1B sequence, and HRV 2 contains 8 changes (table 5.3.). This epitope is therefore highly conserved between these three serotypes, and may be recognised by cells primed against other HRV serotypes which express similar degrees of sequence homology in this sequence. Th cell responses to this epitope in H-2^d mice must therefore contribute to the heterotypic response described for HRV 1A primed cells (chapter 3; Hastings et al., 1991)

5.7. Screening peptides with virus primed cells selects those peptides which can induce virus responsive Th cells

Nine of the 28 VP1 peptides were selected, representing both positive and negative peptides, as determined by peptide screening with HRV 1A primed cells from H-2^k mice (figure 5.3.a.), and these were used to immunise H-2^k mice. The peptides included the two which give strong proliferative responses, three which gave weak responses, and four which gave no response when assayed with HRV 1A primed cells. Responses to the immunising peptide and HRV 1A were determined over a range of antigen concentrations and figure 5.8. shows the proliferative response to a single dose of each antigen. Three response profiles were seen; either i. peptides did not prime Th cells for peptide or virus responses (VP1 1-20 and

201-220), ii. peptides primed for peptide but not virus responses (101-120, 151-170, and 241-260), or iii. peptides primed for both peptide and virus responses (51-70, 111-130, 121-140, and 131-150). Peptides did not induce Th responses to virus without also inducing a peptide specific response. All of the peptides which produced virus specific cellular proliferation had previously given a proliferative response with HRV 1A primed cells (Fig 5.1.c) demonstrating that the screening assays were effective at identifying peptides which elicit a cellular response towards virus. Therefore the peptides identified by the screening assays were representative of those Th cell epitopes arising from the processing and presentation of the native antigen, and these could be expected to immunise for both peptide and virus responsive Th cells.

5.8. Discussion

The Th cell response to protein antigens in mice is often focused on a small number of epitopes (Bixter et al., 1985). The most striking feature of the Th cell response to HRV 1A is its oligoclonal nature. A strong or dominant response was seen towards a total of only nine peptides, three recognised by H-2^b, three by H-2^d and four by H-2^k, only one of which was recognised by more than one haplotype. All of the peptides which gave weak responses with HRV 1A primed cells were specific to a single haplotype. Therefore very few of the

total of 83 peptides representing the entire sequence (x2) of the four HRV 1A viral proteins, were identified as "natural" Th cell epitopes.

The amino acid sequences of the four viral proteins were analysed for the presence of sequences representing predicted Th cell epitopes. The amphipathic alpha helix hypothesis (Delisi and Berzofsky, 1985) was based on the observation that a sperm whale myoglobin (SWM) peptide could adopt an alpha helical conformation. Amino acid residues critical for MHC binding were located on the hydrophobic face of the helix whereas residues important in recognition by TCR were exposed on the hydrophilic face of the helix (Berkower et al, 1986). Th cell epitopes which did not display helical properties in the native protein structure could potentially adopt similar amphipathic helical characteristics when in the form of peptides bound to MHC molecule (Allen et al., 1987). The sequences of a number of identified Th cell epitopes agreed with this algorithm (Spouge et al., 1987) and it was used to predict a Th epitope in the HIV gp120 protein (Cease et al., 1987) and Th epitopes in a number of proteins (Cornette et al., 1989). An alternative predictive method described by Rothbard and Taylor (1988) was based upon the occurrence of a common amino acid sequence motif between known Th cell epitopes and was used to predict Th epitopes in a number of proteins, including the viral proteins of HRV serotype 2 (Francis et al., 1989) and human papilloma virus (HPV) L1 protein (Davies et al., 1990).

Only half of the total number of peptides identified as positive by screening cells primed towards virus for a positive response with peptide, contained a predicted T cell epitope. Conversely, the results obtained by immunising with random HRV 1A VP1 peptides indicated that a considerable number of peptides which were not identified as positive in HRV 1A primed mice, would probably be immunogenic in mice, representing cryptic determinants of HRV 1A capable of generating peptide specific Th cell responses but not cells which would also recognise virus. Therefore, as a number of other research groups have shown, these simple methods of predicting Th cell epitopes in proteins should be only loosely applied to the study of Th cell responses to protein antigens.

Peptides giving no response with HRV 1A primed cells may be able to bind to MHC molecules (Perkins et al., 1991) but might not evoke proliferative responses from virus primed Th cells in that haplotype, possibly due to limitations in the T cell repertoire (Nanda et al., 1991) but more probably due to differences in processing and presentation of the antigen between different MHC backgrounds (Gammon et al., 1987). The role that antigen processing could play in generating the nine strong/dominant Th cell epitopes identified for HRV 1A in the three different mouse strains is discussed in more detail in the following chapter.

HRV is a complex antigen made up of multiple copies of the four structural proteins and the Th cell response was directed towards three of these, VP1, VP2, and VP3. The Th

cell response was not restricted to a single structural protein as has been seen with bovine Th cells which responded only towards VP1 of picornavirus FMDV (Collen et al., 1991). An amino acid sequence from VP1 of poliovirus has been shown to be immunodominant, although the other poliovirus structural proteins were not tested in the study (Leclerc et al., 1990). The lack of response to VP4 of HRV 1A was surprising since an earlier study using peptides based upon the predicted sequences of five Th cell epitopes (Rothbard, 1986) of HRV 2 (Francis et al., 1989) demonstrated that a VP4 sequence was capable of providing T cell help in vivo. However, none of the five peptides representing the predicted Th cell epitopes were recognised by virus specific T cells, possibly due to their short length (only 10 amino acids), or due to not being representative of the "natural" processed Th cell epitopes of the virus. Only one of these HRV 2 epitopes corresponded to a sequence identified as a "weak" HRV 1A Th cell epitope.

The lack of recognition of the same Th cell epitopes by different MHC backgrounds is a disappointing finding in terms of vaccine development since Th epitopes from a number of foreign antigens (Sinagaglia et al., 1987), including viruses (Tindle et al., 1991; Partidos and Steward, 1990) have been shown to bind to different MHC molecules and generate Th cell immunity in different genetic backgrounds. Epitopes recognised by a number of genetic backgrounds would be an essential component of synthetic vaccines if they are to be effective within the population. However, the demonstration that

identical Th epitopes were recognised by cells primed using different adjuvants implies that the virus is processed similarly in the presence and absence of adjuvant and that the epitopes arising from this process are dominant Th epitopes for HRV 1A. An identical epitope was also recognised by Th cells primed against other HRV serotypes, indicating the dominance of that epitope in generating Th cell responses to HRV serotypes. Therefore Th cell epitopes of HRV 1A can be recognised by Th cells which have been primed against different serotypes, agreeing with the finding that HRV 1A raised cellular immune responses which recognise heterologous HRV serotypes. The data summarised above indicates that the epitopes generated in vivo by mice immunised with HRVs are consistently selected irrespective of the adjuvant and the virus serotype used to immunise the mice. This suggests that similar Th cell epitopes may be generated as a result of HRV infection, although it remains to be seen whether the same epitopes are generated when virus enters the host by infection with virus rather than by immunisation. An adapted strain of HRV 2 infectious for mice has been reported (Yin and Lomax, 1986) and this could be used to determine whether there are differences between the Th cell response to HRV infection and immunisation. Studies of Th cell responses to influenza A haemagglutinin (HA) have shown that different epitopes are recognised by mice infected with the virus than were recognised by immunised mice (Mills et al., 1986). This is an important consideration for vaccine design since the

generation of an effective Th cell memory response is as important a feature of a vaccine as is the generation of neutralising antibodies.

The demonstration that specific regions of the VP1 of different HRV serotypes represented conserved Th epitopes agrees with the initial observations discussed in chapter 3, and suggests that different HRV serotypes are processed in vivo in a similar manner such that the conserved epitopes are able to bind class II MHC. This finding is also promising for the design of synthetic vaccines based upon HRV Th cell epitopes, with the conserved epitopes able to generate Th cell responses towards HRV which would be boosted by subsequent contact with virus serotypes exhibiting a similarly processed Th cell epitope.

Figure 5.1. Response of HRV 1A primed cells to VP1 peptides

Proliferative responses of HRV 1A primed lymph node cells from H-2^b (figure 5.1a), H-2^d (5.1b), & H-2^k (5.1c) were assayed with single concentration (100 μ g/ml) of each of the VP1 peptides. Peptides were 20 amino acids in length and are numbered from the first amino acid at the N-terminus. ³HT incorporation for each peptide was measured in three separate culture wells, the mean taken and the background c.p.m., obtained from cells cultured with medium alone, was subtracted. Peptides which gave a c.p.m. value lower than the background c.p.m. were given a value of zero. The dotted line represents a value twice the level of background proliferation.

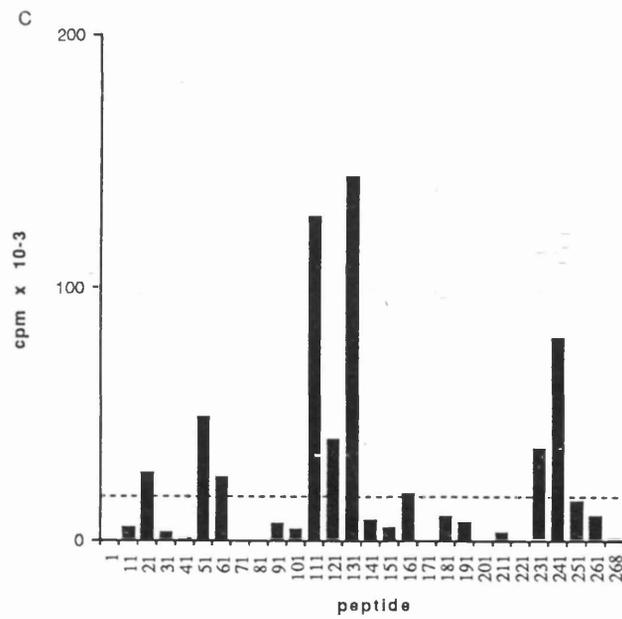
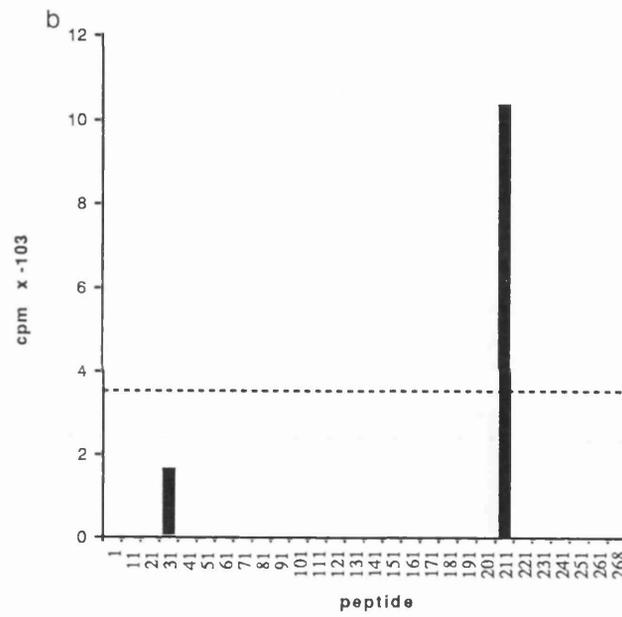
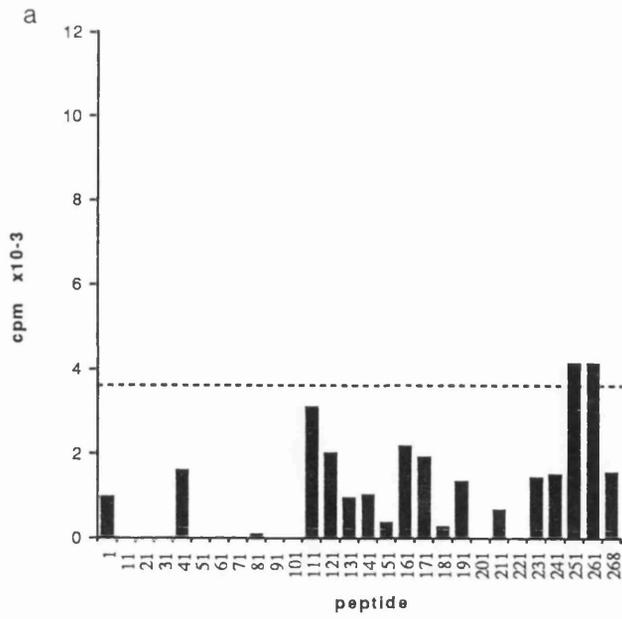


Figure 5.2. Response of HRV 1A primed cells to VP2 peptides

Proliferative responses of cells from H-2^b (figure 5.2.a), H-2^d (5.2.b), & H-2^k (5.2.c) mice. Peptides were added at a concentration of 100µg/ml and ³HT incorporation was determined in three separate culture wells for each peptide, the mean taken and the background c.p.m., obtained from cells cultured with medium alone, was subtracted. Peptides which gave a c.p.m. value lower than the background c.p.m. were given a value of zero. The dotted line represents a value twice the level of background proliferation.

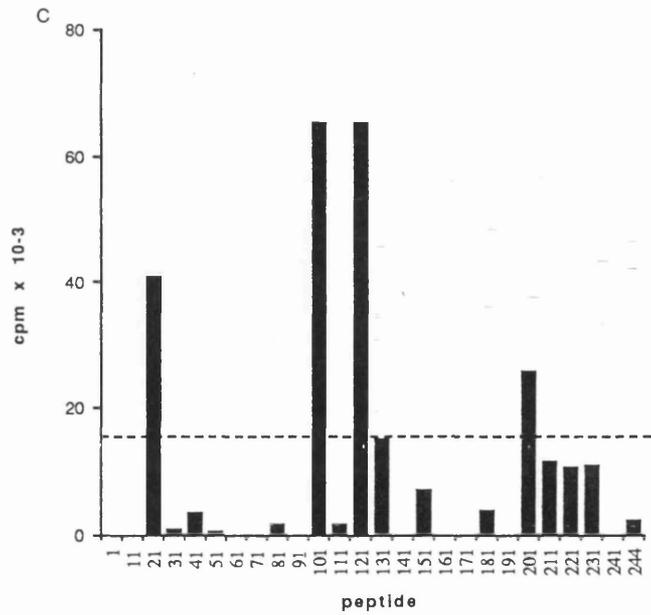
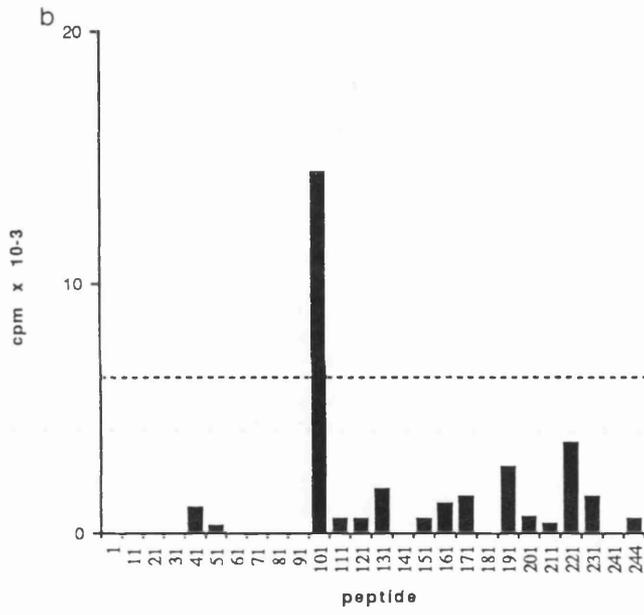
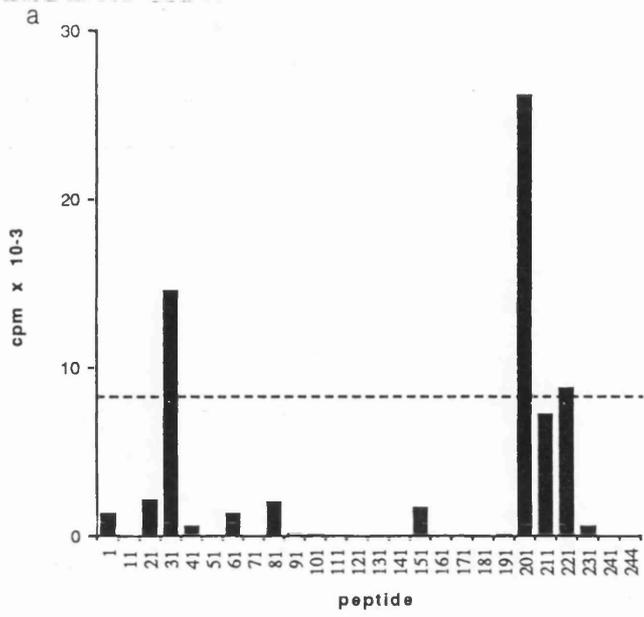


Figure 5.3. Response of HRV 1A primed cells to VP3 peptides

Proliferative responses of cells from H-2^b (figure 5.3.a), H-2^d (5.3.b), & H-2^k (5.3.c) mice. Peptides were added at a concentration of 100 μ g/ml and ³HT incorporation was determined for three separate culture wells for each peptide, the mean taken and the background c.p.m., obtained from cells cultured with medium alone, was subtracted. Peptides which gave a c.p.m. value lower than the background c.p.m. were given a value of zero. The dotted line represents a value twice the level of background proliferation.

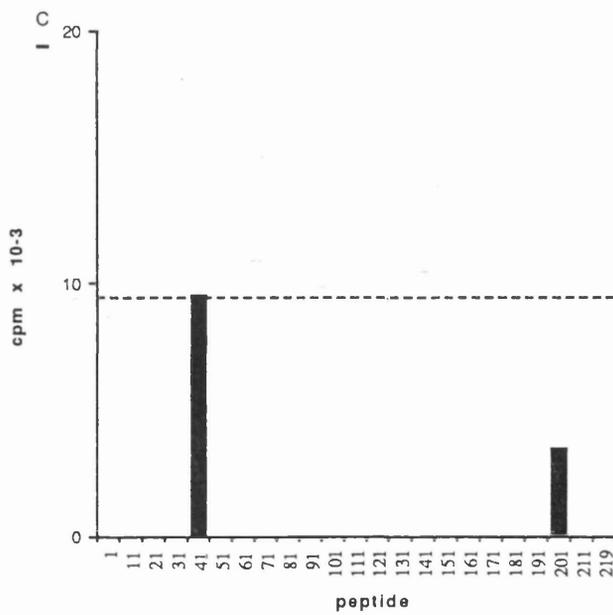
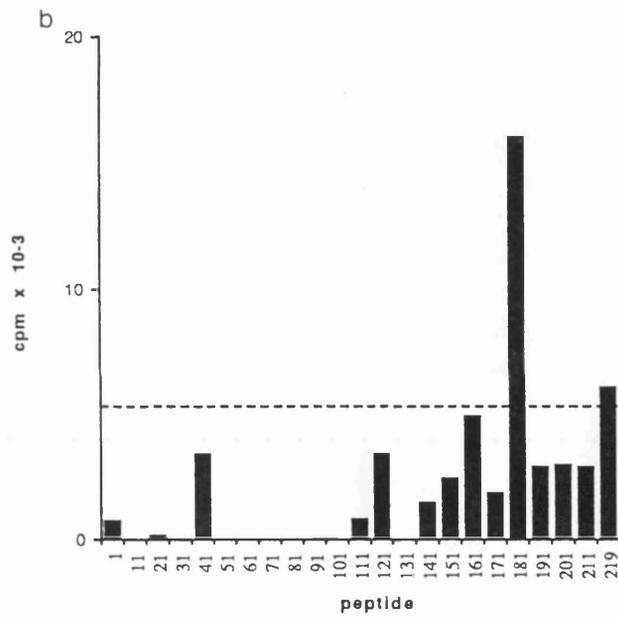
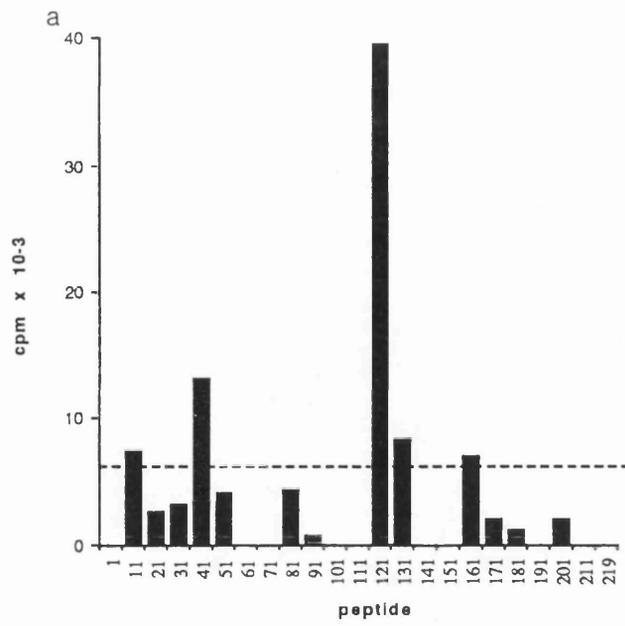


Figure 5.4. Response of HRV 1A primed cells to VP4 peptides

Proliferative responses of HRV 1A primed cells to VP 4 peptides at 100 μ g/ml. 3 HT incorporation was measured for each of the peptides and the background c.p.m. was subtracted from the mean of three individual determinations of the level of 3 HT incorporation.

□ H-2^b, ▨ H-2^d, and ■ H-2^k.

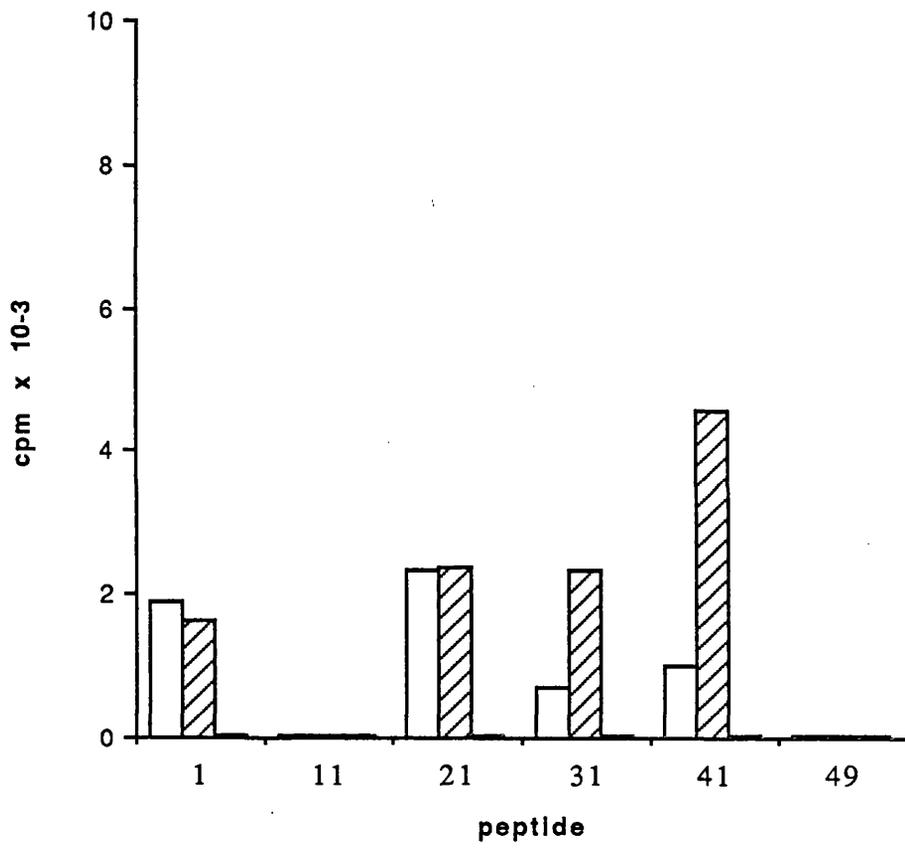


Figure 5.5. Inhibition of Con A stimulated cells by synthetic peptides

Con A was used to stimulate H-2^d cells primed with FCA alone. Synthetic peptides were added at a concentration of 100 μ g/ml (10 μ g/well) to three culture wells and the mean ³HT incorporation was determined. Results were expressed as a percentage of the proliferation obtained with cells cultured with Con A alone (taken as representing 100% proliferation). The dotted line represents 100% cellular proliferation.

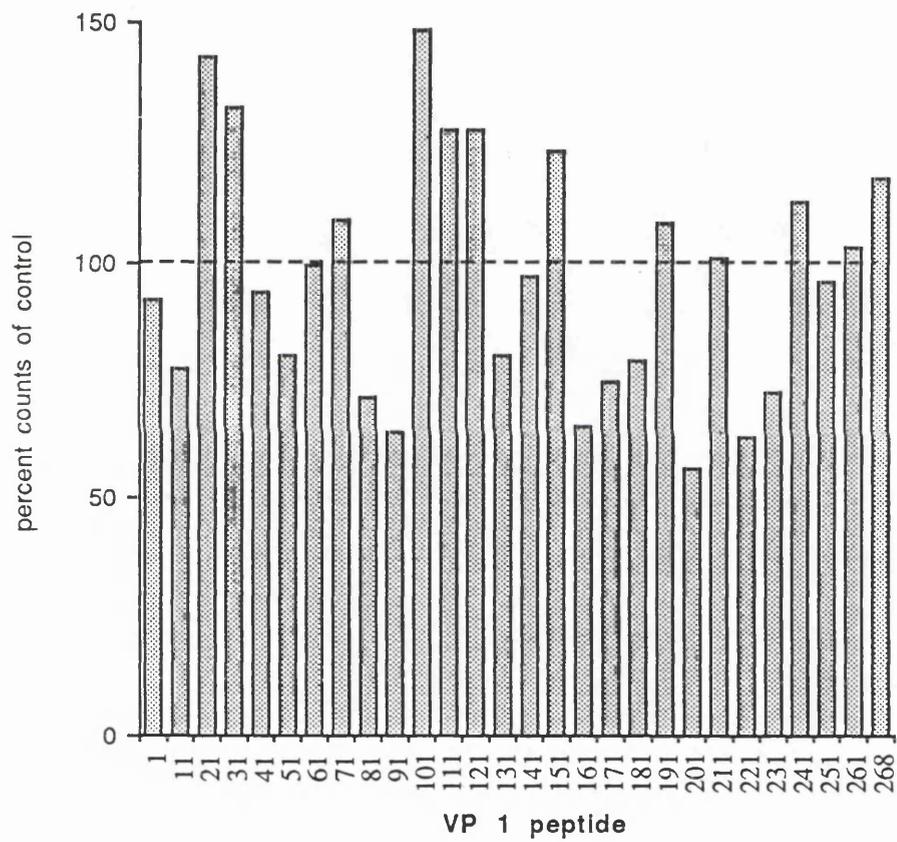


Figure 5.6. Response of cells from H-2^d mice immunised with HRV 1A in different adjuvants to VP 1 peptides

Results are expressed as the mean of three separate determinations of ³HT incorporation for each of the peptides, at 100µg/ml, minus the mean c.p.m. of cells cultured in medium alone. Cells were also assayed with HRV 1A at 0.2µg/ml. The dotted line shows the value of 2x the cell background. Mice were immunised with 0.2µg per mouse HRV 1A in 100µl of; a, Freund's complete adjuvant (FCA); b, Freund's incomplete adjuvant (FIA); c, aluminium hydroxide; & d, phosphate buffered saline (PBS).

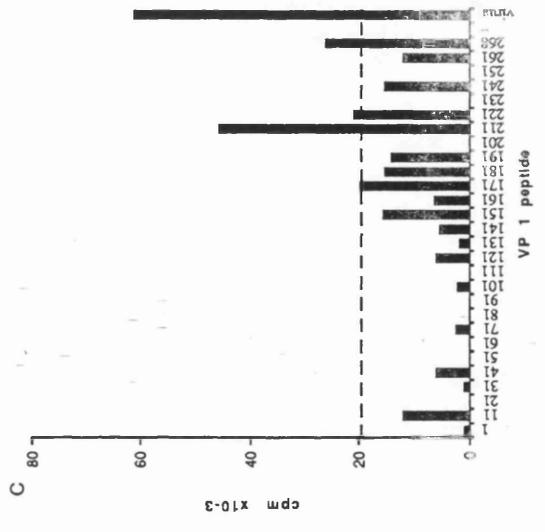
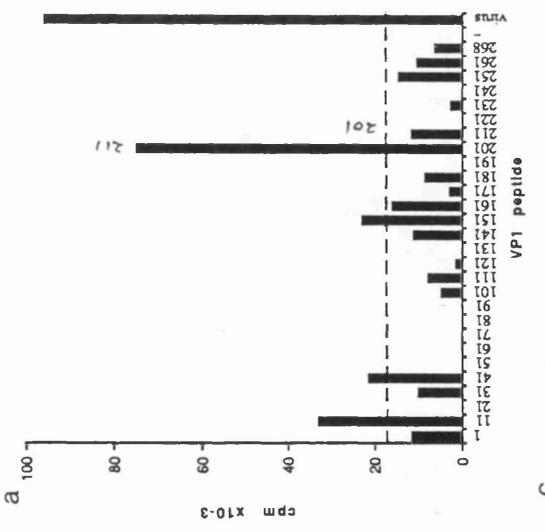
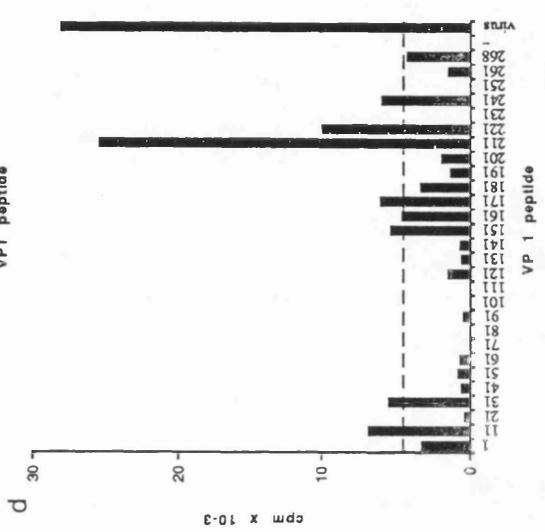
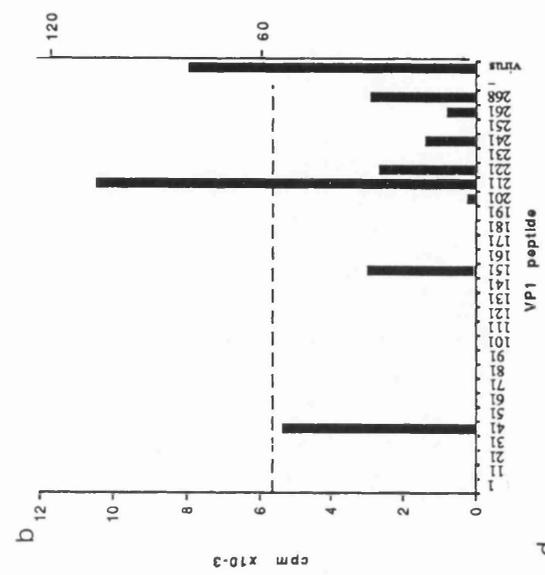


Figure 5.7. Proliferative responses of cells primed with other HRV serotypes towards HRV 1A peptides

H-2^d mice were immunised with 0.2 μ g of HRV 1B (5.7.a) or HRV 2 (5.7.b) in FCA. The proliferative response to each of the VP1 peptides was determined for three culture wells and the results expressed as the mean c.p.m. minus the mean c.p.m. of cells cultured with medium alone. The dotted line represents 2x the background c.p.m. Both groups of animals responded to the heterologous virus and to HRV 1A.

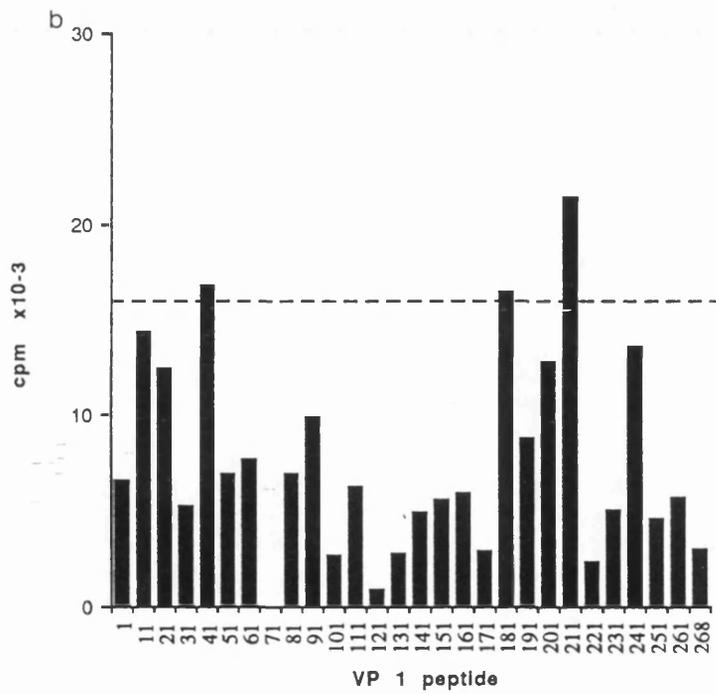
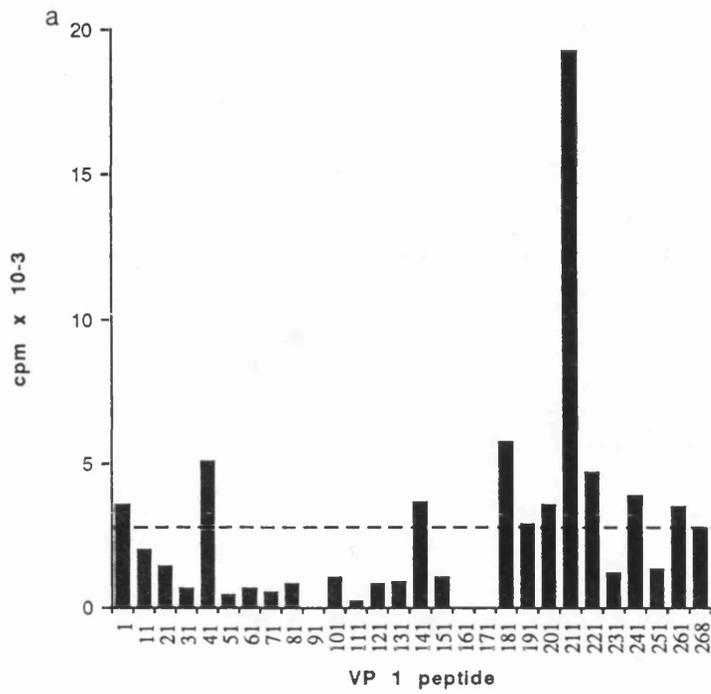


Figure 5.8. Peptides prime for virus specific Th cell responses in H-2^k mice

Groups of 4 H-2^k mice were immunised with 50 μ g of each of the VP1 peptides emulsified 1:1 with FCA. The level of cellular proliferation in response to 100 μ g/ml of peptide in vitro was determined for three individual wells and the mean ³HT incorporation was calculated. The results show the mean c.p.m. minus the mean background proliferation obtained from cells cultured with medium alone.

peptide, virus.

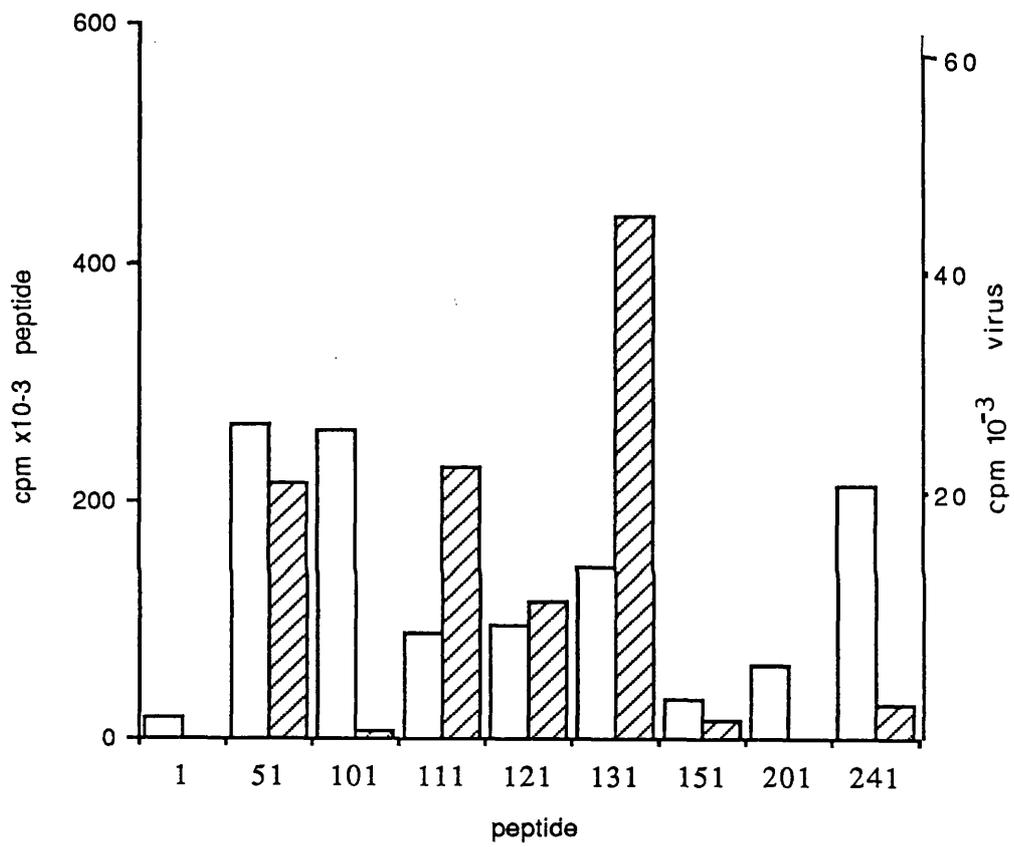


Table 5.1. Proliferative responses of HRV 1A primed cells to synthetic peptides

Results are expressed as the mean stimulation index (S.I.) from two separate determinations of the proliferative response to peptides (representative experimental results are shown in figures 5.1-5.4.). - negative response (S.I.<2), + weak response (S.I. 2-5), +* weak but dominant response for the peptide series (S.I. 2-5), and ++ strong response (S.I. 5-10) & +++ strong response (S.I.>10).

viral protein	peptide	mouse strain		
		Balb/c (H-2d)	cba (H-2k)	c57/bl (H-2b)
VP1	51-70	-	+	-
	111-130	-	+++	-
	121-140	-	+	-
	131-150	-	+++	-
	211-230	+	-	-
	241-260	-	+	-
	VP2	21-40	-	+
31-50		-	-	++
91-110		-	-	+
101-120		++	++	-
121-140		-	++	-
201-220		-	-	+++
211-230		-	-	+
221-240		-	-	+
VP3	41-60	-	+	-
	121-140	-	-	+
	161-180	+	-	-
	181-200	++	-	-
	201-220	-	+	-

Table 5.2. Protease inhibitors reduce the cellular proliferative response to virus

Results are expressed as the percentage proliferation of the positive control, antigen at the given concentration cultured in the absence of proteinase inhibitor. The control level of proliferation was determined by the mean of three determinations of the amount of ^3HT incorporated by cells cultured with antigen but in the absence of protease inhibitor. The toxic effect due to the addition of protease inhibitors was determined by measuring the percentage proliferation of cells, in triplicate assay wells, in the presence of inhibitor compared to that of cells in media alone. In table 5.2.a $0.0002\mu\text{g}$ HRV 1A gave 15 341 c.p.m. and $1\mu\text{g}$ peptide gave 97 025 c.p.m., representing 100% proliferation, and in table 5.2.b $0.002\mu\text{g}$ HRV 1A gave 105 544 c.p.m. and $0.1\mu\text{g}$ peptide gave 98 589 c.p.m.

Table 5.2.a. Virus immunised mice

inhibitor		antigen	
pepstatin (ug/ml)	virus (0.0002ug/ml)	peptide (1.0ug/ml)	cells (no antigen)
40	38.07	84.66	62.65
20	77.93	91.45	71.96
10	77.46	79.22	N.D.
E64 (ug/ml)			
40	38.97	52.95	73.19
20	55.07	54.28	110.79
10	71.76	53.78	97.51

Table 5.2.b. Peptide immunised mice

inhibitor		antigen	
pepstatin (ug/ml)	virus (0.0002ug/ml)	peptide (0.1ug/ml)	cells (no antigen)
40	63.25	87.12	102.99
20	57.62	134.64	116.42
10	72.05	139.01	122.43
E64 (ug/ml)			
40	65.05	56.64	77.89
20	60.15	85.10	103.54
10	67.81	82.57	109.80

Table 5.3. Sequence homology of the VP1 protein from amino acids 211-230 between HRV serotypes

Amino acids homologous to the sequence of HRV 1A are shown in capital letters; residues which differ from the HRV 1A sequence are shown in lower case letters.

HRV 1A VP1, amino acids 211-230

1A SVVTNDMGTICSRIIVTEKQK
1B SiVTNDMGTICSRIIVTEKQe
2 taⁿTNⁿMGslCSRIIVTEKhi
14 itV~~T~~NDMGTmaSRIV~~T~~Ehde
15 iVVTNDMGTlCSRIIVTEehg
49 iVsTNDMGslCSRIIVTEKhi
9 iiVTNDMGslCSRIIVTEehg
39 ssVTNDMGTlCtRIVTnqQK
85 tsVTNhMGTlCSRIIVTnKQq
89 SVVTNDMGTICvRIVTskQK

Chapter 6. Immunogenicity of HRV 1A peptides

6.1 Introduction

To stimulate a lasting T cell immunity, the Th cell epitopes incorporated into synthetic vaccines should not only prime for a Th cell response, but that response should also be re-stimulated by subsequent contact with the virus. Peptides likely to contain Th epitopes representative of those generated by processing and presentation of the native virus were selected by the screening procedure described in the previous chapter. Nine peptides were identified as giving either strong or dominant responses in the three different mouse strains tested. These peptides were used as immunogens and their ability to induce both peptide and HRV 1A specific Th cell responses in the three haplotypes was investigated. The ability of these peptide primed virus specific Th cells to respond to heterologous virus serotypes was studied. Peptides were also studied for their ability to act as functional Th epitopes in vivo, by providing T cell help for antibody production by B cells. Finally, the structural location of these epitopes within the three dimensional structure of HRV 1A (Kim et al., 1989) and their relationship to the known B cell determinants of HRV (Appleyard et al., 1990; Sherry et al., 1986) was examined.

6.2. Peptides prime Th cells which are specific for peptide and virus

The nine strong/dominant peptides described in chapter 5 were used to immunise mice of each haplotype, and both peptide and virus specific cellular proliferation was measured over a range of antigen concentrations (Figures 6.1-6.3). Stimulation indices for the antigen concentration giving the maximum proliferation (100 or 10 μ g/ml for peptide and 0.2 or 0.02 μ g/ml for HRV 1A) were calculated and table 6.1 provides a summary of the results obtained. Peptides generally induced peptide specific Th responses in the mouse strain in which they were originally identified as positive. Six of the peptides (referred to as A, B, C, D, E, and F; table 6.1) also primed for peptide specific responses in one other haplotypes and two peptides (G and H; table 6.1) induced peptide responses in all three haplotypes. These results indicated that peptides demonstrated different levels of degeneracy in their ability to bind MHC.

Peptides which primed Th cells recognising HRV 1A exhibited a greater degree of MHC restriction. The majority of peptides stimulated virus reactive Th cells only in the mouse strain in which they were originally identified. There were however two exceptions to this finding, VP1 peptide 111-130 (A) which induced proliferative responses in H-2^b mice as well as the original H-2^k strain, and VP2 peptide 121-140 (F), which induced a peptide response but did not raise a

significant virus response in H-2^k mice (where it was originally identified as positive) but which did induce both peptide and virus specific Th cells in H-2^d mice.

6.3. Immunisation with a mixture of peptides primes for virus specific Th cell responses in all three haplotypes

Mice of the three inbred strains were immunised with a mixture of peptides B, H, and I in FCA. These peptides were selected because each peptide was capable of priming virus specific Th cell responses in only one haplotype, although peptide B also gave peptide specific responses in H-2^b mice and peptide H gave peptide specific Th cell responses in all three mouse strains. All three mouse strains mounted a virus specific Th cell response (figure 6.4), although the level of proliferation seen in H-2^b mice was less than expected from previous experiments where mice were immunised with a single peptide (table 6.1). This reduced level of proliferation could be due to the lower immunising dose of peptide (30 μ g rather than 50 μ g) or an effect of immunising H-2^b mice with two peptides, each of which were able to prime a peptide specific Th cell response in H-2^b mice. The two peptides could possibly compete for the MHC class II binding sites, and this competition could result in dominance effects such as those described by Adorini et al. (1988).

6.4. HRV 1A peptides prime Th cells which respond to heterologous HRV serotypes

Three of the nine peptides used in this study, A, B and I, were used to immunise mice and the cellular proliferative response to nine HRV serotypes, including HRV 1A, was determined over a range of virus concentrations. Th cells from each group recognised all nine virus serotypes tested (figures 6.5-6.7). The proliferative response to minor group HRVs could be distinguished from that to major group HRVs by the fact that major group serotypes required higher in vitro concentrations to give maximum cell proliferation, a similar finding to that seen with cells from virus immunised mice (chapter 3, figures 3.5 and 3.6). VP1 peptide A (111-130) primed for a cross-serotype virus response in H-2^k and H-2^b mice, the two strains in which it had previously been shown to prime for HRV 1A Th cell responses.

6.5. Sequence homology of the selected Th epitopes between HRV serotypes

Ten HRV serotype sequences were available for VP1, and eight for VP2 and VP3. (Palmenberg 1989). Most of the peptides showed considerable sequence conservation between serotypes (figure 6.8). Peptides A, B, C, E, G, and I contained a core of four or more totally conserved residues, whilst other residues often displayed semi-conservative changes such as

isoleucine to leucine or valine. None of the peptides contained the hypervariable sequences which are associated with the neutralising antibody (NIm) combining sites of HRV.

6.6. Priming with peptide enhances the titre of HRV 1A specific antibodies

To demonstrate that the peptides identified as Th cell epitopes by cellular proliferation assays could act as functional Th cell epitopes in vivo, their ability to provide T cell help for antibody production was investigated. Four peptides, which had been previously identified as positive Th cell epitopes for H-2^k mice, were selected for use in in vivo assays in CBA (H-2^k) mice. The mice were immunised with peptide (chapter 2) so as to prime for peptide specific HRV 1A responsive Th cells. Twenty one days later the mice received a sub-optimal dose of HRV 1A (0.002 μ g) administered in FIA. This dose of virus had previously been shown not to induce a primary virus specific antibody response in mice (chapter 4, figure 4.4.a.). The mice were bled at intervals and the titre of HRV 1A specific IgG of individual mice was measured by ELISA. The aim of this experiment was to demonstrate that a pre-primed population of virus specific Th cells (generated by immunisation with peptide) would be capable of enhancing the antibody response to HRV 1A by providing pre-primed T cell help. The specific antibody response of these groups of mice would be enhanced compared to that seen in mice which had not

received peptide priming, or which had been primed with a peptide known not to elicit a virus specific Th cell response. Therefore, as well as priming mice for Th cell responses towards the four peptides A, B, E, and F identified as Th epitopes for HRV 1A, groups of mice were also primed with a negative peptide VP2 31-50 (peptide Z) and with FCA emulsified with PBS (i.e. not pre-primed towards peptide).

Table 6.2. shows the mean HRV 1A specific IgG titres of the groups of five mice immunised with peptides A, B, E, and F and of the control groups. Three of the groups of mice which had been primed with the positive Th cell peptides A, B, and E showed an enhanced antibody response towards HRV 1A after receiving the sub-optimal dose of virus, but mice immunised with peptide F did not exhibit an enhanced antibody response towards HRV 1A. Priming mice with a control peptide, VP2 31-50, or with FCA alone, did not boost virus specific antibodies. Therefore the T cells generated by immunisation with the three peptides A, B, and E also cross-react with the Th epitopes generated from native virus in vivo giving rise to enhanced anti-virus antibody responses in these mice compared to the antibody responses seen in mice which have not been primed towards any peptide or which have been primed towards a peptide which HRV 1A primed T cells do not recognise. In this experiment peptide F failed to elicit T cells capable of recognising HRV 1A in vivo and did not show enhanced antibody titres towards HRV 1A. This finding was in agreement with the results described above (chapter 6.2.) in which peptide F was

shown to be recognised by HRV 1A specific Th cells from H-2^k mice but was not able to prime a peptide specific Th cell response which recognised virus. Therefore three of the four peptides identified as T cell epitopes by cellular proliferation assays were able to provide a significant degree of T cell help in vivo. These peptides were specifically those which primed Th cells specific for intact virus in vitro. This experiment therefore demonstrates not only that the epitopes derived from studies of in vitro proliferation are indeed "helper" epitopes in the formal sense, but also that these epitopes can be generated in vivo following virus immunisation and are therefore representative of the "natural" Th epitopes arising from in vivo processing and presentation of native HRV 1A. Similar experimental evidence was provided by Millich et al., (1987) for the pre S1 antigen of hepatitis B virus surface antigen (HBsAg) where mice primed with Th cell peptides exhibited enhanced antibody responses towards HBsAg when immunised with low amounts of virus.

Interestingly, primary immunisation with peptides B, E, and F elicited strong anti-peptide responses (data not shown), suggesting that these peptides expressed B cell determinants. It was possible that the high anti-HRV 1A titres seen with peptides B and E could have been due to a cross-reactivity of the peptide specific antibodies with HRV 1A, and/or of boosting of these responses by secondary immunisation with HRV 1A. However, peptide A did not give a peptide specific antibody response but gave a good HRV 1A specific antibody

response. Conversely, peptide F elicited anti-peptide antibodies but only exhibited a weak virus specific antibody response demonstrating that the enhanced HRV 1A specific antibody response in mice primed with positive Th cell peptides was due to the Th cell response elicited in these animals.

6.7. Virus primes for an antibody response to haptened HRV 1A peptides

Having demonstrated that three of the four peptides A, B, E, and F identified as proliferative HRV epitopes in H-2^k mice could also act as functional Th cell epitopes in vivo (see above), the ability of these four epitopes in the context of intact virus, to provide T cell help for antibody production against an independent B cell epitope, the hapten trinitrophenol (TNP), was investigated. In these experiments a population of HRV 1A primed Th cells was elicited by immunisation with 0.2 μ g of HRV 1A in aluminium hydroxide. Fourteen days later the mice received 50 μ g of haptened peptide (chapter 2), administered i.p. in aluminium hydroxide) and the IgG response to TNP was measured at various time intervals by ELISA.

As well as the four peptides used in the preceding set of experiments (chapter 6.6.), two control groups of mice were used. One group received a haptened peptide (B) but were not pre-immunised with HRV 1A. The second control group were pre-

immunised with HRV 1A, but were boosted with a peptide which did not cross-react with HRV 1A primed Th cells from H-2^k mice (VP1 241-260). The results are shown in figure 6.9., and for time points 14, 21 and 70 days post primary immunisation table 6.3. shows the experimental error expressed as S.E.M. At late time points both control groups and two experimental groups showed high TNP-specific responses presumably reflecting an intrinsic helper activity of the boosting peptides. However, at earlier time points (2-4 weeks), the groups primed with HRV 1A and boosted with peptides B, E, and F gave enhanced TNP-specific antibody responses. Peptide A did not elicit a specific antibody response and Peptide E elicited a smaller increase in antibody titre than did peptides B and F. Mice immunised with peptide E also exhibited a high background titre against HRV 1A.

These experiments showed two unexpected findings. Peptide A which had previously been shown to act as a Th cell epitope in vivo, providing help for a HRV 1A specific antibody response (chapter 6, section 2.), was negative in this experiment failing to generate TNP specific antibodies in mice pre-immunised with native virus. This difference between the two assays could result if haptenisation of the peptide had inhibited its ability to bind MHC molecules. Secondly, peptide F, which failed to provided help for the production of a significant HRV 1A antibody response (chapter 6, section 6), did help in the generation of an anti-TNP response. This is a similar finding to that described for the cellular

proliferative response of peptide F primed Th cells (chapter 6, section 1), in which peptide F was identified as positive by screening with HRV 1A specific Th cells but failed to elicit Th cell which responded to virus. These results suggest that differences between the processing of virus in vivo and in vitro (as proposed in chapter 6, section 2) do not explain this finding. Rather, the results suggest that the dominant population of T cells stimulated in response to synthetic peptide F have a distinct specificity from those generated from natural processing of virus. However processing of virus stimulates a response to a closely related epitope contained in the amino acid sequence of peptide F and these cells can recognise peptide F in the context of MHC.

The ability of HRV 1A primed Th cells to recognise the peptide Th cell epitopes in vivo, again demonstrates that the Th cell peptides selected in the proliferation assays represent the natural Th epitopes of HRV 1A. The results also demonstrate that peptides representing the Th cell epitopes of HRV 1A can provide help for B cell epitopes from "foreign" proteins. Therefore, the Th cell epitopes could be used as components of vaccines against viruses other than HRV 1A (see below).

6.8. Positions of the selected peptides within the structure of the HRV 1A viral proteins

The structures of a number of picornaviruses including

two serotypes of HRV, HRV 14 (Rossmann et al., 1985) and HRV 1A (Kim et al 1989), have been solved using X-ray crystallography. The tertiary structures of VP1, 2, and 3 consist of two anti-parallel β sheets forming a β barrel core structure (figure 1.f). Plotting the coordinates of the peptides A-I on the structure of HRV 1A revealed that large portions of these epitopes, and of the peptides identified as weak Th cell epitopes, were located on the anti-parallel β sheets making up the core of the viral structural proteins. In contrast, the regions of the viral proteins involved in the generation of neutralising antibodies have been shown to be located at the surface of the virus (Sherry et al., 1986; Appleyard et al., 1990). Therefore the Th epitopes appear to be situated away from the B cell sites of the virus and are probably protected from any effects of antibody binding, such as virus aggregation, conformational changes induced by antibody binding, or antibody induced mutation in the primary sequence. The buried nature of the Th epitopes strongly suggests a role for antigen processing of the viral proteins in their generation. The observation that the nine Th epitopes identified in this present study are all located on the β strands of the viral proteins may also suggest that the proteins are processed in a similar manner, possibly by proteolytic enzymes clipping off the loops which join the β strands. Two peptides VP1 101-120 and VP1 241-260 which represent cryptic determinants for HRV 1A VP1 in H-2^k mice (chapter 5.7.), are located on surface exposed regions of the

VP1 protein. These epitopes would be more exposed to the action of proteolytic enzymes and may be destroyed during the various stages involved in the processing of native virus.

The amino acid sequences represented by the four peptides A, B, E, and F, identified as strong Th cell epitopes of HRV 1A in H-2^k mice, were plotted onto the molecular model of HRV 1A (figure 6.10.). The two VP1 peptides are highlighted in blue and the VP2 peptides in green. An interesting feature of the epitopes on both VP1 and VP2 is that the two positive peptides are consecutive in the protein sequence (VP1 peptides 111-130 & 131-150 and VP2 peptides 101-120 & 121-140) and each peptide spans most of the length of one of the β strands making up the tertiary structure of the two viral proteins and each set of peptides on each protein appears to form two sides of a "hairpin" bend (figure 6.10.). All four peptides are recognised by HRV 1A primed Th cells. However, the peptide representing the overlap between the two VP1 peptides (VP1 121-140) was recognised to a much lesser degree and the overlapping peptide for the VP2 peptides, VP2 111-130, was not recognised by HRV 1A primed Th cells. VP1 121-140 was shown generate T cells responsive to both peptide and virus, but the levels of response were lower than those generated by immunisation with VP1 111-130 and 131-150 (figure 5.8). Therefore peptide 121-140 may contain a functional Th epitope similar to either of those located in the strong response peptides, or may contain a novel Th epitope which is also generated from processing of HRV 1A and recognised by HRV 1A

Th cells. Detailed peptide mapping studies would help to resolve this matter. The other peptides containing the N and C terminal 10 amino acids from the positive peptides (VP1 101-120, VP1 141-160, VP2 91-110 and VP2 131-150) were also negative when assayed for proliferation with HRV 1A primed Th cells.

These results suggest that either the T cell epitope in each of the four positive peptides is located towards the centre of the peptide sequence and is therefore not present in its entirety in overlapping peptides containing the C or N terminal amino acids, or that the location of the peptide in the context of the virus structure may influence the selection of the T cell epitopes of HRV. The two VP1 peptides (figure 6.11) are located on linear stretches of two anti-parallel β -sheets forming the β barrel core of VP1 (as are the two VP2 peptides shown in figure 6.10), whereas the overlapping peptide for each pair represents the "bend" formed by the two β strands. It is possible that during the process of virus internalisation and antigen processing by cells that the epitope representing the "bend" is more exposed to the action of intracellular proteolytic enzymes and is more easily destroyed than the internal β strands which form the core structure of the protein. It is possible that epitopes located on the β strands are the major regions of the viral proteins which remain intact during cellular processing such that they are available to interact with class II MHC molecules and generate a virus specific T cell response.

6.9. Location of Th epitopes on the virus structure with respect to the neutralising antibody determinants

The antibody combining sites of HRV 2 and HRV 14 have been defined using monoclonal antibody escape mutants. Four sites NIm, Ia, Ib, II and III (Sherry and Reukert, 1984; Sherry et al., 1986) were identified for HRV 14, and three sites, A, B, and C for HRV 2 (Appleyard et al., 1990). HRV 2 belongs to the same receptor group as HRV 1A and the sequence and structure of serotype 2 are also more similar to HRV 1A than those of HRV 14. Therefore the amino acids involved in forming the neutralising antibody combining sites of HRV 2 were superimposed on the structural model of HRV 1A and the structural relationship of the nine Th cell epitopes was examined. (Figure 6.12). None of the nine strong/dominant peptides demonstrated any overlap with the B-cell epitopes, although one of the weak peptides (VP2 221-240) did overlap with HRV 2 site C.

6.10. Discussion

Immunisation with HRV 1A was shown to generate a Th cell response to a limited number of epitopes. The establishment of this epitope hierarchy was further investigated using the nine strong/dominant peptides. Six peptides raised peptide specific responses in another haplotype to the one in which the peptide had been identified and two peptides elicited peptide specific

Th cells in all three haplotypes, indicating a degree of degeneracy in their ability to bind MHC (Buus et al., 1987). In contrast, peptides generally raised virus responsive Th cells only in the haplotype in which the peptide was originally identified as positive.

The results described in this current chapter and in chapter 5 shed some light on the possible mechanisms involved in the generation of T cell epitopes. These results clearly demonstrate that the qualitative ability to bind MHC, per se, is not sufficient to explain the MHC-linked genetic control of immune responsiveness. In the case of peptides which cross react with virus primed T cells in at least one haplotype (chapter 6.2.) direct processing effects alone such as destruction of an epitope, or inability to generate an epitope cannot easily explain the results obtained. However it is possible that processing in combination with particular MHC haplotypes results in production of specific epitopes. Such MHC directed processing could result from protection of epitopes from degradation by MHC binding. Alternatively, the hierarchies obtained could result directly from differences in quantitative parameters of peptide/MHC interaction, under the conditions of stringent competition for MHC binding sites which occur during intracellular antigen processing of the virus. These quantitative differences could be easily converted into qualitative differences in response to individual peptide epitopes.

In contrast to these MHC dependent differences, the

failure to stimulate responses from virus primed Th cells from any haplotype by peptides, which were shown to stimulate responses towards themselves (figure 5.8), may result directly from processing defects; either an inability to release this epitope or "over-processing" and consequent destruction of these epitopes. Even more subtle effects of processing, leading to asymmetric patterns of response to peptide and virus (e.g. as described for peptide F in this chapter) have been discussed above. In conclusion it is clear that the factors which determine epitope hierarchy in response to a complex antigen such as HRV are extremely complex and that our studies have only begun to reveal some of the factors involved.

MHC restriction could be overcome by using a mixture of peptides, each one specific for the induction of virus responsive Th cells in a single haplotype. This is not a practical approach to the design of synthetic vaccines if individual Th peptides were required for each different genetic background, but it does demonstrate the principle that mixed peptides could effectively prime for a virus specific response in different MHC backgrounds. The inclusion of peptides which prime for Th cell responses in a number of genetic backgrounds (Panina-Bordignon et al., 1989; Tindle et al., 1991) in vaccines, either as simple mixtures, synthesised co-linearly, or presented as different epitopes on multiple antigen peptides (Tam and Lu, 1989) may be an important feature in the design of novel vaccines.

Peptides also primed Th cells which recognised all the HRV serotypes tested, including both minor and major receptor group viruses, demonstrating that the peptides represented the "natural" Th determinants of different HRV serotypes. The protein sequences of the peptides were highly conserved between serotypes and this suggested that these conserved Th epitopes were also involved in the heterotypic responses demonstrated by immunisation with virus (chapter 3). This finding was encouraging as it implied that infection with one HRV serotype could generate a Th cell response which would be responsive to other HRV serotypes. Indirect evidence to support this comes from a study using human volunteers who were infected experimentally with HRV (Hsia et al., 1990). Lymphocytes from these individuals demonstrated a pre-existing specificity towards the virus, although they did not have virus specific antibodies, indicating that they had not been previously infected with that serotype.

A second question addressed in this chapter was whether the cellular proliferative response to peptides described in chapter 5 corresponded to genuine T helper cell responses, and to test two models in which these peptide specific responses could be useful in generating antibody specific responses. In the first model, peptide primed mice were re-stimulated by native HRV 1A in vivo. Priming with peptide was demonstrated to prime and enhance T cell help for the production of a HRV 1A specific antibody response. This model illustrates the potential use of peptide immunisation in the development of a

HRV specific vaccine, although it does not directly address the problem created by the large number of HRV serotypes. The results were similar to findings reported for peptide priming for enhanced antibody responses to a hepatitis B virus protein, HBsAg (Millich et al., 1987), but the results from the HRV 1A experiment are of more relevance to the study of whether peptides can prime for Th responses which recognise and are re-stimulated by the native infectious agent. Since the HRV 1A peptides primed for Th responses to heterologous HRV serotypes, by implication these peptide primed Th cells could also provide help for antibody production against other HRV serotypes.

In the second, complementary, model we tested the ability of HRV 1A primed Th cells to recognise the peptide epitopes in vivo by demonstrating their ability to prime for help for the generation of an antibody response to a hapten carried by the peptide. This experiment demonstrated that the peptides could provide T cell help for an independent non-HRV B cell epitope. Therefore HRV 1A Th cell peptides could be used to provide help for B cell epitopes from other viruses, such as HPV, as well as for HRV B cell epitopes (Francis et al., 1987; Francis et al., 1989). It is estimated that humans become infected, either clinically or sub-clinically, with at least two HRV infections per annum. HRV specific T cells therefore provide an attractive "reservoir" of T cell help, which could be used to stimulate or boost antibody responses to a variety of epitopes, by chemically linking these epitopes to the HRV

Th cell epitopes. More complex vaccines composed of B cell epitopes from other infectious agents, e.g. HPV L1 protein, a Th cell epitope from the same infectious agent and a HRV Th cell epitope, can be envisaged, which would generate memory responses to both the B and T cell epitopes of HPV, whilst allowing the HRV Th epitope to provide an immediate Th cell response directed towards the peptide construct. Vaccines aimed towards generating CTL responses could also receive additional help from the inclusion of HRV Th epitopes which would act in a similar manner, generating help from a population of pre-primed HRV specific Th cells. The finding that the functional Th epitopes of HRV 1A were buried within the virus structure suggests that intracellular antigen processing plays an important role in determining the protein sequences which act as the Th cell epitopes of the virus. The route by which virus enters cells may influence the way in which antigens are processed, with entry to B cells via surface antibody capture possibly providing protection for the surface epitopes (Davidson and Watts, 1989) and entry to cells via virus binding to its specific cellular receptor causing conformational changes (Skern et al., 1991) which may facilitate processing.

The Th epitopes of HRV were shown to be remote from the external regions of the native virus which make up the antibody combining sites (Sherry et al., 1986; Appleyard et al., 1990). Therefore antibody bound to the virus would not be expected to directly affect the selection of the Th epitopes

by protecting epitopes from processing. HRV binds to its cellular receptors via a surface depression, the "canyon" running around the five fold axis of the virus (formed by five VP1 molecules). There is evidence to suggest that binding causes conformational changes in the virus (Neubauer et al., 1988), and any such changes could influence the generation of the processed Th epitopes. Neutralising antibody, by preventing virus binding to cellular receptors, could therefore influence the selection of the Th cell determinants arising from processing of HRV by preventing receptor mediated uptake. The finding that the Th cell epitopes of HRV 1A were highly conserved between other HRV serotypes also indicates that their position within the virus structure protects them from external influences such as antibody mediated sequence mutations, and implies that the regions may need to be conserved so as to maintain the correct structure of the viral proteins. However, the fact that strong Th responses are generated in the host in response to HRV, and that the response is cross-reactive with a number of different virus serotypes also suggests that a specific helper T cell response towards HRV may not directly affect the ability of virus to replicate and cause disease within a population (see below). Also, although epitopes appear to be conserved between serotypes, different HRV serotypes may stimulate different epitope hierarchies, resulting in populations of Th cells responding to dominant peptides.

The structural information on the relationship of the

known Th and B cell epitopes of HRV suggests that it is unlikely that antigen processing in vivo of HRV would result in the generation of peptides containing both functional Th cell and virus neutralising B cell epitopes as has been described for other picornaviruses, poliovirus (Leclerc et al., 1991) and FMDV (Francis et al., 1987) and for hepatitis B (Millich et al., 1986). Therefore vaccines designed specifically for generating immune responses to HRV would require at least two separate components, an identified HRV B cell epitope, such as that described by Francis et al. (1987) and Hastings et al. (1990), and a Th cell epitope(s) functional in a range of different genetic backgrounds so that the vaccine would be effective in a broad range of the population.

Figure 6.1. Cellular proliferative response of peptide immunised H-2^b mice to peptide and HRV 1A

Groups of four mice were immunised with 50 μ g of peptide emulsified 1:1 in FCA. The cellular proliferative response towards the immunising peptide (figure 6.1.a) and virus (figure 6.1.b) was measured over a range of antigen concentrations. The mean ³HT incorporation from three individual cultures was taken and the background ³HT incorporation by cells cultured in medium alone was subtracted.

Mice were immunised with;

- peptide A (VP1 111-130)
- peptide B (VP1 131-150)
- △— peptide C (VP1 211-130)
- peptide D (VP2 31-50)
- peptide E (VP2 101-120)
- ▲— peptide F (VP2 121-140)
- peptide G (VP2 201-220)
- peptide H (VP3 121-140)
- △-- peptide I (VP3 181-200)

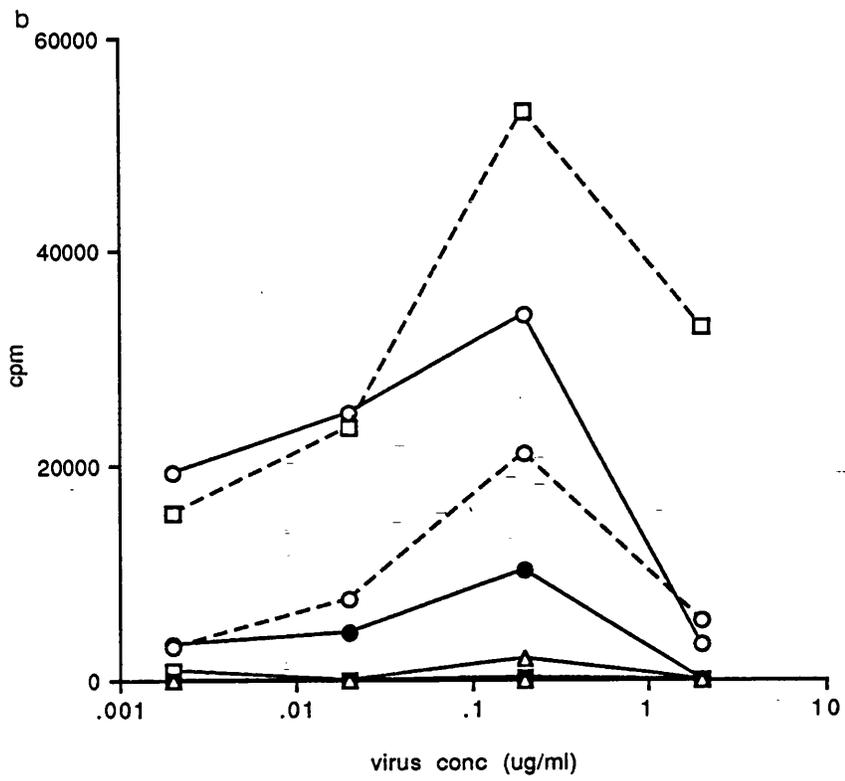
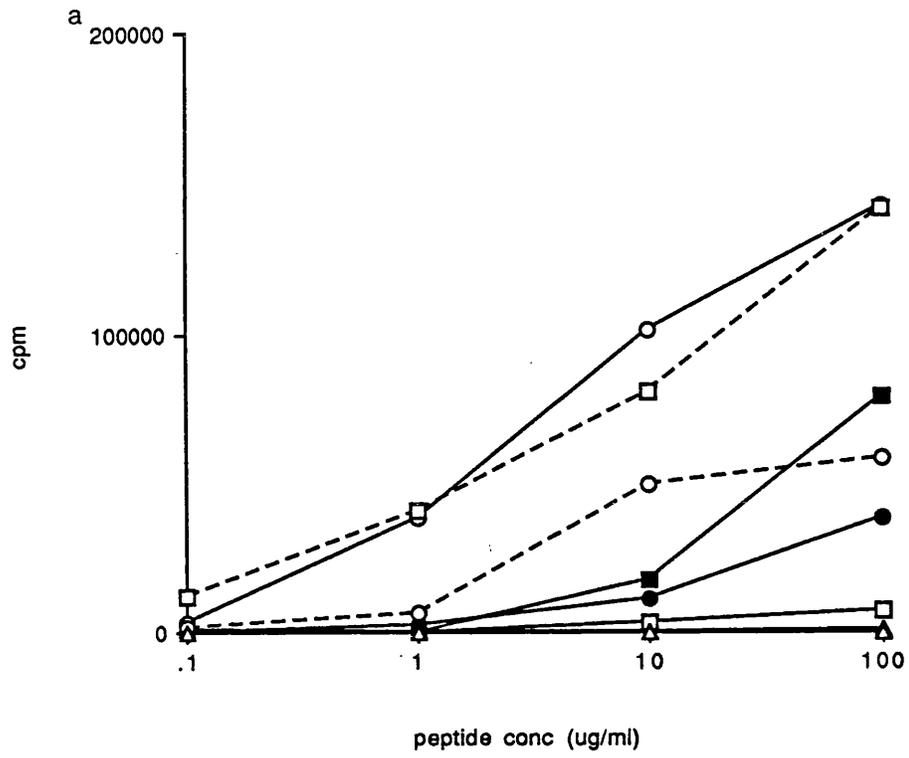


Figure 6.2. Cellular proliferative response of peptide immunised H-2^d mice to peptide and HRV 1A

Groups of four mice were immunised with 50 μ g of peptide emulsified 1:1 in FCA. The cellular proliferative response towards the immunising peptide (figure 6.2.a) and virus (figure 6.2.b) was measured over a range of antigen concentrations. The mean ³HT incorporation from three individual cultures was taken and the background ³HT incorporation by cells cultured in medium alone was subtracted.

Mice were immunised with;

- peptide A (VP1 111-130)
- peptide B (VP1 131-150)
- △— peptide C (VP1 211-130)
- peptide D (VP2 31-50)
- peptide E (VP2 101-120)
- ▲— peptide F (VP2 121-140)
- peptide G (VP2 201-220)
- peptide H (VP3 121-140)
- △-- peptide I (VP3 181-200)

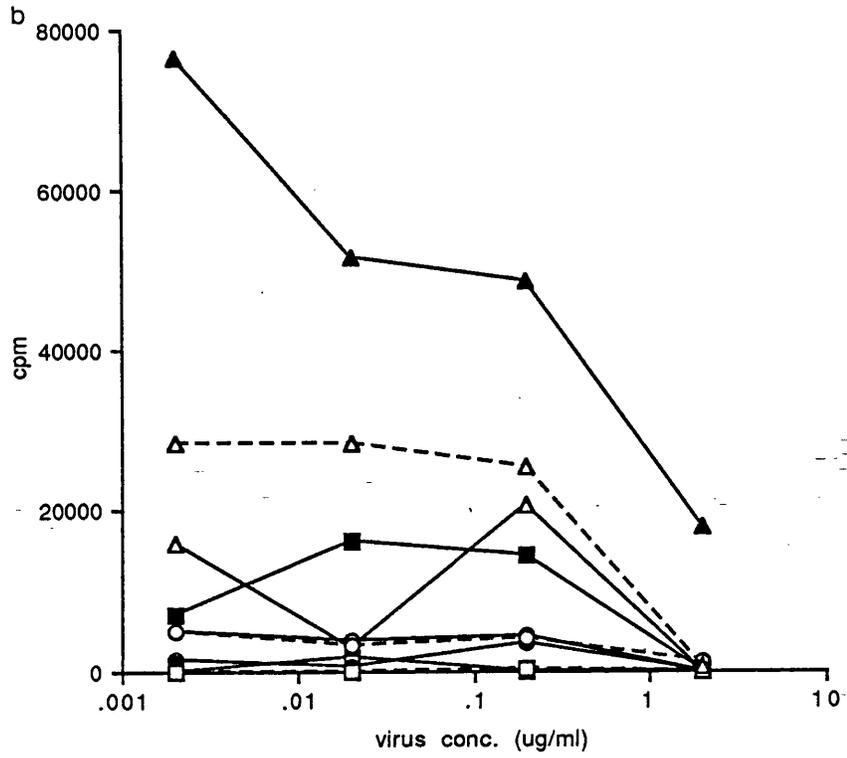
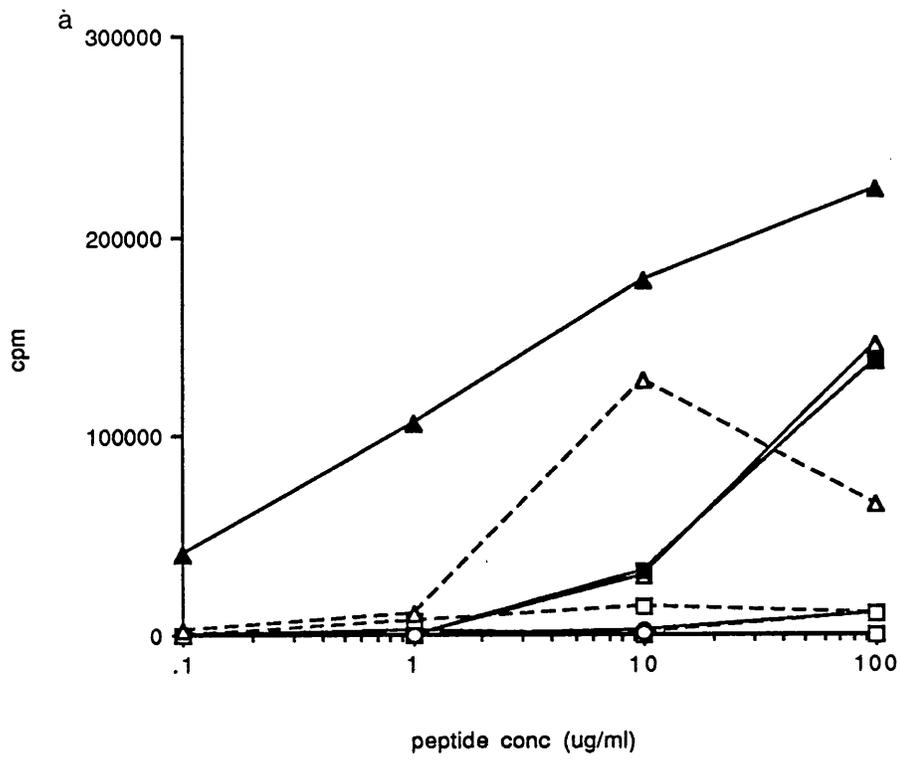


Figure 6.3. Cellular proliferative response of peptide immunised H-2^k mice to peptide and HRV 1A

Groups of four mice were immunised with 50 μ g of peptide emulsified 1:1 in FCA. The cellular proliferative response towards the immunising peptide (figure 6.2.a) and virus (figure 6.2.b) was measured over a range of antigen concentrations. The mean ³HT incorporation from three individual cultures was taken and the background ³HT incorporation by cells cultured in medium alone was subtracted.

Mice were immunised with;

- peptide A (VP1 111-130)
- peptide B (VP1 131-150)
- △- peptide C (VP1 211-130)
- peptide D (VP2 31-50)
- peptide E (VP2 101-120)
- ▲- peptide F (VP2 121-140)
- peptide G (VP2 201-220)
- peptide H (VP3 121-140)
- △- - peptide I (VP3 181-200)

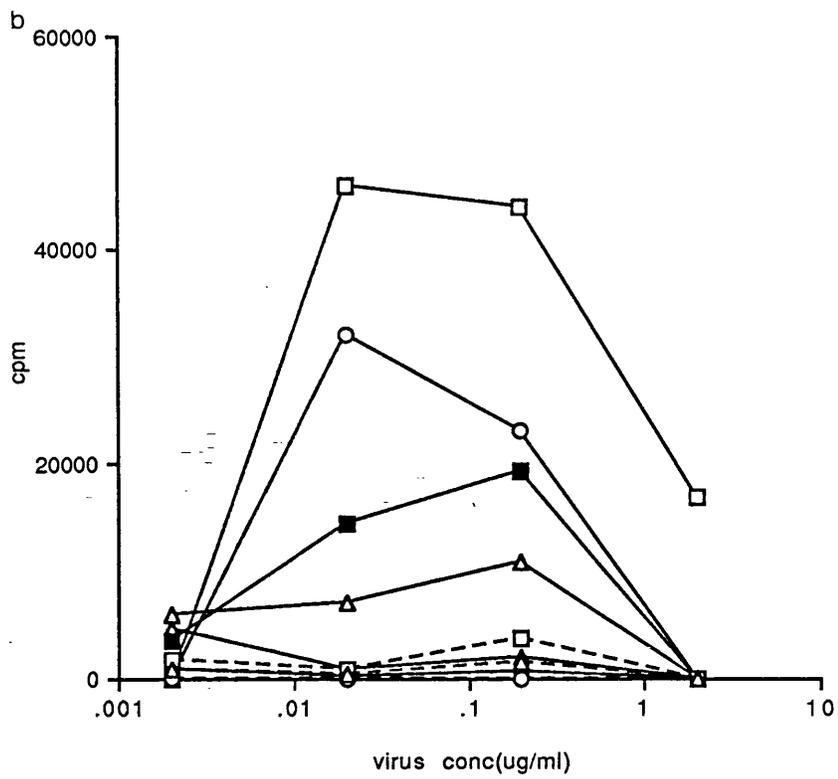
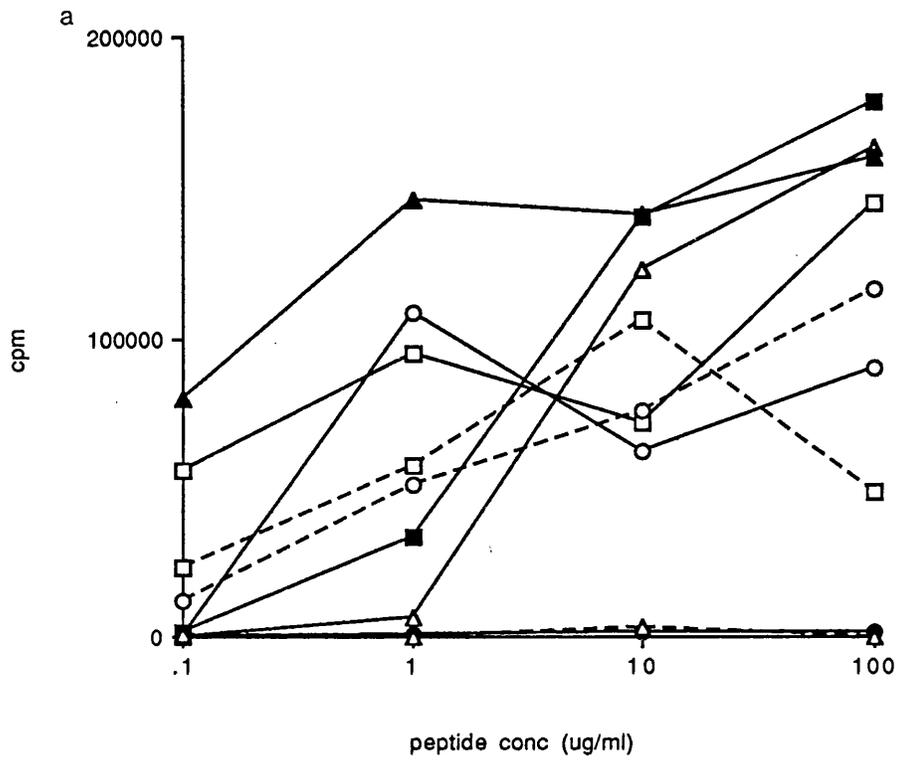


Figure 6.4. Cellular proliferative response of mice immunised with a mixture of HRV 1A peptides

Mice from each haplotypes were immunised with a mixture of three peptides (VP1 131-150, VP3 121-140 and VP3 181-200) emulsified 1:1 in FCA such that each mouse received 30 μ g of each peptide. Cellular proliferation in response to peptide at 10 μ g/ml and HRV 1A at 0.02 μ g/ml was measured in three individual cultures, and the mean 3 HT incorporation was determined. Stimulation indices were calculated by dividing the mean 3 HT incorporation by background incorporation of radiolabel seen with cells cultured with medium alone.

□ H-2^b, ▨ H-2^d, ▩ H-2^k.

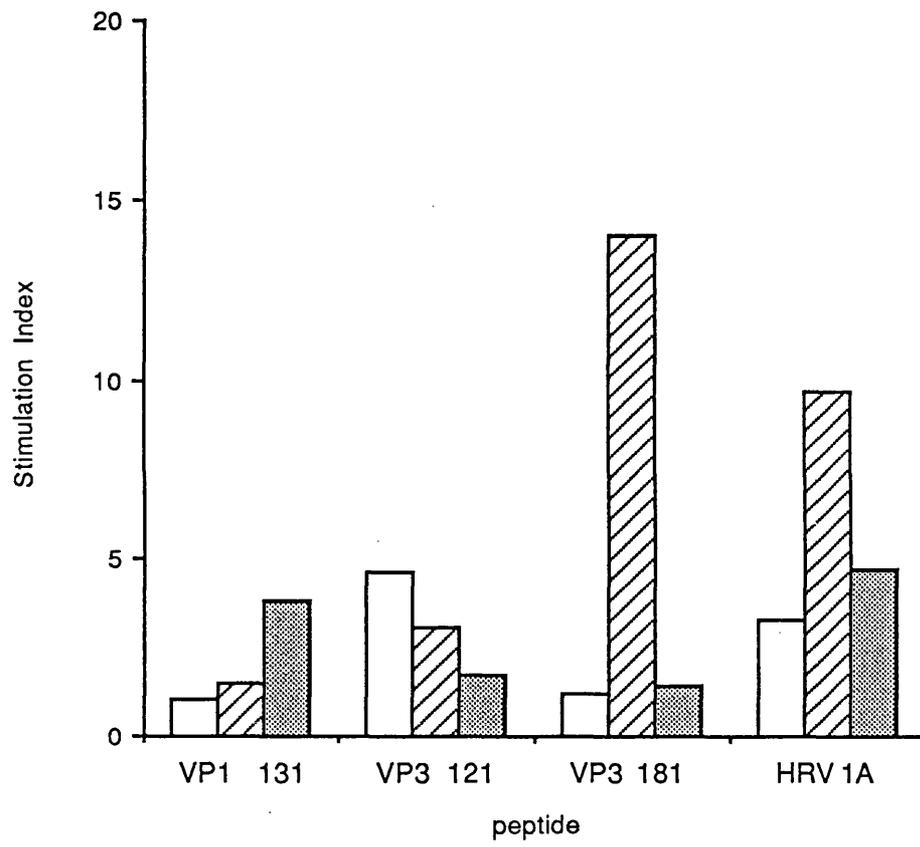


Figure 6.5. Proliferation of VP1 peptide 111-130 (A) primed cells with HRV serotypes

Groups of 10 mice were immunised with 50 μ g of peptide A (VP1 111-130) emulsified 1:1 in FCA. The mean c.p.m. was determined for three assay wells at each of the virus concentrations and the background c.p.m. subtracted. Figure 6.5.a shows the response of lymph node cells from H-2^b mice and figure 6.5.b shows the response of H-2^k cells. HRV serotypes used were as follows;

- HRV 1A
- HRV 1B
- △— HRV 2
- HRV 3
- HRV 7
- ▲— HRV 14
- HRV 15
- HRV 29
- △-- HRV 49

Figure 6.6. Proliferation of VP1 peptide 131-150 (B) primed H-2^k mice with HRV serotypes

Groups of ten CBA mice were immunised with 50 μ g of peptide B (VP1 131-150) emulsified 1:1 in FCA. Cellular proliferation was determined by measuring the ³HT incorporation in three individual wells. The mean was taken and the background cellular proliferation from cells cultured in medium alone was subtracted. Figure 6.6.a. shows the response of peptide primed cells to minor receptor group viruses and figure 6.6.b. shows the response to major receptor group HRV serotypes. The HRV serotypes used were as follows;

- HRV 1A
- HRV 1B
- △— HRV 2
- HRV 3
- HRV 7
- ▲— HRV 14
- HRV 15
- HRV 29
- △-- HRV 49

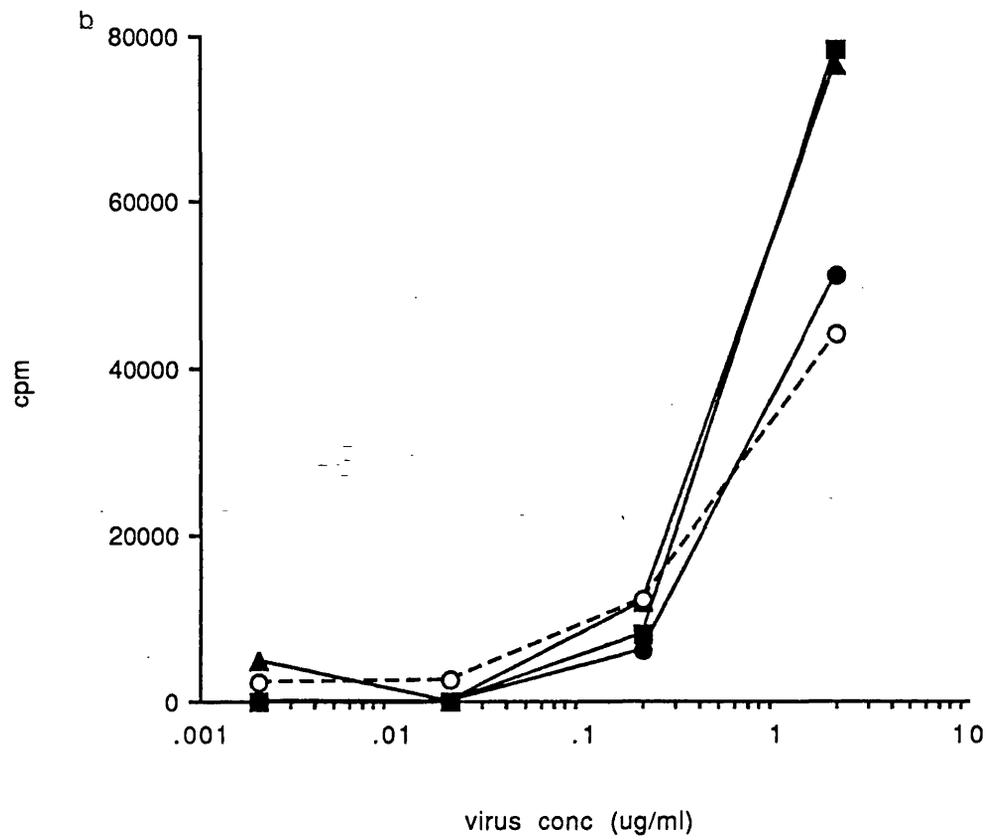
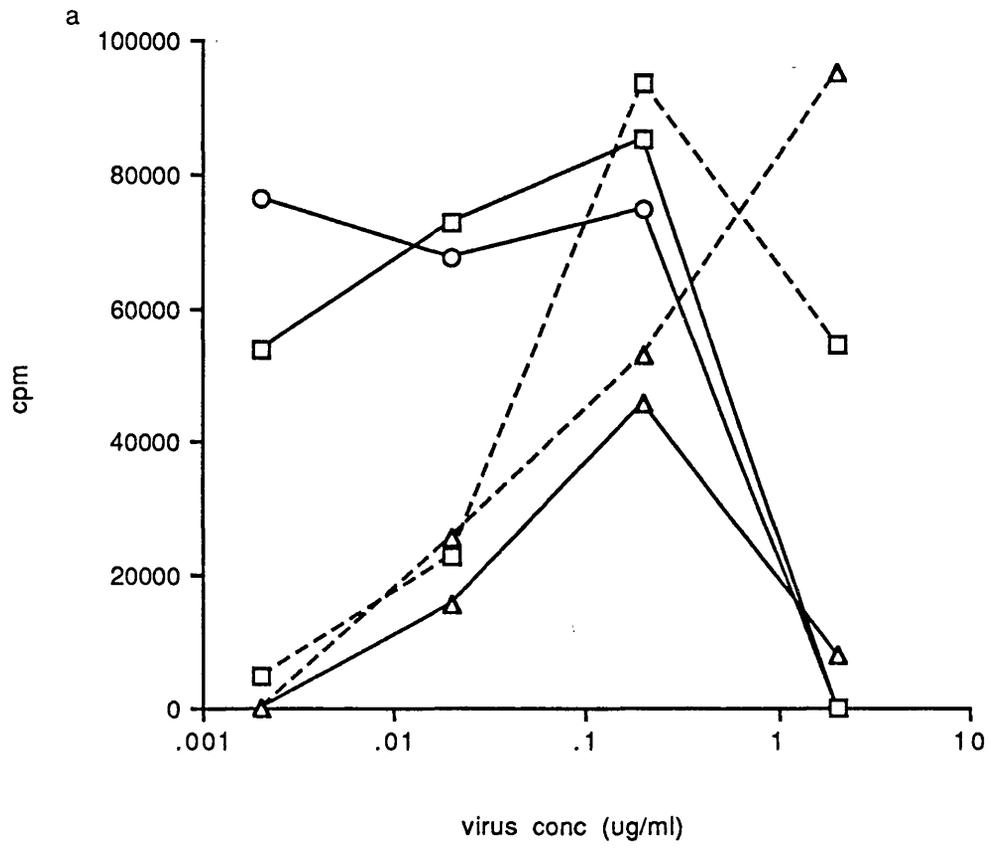


Figure 6.7. Proliferation of VP3 peptide 181-200 (I) primed H-2^d mice with HRV serotypes

Groups of ten CBA mice were immunised with 50µg of peptide I (VP3 181-200) emulsified 1:1 in FCA. Cellular proliferation was determined by measuring the ³HT incorporation in three individual wells. The mean was taken and the background cellular proliferation from cells cultured in medium alone was subtracted. Figure 6.7.b. shows the response to major receptor group HRV serotypes. The HRV serotypes used were as follows;

- HRV 1A
- HRV 1B
- △— HRV 2
- HRV 3
- HRV 7
- ▲— HRV 14
- HRV 15
- HRV 29
- △-- HRV 49

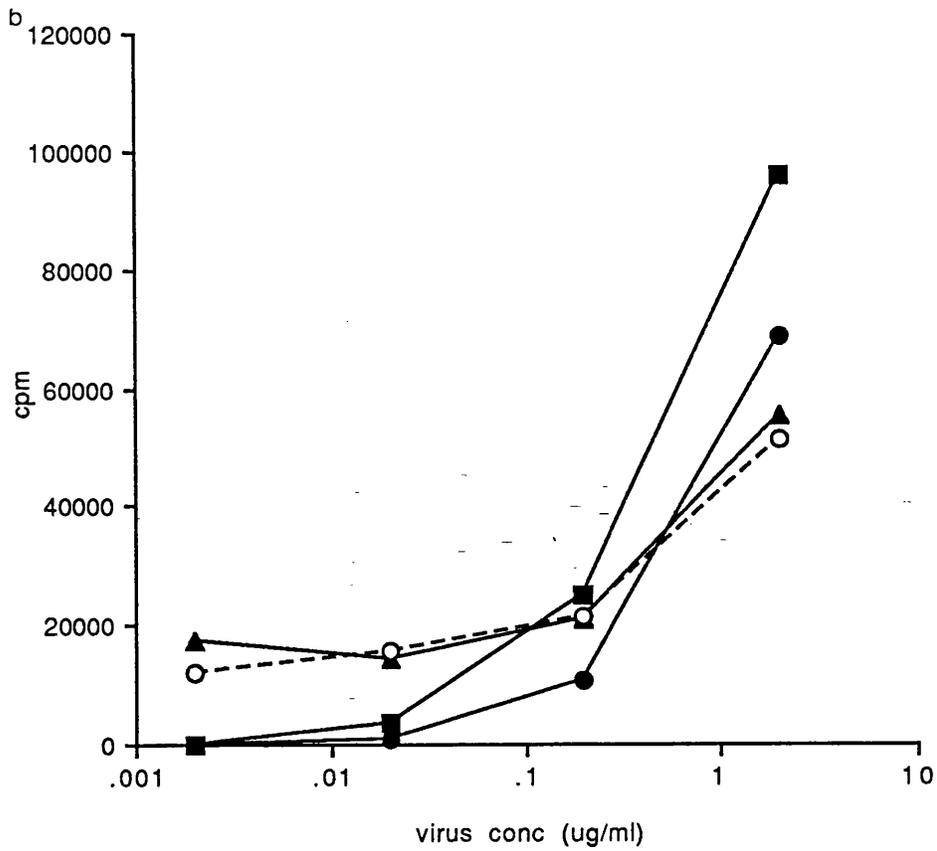
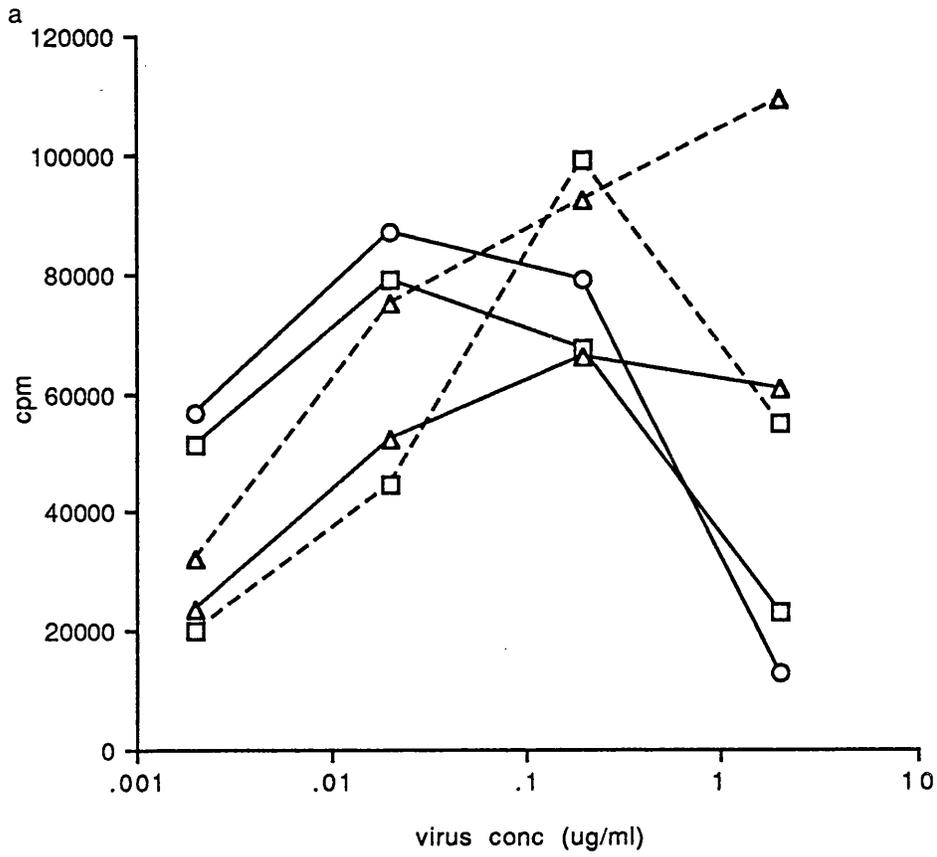


Figure 6.8. Amino acid sequences of HRV serotypes in the regions defined using peptides as T cell epitopes for HRV 1A

Amino acid sequences for HRV serotypes 1A, 1B, 2, 14, 39, 49 and 89 are those described by Palmenberg (1988). The primary amino acid sequence of HRV 15 was obtained from Dr. B.E. Clarke, Wellcome Foundation. Differences from the amino acid sequence of HRV 1A are shown in lower case letters.

HRV 1A VP1

111-131

1A RKFELFTYVRFDSEITLVPC
1B RKFELFTYVRFDSEvTLVPC
2 RKFELFTYtRFDSEITLVPC
14 kKlELFTYVRFDSEyTilat
15 RklELFTYVRFDSEvTLVPC
49 RKFELFTYtRFDSEITLVPC
9 RKFELFTYaRFDSEITLVPC
39 RKFEmFTYVRFDSEITLVPC
85 RKFEEFTYtRFDSEITLVPC
89 RKyELFTYtRFDSEITItaa

131-150

1A IAGRGDDIGH IVMQYMYVPP
1B IAGRGDDIGH vVMQYMYVPP
2 IsaLsqDIGH ItMQYMYVPP
14 asq p DsanysnlVvQAMYVPP
15 IAaKsDnIGH vVMQYMYVPP
49 IsaLskDIGH ItMQYMYVPP
9 IAaEsesvGH vVMQYMYVPP
39 IAGRGeDIGH IVMQYMYVPP
85 IAGKGDDIGH IVMQYMYVPP
89 aAq GnDsGH IVlQFMYVPP

211-230

1A SVVTNDMGTICSRIVTEKQK
1B SiVTNDMGTICSRIVTEKQe
2 tanTNnMGslCSRIVTEKhi
14 itVlNhMGsmafRIVNEhde
15 tVVTNDMGTlCSRIVTEehg
49 tVsTNnMGslCSRIVTEKhi
9 tiVTNDMGslCSRIVTEehg
39 vsVTNDMGTlCtRIVTnqQK
85 tsVTNhMGTlCSRIVTnKQq
89 SVVTNDMGTICvRIVTsnQK

HRV1A VP2

031-051

1A AVVGYGVWPHYLTPQDATAI
1B AVVGYGVWPHYLTPQDATAI
2 AiVaYGVWPHYLsSkDAsAI
14 AVVCY AeWPeYLpdvDAsdi
15 AVVGYGVWPHYLyPeDATAI
9 AVVGYGVWPHYLTPQDATAI
85 AVVGYGVWPHYLTPQDATAI
89 AVVAYGVWPsYLTPdDATAI

101-120

1A LGRSGYTVHVQCNASKFHQG
1B LGRSGYTVHVQCNASKFHQG
2 LGRSGYTihVQCNASKFHQG
14 LGRSGYTVHVQCNA tKFHsG
15 LGRSGYTVHVQCNaSKFHQG
9 LGRSGYTVHVQCNaSKFHQG
85 LGRSGYTVHVQCNA sKFHQG
89 LGRSGYTihVQCNS SKFHQG

121-140

1A TLLVAMIPeHQlASAKHGsv
1B TLLVAMIPeHQlASAKnGsv
2 TLIVAlIPEHQIASAlHGnV
14 cLlVvvIPEHQlAShegGnV
15 TLIVAMIPeHQlASAEkGni
9 TLIVAMIPeDQlAaAerGnV
85 TllVvMIPEHQlASAtkGnV
89 llIVvaIPEHQlASAtsGnV

201-220

1A LIVPYVNAVPMDSMLRHNNW
1B LIVPYVNAVPMDSMLRHNNW
2 iIaPYVNAVPMDSMrSHNNW
14 iviPYiNsVPiDSMtRHNNv
15 LIVPYVNAVPMDSMLRHNNW
9 iIVPYVNAVPMDSMLRHNNW
85 iIVPYVNAVPMDSMLRHNNW
89 LIlPYVNAVPMDSMLRHNNW

HRV 1A VP3

121-140

1A GTANTTLKLLLAYTPPGIDE
1B GTANTTLKLLLAYTPPGIDk
2 GTANTTvkLLLAYTPPGIaE
14 GpAlssaKLiLAYTPPGarg
15 GTANTTLKLLLAYTPPGIak
9 GsAssTLKLLLAYTPPGIak
85 GTANTTLKLLLAYTPPGIDk
89 GsAssTLKLLiAYTPPGvgk

181-200

1A NKYSMAGYITCWYQTNLVVP
1B NKYSMAGYITCWYQTNLVVP
2 grsts.GYITCWYQTrLViP
14 dtYtsAGflsCWYQTSLiP
15 dtfSsAGYIsCWYQTNLVVP
9 dKYSlAGfITCWYQTNLVVP
85 dtYSrAGYITCWYQTNfvfP
89 dtYSsAGYITCWYQTNfvVP

Figure 6.9. Antibody response of virus primed mice to haptened peptides

Groups of five H-2^k mice were primed with 0.2 μ g HRV 1A in aluminium hydroxide sub-cutaneously at the tail base. Fourteen days after primary immunisation the mice were boosted i.p. with 50 μ g TNP-peptide. The antibody response towards TNP was measured by ELISA at 14, 21, 28, 42, and 70 days after primary immunisation with virus. The anti-TNP response was measured for individual mice and used to calculate the mean endpoint titre (chapter 2), expressed as the reciprocal dilution. Mice were immunised as follows;

- peptide A (VP1 111-130)
- peptide B (VP1 131-150)
- ▲ peptide E (VP2 101-120)
- peptide F (VP2 121-140)
- negative peptide (VP1 241-260)
- △ no virus priming, followed
by peptide B

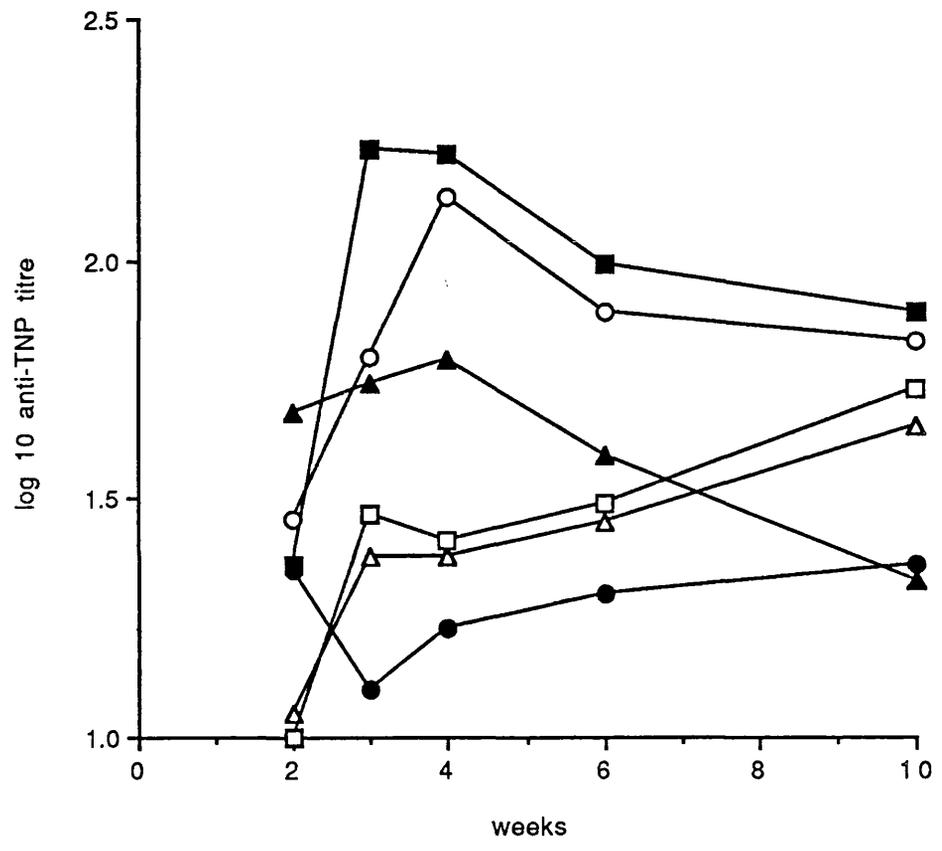


Figure 6.10. Location of the four peptides identified as dominant Th epitopes for HRV 1A VP1 and VP2 in H-2^k mice

The four peptides (A, B, E, and F) identified as dominant in H-2^k mice were plotted onto the molecular model of HRV 1A. A pentameric subunit, comprising five protein trimers (1 copy each of VP1, 2, and 3) of the virus is shown, the figure to the left of the page shows a view looking down onto the five-fold axis of the virus where the five VP1 molecules meet, and the figure to the right shows an orthogonal view of the pentamer. In this view the outer surface of the virus is towards the left hand side, and the inner surface is towards the right hand side. The two VP1 peptides are highlighted in blue and the VP2 peptides are highlighted in green.

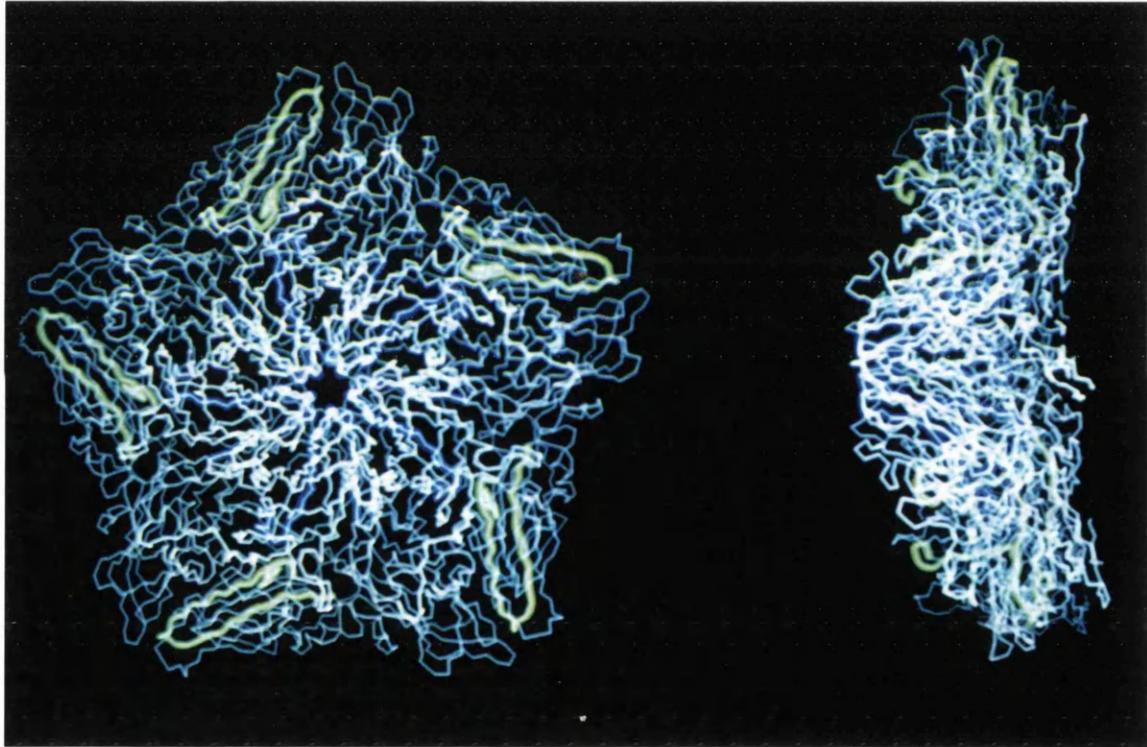


Figure 6.11. Structural location of the two VP1 peptides 111-130 and 131-150 identified as dominant Th epitopes in H-2^k mice

A single protein trimer of HRV 1A is shown with VP1 coloured blue, VP2 green and VP3 coloured red. The positions of the two VP1 peptides, 111-130 (A) & 131-150 (B) are highlighted on the molecular backbone of the structural proteins of HRV 1A.

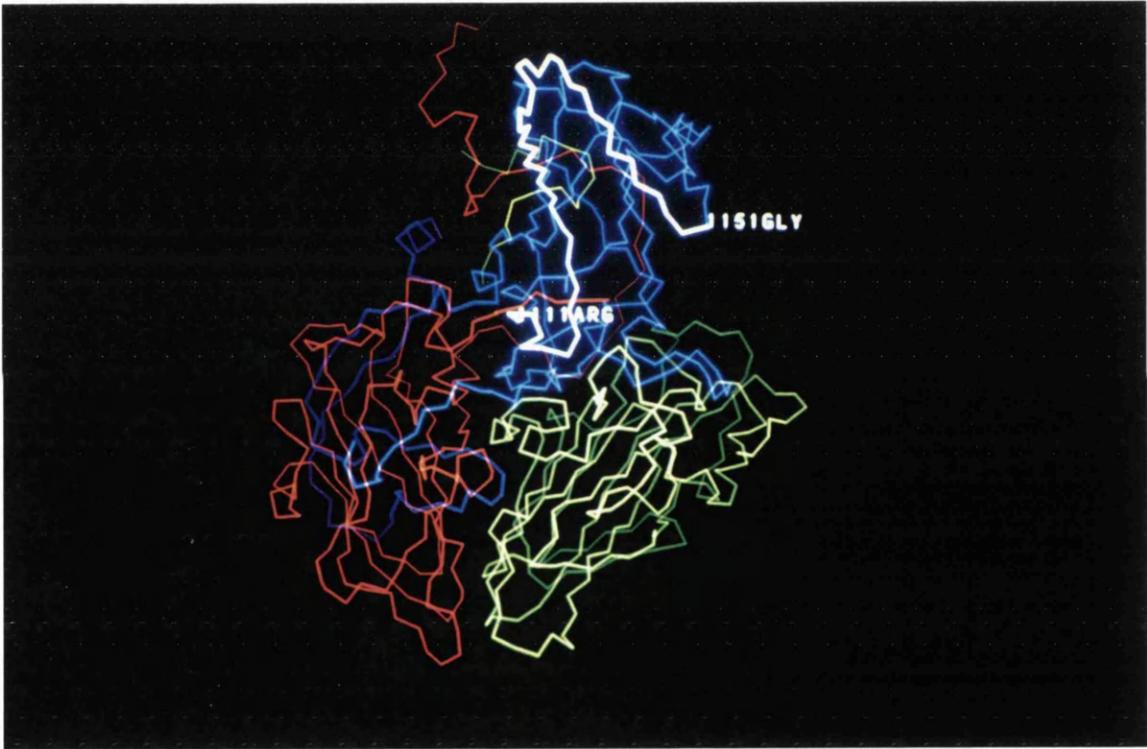


Figure 6.12. HRV 1A Th epitopes are internal within the virus structure and are remote from the B cell epitopes

The three dimensional structure of HRV 1A (Kim et al, 1989) was used to locate the positions of Th cell epitopes of HRV 1A and the B cell determinants of HRV 2 (Appleyard et al., 1990). The amino acids involved in forming the B cell sites are represented by spheres coloured according to the viral protein on which they are located, blue VP1, green VP2, and red VP3. The Th cell epitopes are coloured according to the mouse strain in which they were identified positive, cyan H-2^d, magenta H-2^k, and yellow H-2^b. The outer surface of the virus is towards the right of the figure and the inner surface is towards the left of the figure.

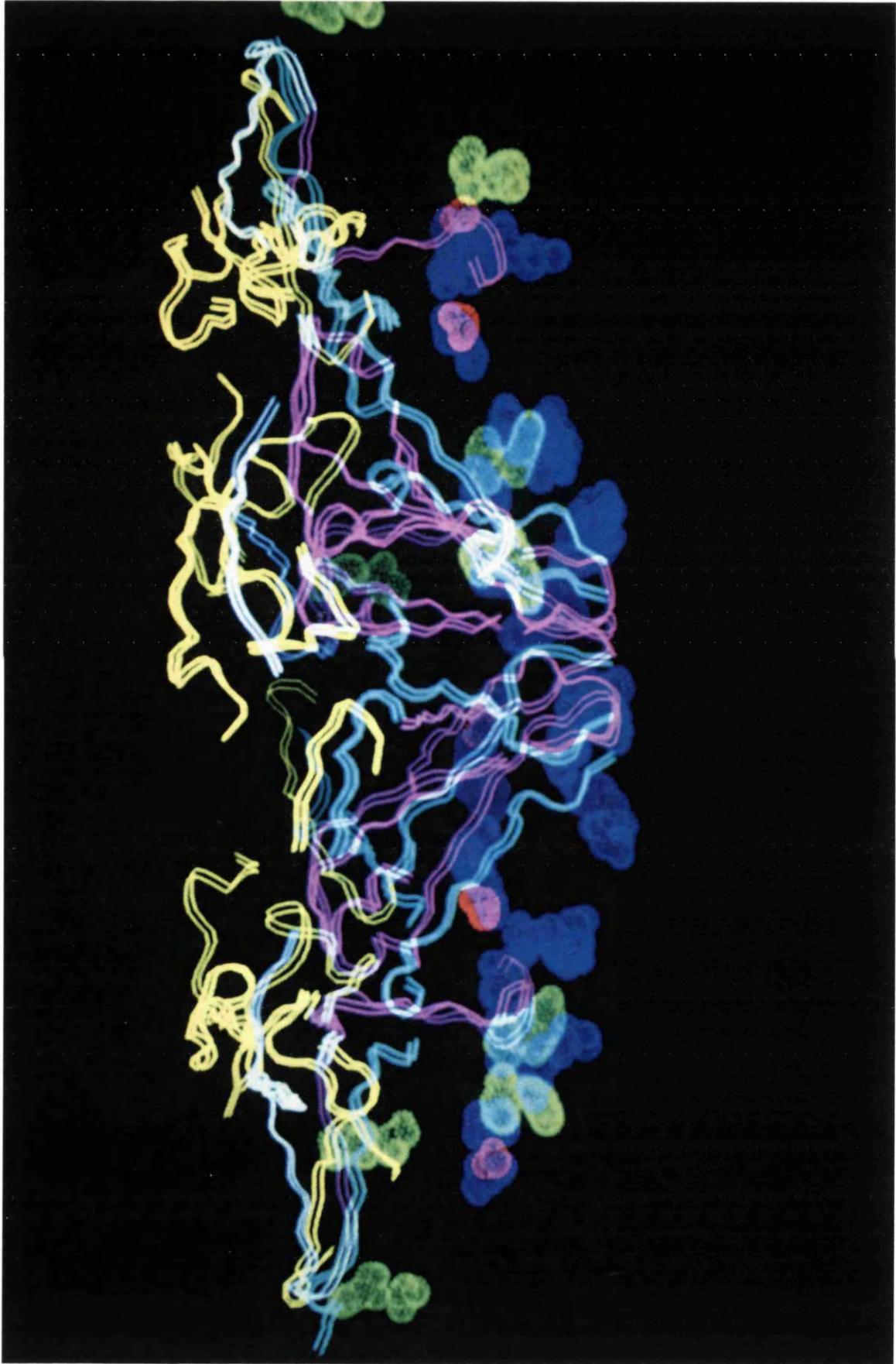


Table 6.1. Proliferative responses of peptide-primed mouse cells to peptide and HRV 1A

The data shown in figures 6.1-6.3 was used to compile table 6.1. The stimulation index was calculated for each of the three mouse strains immunised with each of the nine peptides (A-I) at the concentration of peptide and virus giving the maximum cellular proliferation (data shown in figures 6.1.-6.3.).

(-) S.I.< 2.5; (+) 2.5-5; (++) 5-10 and (+++) > 10.

mouse strain

viral protein	immunising peptide	mouse strain						
		letter	Balb/c	H-2d	cba	H-2k	C57/bl	H-2b
		peptide	peptide	virus	peptide	virus	peptide	virus
1	111-130	A	-	-	++	+	+++	+++
	131-150	B	-	-	++	++	++	-
	211-230	C	+++	+	+++	-	-	-
2	31-50	D	+	-	-	-	+++	+
	101-120	E	+++	+	++	+	+++	-
	121-140	F	+++	+++	+++	-	-	-
	201-220	G	+	-	+++	+	+++	+++
3	121-140	H	++	-	+++	-	++	++
	181-200	I	+++	+++	-	-	-	-

Table 6.2. Priming with peptide containing HRV 1A Th cell epitopes enhances virus specific antibody titres

Groups of five H-2^k mice were immunised with 50µg peptide emulsified 1:1 in FCA sub-cutaneously at the tail base. 21 days later mice received a sub-optimal dose of virus in FIA (0.002µg per animal). The anti-virus antibody titre was measured by ELISA at 21, 28 and 42 days after primary immunisation with peptide. The end point titre was determined for individual mice and used to calculate the mean end point titre, expressed as the reciprocal dilution, for the group. The standard error (expressed as S.E.M.) was also calculated.

viral protein	peptide	letter	antibody titre at days post immunisation		
			21	28	42
1	111-130	A	34.4± 10.1	202.1± 12.6	126.0± 19.2
1	131-150	B	<10	238.0± 89.2	124.0± 26.2
2	101-120	E	19.1± 2.3	339.7±103.5	107.2± 27.1
2	121-140	F	10.7± 0.7	20.7± 3.6	20.6± 2.9
2	31-51	Z	12.5± 0.8	17.4± 2.6	24.0± 2.1
none	none	none	11.2± 0.6	<10	11.0± 0.7

Table 6.3. Anti-TNP antibody response of groups of mice primed towards HRV 1A and boosted with haptened peptides

The results show the mean titre of anti-TNP specific IgG, expressed as the reciprocal dilution at days 14, 21 and 70 post primary immunisation of H-2^k mice. This information was used in the compilation of figure 6.9. This table also shows the standard error, expressed as S.E.M., for each of the groups of five mice at the given time points.

viral protein	peptide	letter	antibody titre at days post immunisation		
			14	21	70
1	111-130	A	22.4± 7.4	24.0± 9.7	22.9± 12.4
1	131-150	B	22.9± 7.8	208.9 ± 46.5	77.6± 32.9
2	101-120	E	47.9± 22.4	55.0± 17.2	21.4± 5.3
2	121-140	F	28.8± 11.6	63.1± 17.0	67.6± 9.5
2	241-260	X	11.2± 1.0	21.4±11.2	44.7± 31.2
1	131-150	B	<10	29.5± 9.6	53.7± 3.3

Chapter 7. General discussion

7.1. Summary of the results

HRV elicits strong antibody responses, after infection of the natural host (Barclay et al., 1988) and when used to immunise experimental animals (Hastings et al., 1990). The studies described in this thesis have shown that HRV also exhibits a high level of immunogenicity at the level of virus specific T cell responses. The major findings of this thesis can be summarised as follows;

1. HRV exhibits an intrinsic immunogenicity.
2. Helper T cell responses to HRV were heterotypic in three different mouse haplotypes.
3. T cells primed towards minor group serotype 1A recognised other minor receptor group serotypes preferentially to major group HRV serotypes.
4. The Th epitopes could be defined using synthetic peptides; however the response of HRV 1A primed Th cells to the peptides in three different mouse strains was mostly haplotype specific.
5. Peptides identified in 4. primed for virus specific Th cell responses towards the nine HRV serotypes tested.
6. Positive peptides provided T cell help for antibody production in vivo.
7. The T cell epitopes for HRV 1A were buried within the virus structure and were remote from the neutralising antibody sites.

7.2. Immune responses to HRV infection

The high titres of serotype specific neutralising antibodies seen in infected humans and experimental animals suggest that the virus is highly immunogenic. Virus neutralising antibodies were shown to be directed towards a relatively small number of determinants, four for HRV 14 (Sherry and Reukert, 1984), and three for HRV 2 (Appleyard et al., 1990). The factors responsible for the strong immunogenicity of HRV, and for directing the antibody response to these restricted epitopes areas of the HRV surface remain unclear. However, a single virus particle expresses 60 copies of each of the viral proteins, presenting multiple copies of the antigenic determinants on the virus surface. Such an arrangement should provide an efficient delivery system for B cell epitopes and is similar to that of genetically engineered expression systems such as hepatitis B virus core antigen protein (HBcAg). HBcAg can be modified so that it expresses antigenic determinants from other proteins on the surface of the protein particles that it forms (Clarke et al., 1987), and has been shown to raise antibody responses to such epitopes more efficiently than synthetic peptides representing those epitopes (Francis et al., 1990).

The finding that minor receptor group viruses were more immunogenic than major group viruses in mice, which only express the minor HRV receptor, suggests that a special mechanism exists to bring about this difference in

immunogenicity. Since human cells express both major and minor HRV receptors, this mechanism would be expected to occur for all virus serotypes. The facilitated uptake of virus by cells expressing HRV receptors, leading to the efficient processing and presentation of the helper T cell epitopes of the virus, may enhance the production of virus specific antibody. The ability of HRV to direct an immune response favouring the generation of virus specific antibodies may also confer an evolutionary advantage to HRV, which is able to mutate its protein capsid under antibody pressure such that it is no longer recognised by that specific antibody and eventually becomes a new infectious serotype.

7.3. Identification of Th cell epitopes of HRV 1A using synthetic peptides.

Identification of the Th cell epitopes of HRV 1A using synthetic peptides revealed that only a small number of epitopes were involved in the generation of the Th response. Moreover, the epitopes were mostly specific to a particular genetic background. Peptides identified as positive were shown to represent the natural Th epitopes, which arise from processing and presentation of the native virus, by their ability to prime for virus specific Th cell responses. Moreover, the Th responses elicited by peptides were heterotypic for other serotypes, suggesting that Th epitopes were conserved between serotypes and a comparison of the

available sequences for HRV serotypes revealed that there was a high degree of sequence homology within the regions represented by the positive peptides.

7.4. Position of the identified T cell peptides within the three dimensional structure of HRV 1A

Plotting the coordinates of the peptides on the three dimensional structure of the virus (Kim et al., 1989) revealed that these epitopes were buried within the overall virus structure and were remote from the surface B cell determinants. Two peptides from HRV 1A VP1, which generated T cells recognising peptide but not virus, were found to be located on surface accessible regions of the virus structure. Thus the requirement for antigen processing of HRV 1A, which was demonstrated experimentally, could be explained by the finding that the functional Th cell epitopes of HRV 1A were located internally within the three dimensional structure of the virus particle, implying that antigen processing plays an important role in determining the T cell epitopes of the virus.

The studies we have carried out have been confined to murine systems despite the consequent disadvantage that HRV does not cause a productive infection in these animals. However the animal studies have allowed us to study the parameters involved in stimulating HRV specific T cell responses in detail. Furthermore, they have allowed a

preliminary characterisation of T cell epitope recognition in relation to viral structure, and have allowed us to test the potential of peptide antigens from HRV 1A as in vivo immunogens. These studies have provided a good base for further studies of HRV immunogenicity in man, which are already in progress.

7.5. Immunisation with synthetic peptides primes for T cell help in vivo

The peptides were also shown to represent the "natural" T cell epitopes of the native virus by their ability to provide functional T cell help in vivo. Antibody production towards virus was enhanced by pre-priming animals with peptides prior to boosting with HRV 1A, demonstrating that peptide specific Th cells recognised and responded to the T cell determinants derived from the native virus. The reciprocal experiment, in which priming with HRV 1A was shown to provide T cell help for antibody production towards an unrelated B cell epitope linked to the HRV 1A T cell peptides, demonstrated that pre-existing virus specific T cells could be stimulated by peptides. These findings have a particular significance to the development of a peptide vaccine based upon the T cell epitopes of HRV (see below).

7.6. Potential use of Th cell peptides in the development of a synthetic HRV vaccine

The results presented in this thesis are relevant to the development of a peptide vaccine against HRV. A peptide representing the linear portion of a B cell epitope for HRV 2 (Francis et al., 1987) has been successfully used to elicit virus neutralising antibodies when coupled to defined Th cell epitopes (Francis et al., 1989) or when expressed in hepatitis B core antigen particles (Francis et al., 1990). However, the anti-peptide antibodies did not cross-neutralise other HRV serotypes. The lack of sequence homology between serotypes in the regions containing the B cell epitopes argues against the development of a synthetic peptide vaccine effective against all HRV serotypes. However, attempts to produce antibodies against other epitopes, which are more conserved than those against which natural antibody responses are directed (McCray and Werner, 1988), may prove promising. Conversely, the helper T cell epitopes of HRV 1A have been shown to be conserved between serotypes and peptides representing the "natural" Th epitopes prime experimental animals for heterotypic virus responses and act as functional Th cell epitopes in vivo (chapters 5 and 6). Therefore these epitopes would be ideal for inclusion in synthetic peptide vaccines comprising B and T cell epitopes. However, the specific nature of the response to peptides by different mouse strains suggests that different epitope hierarchies exist in different genetic backgrounds and

that a number of Th epitopes may be required in order to cover a broad range of the population.

Little is known about the CTL response mounted in response to virus infection. Studies with infected human volunteers indicated that cell killing was occurring but was possible attributable to the action of NK cells rather than virus specific CTLs. It is possible that the strong Th cell proliferative response to HRV is detrimental to the host since the immune system may be diverted from mounting an effective virus specific CTL response. Studies in animals have not been possible due to the species specificity of HRV for humans. However a variant of HRV 2 has been derived (Yin and Lomax, 1986) which is infectious for mice and this could be used to investigate whether virus specific CTL responses are generated. Also, the symptoms of a rhinovirus common cold infection are mostly caused by the host immune response to the virus (reviewed by Tyrrell, 1988) and therefore generation of strong cellular responses against HRV by immunisation may cause discomfort to the vaccinee.

An alternative approach to the use of defined Th epitopes of HRV in synthetic peptide vaccines would be to incorporate these epitopes into vaccines against infectious agents other than rhinovirus, providing T cell help for epitopes derived from unrelated viruses (Francis et al., 1987b). HRV is a common infection in man which is relatively harmless, and repeated infection with different HRV serotypes results in the generation of a memory population of virus specific Th cells

(Hsia et al., 1990). This memory T cell population could be exploited by including HRV T cell epitopes in synthetic vaccines to provide an immediate helper T cell response to the non-HRV epitopes of the vaccine.

7.7. Future studies

Further work arising from this study will include an investigation of the Th response to HRV in humans. Peripheral blood lymphocytes (PBLs) and T cell lines derived from normal volunteers will be used to examine whether heterotypic HRV responses are observed in humans, as has been suggested by Hsia et al., (1990). The specific Th cell epitopes involved in the generation of the Th response to virus will also be identified using synthetic peptides and the sequences can be compared to those identified as positive for virus used to immunise mice. The response of cells derived from human tonsils may also be investigated as these may provide a better source of T lymphocytes since they would be obtained from draining mucosal tissues and would be more representative of the memory population that a synthetic vaccine Th epitope would be designed to stimulate. The relationship of T and B cell epitopes within the protein structure of the virus could also be interpreted using epitopes identified as positive for human Th cells. These findings could also be applied to other picornaviruses which are known to exhibit a great deal of structural similarity, and which cause number of important

diseases in animals and man. A comparison of the similarities, and differences arising from the study of the immunogenicity of HRV in mouse and man would contribute to the overall knowledge of the factors governing the immune response to an antigen, and in particular to a complex antigen such as HRV.

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Appendix

The following papers have been published or are in preparation in connection with the work described in this thesis;

Hastings, G.Z., Rowlands, D.J., and Francis, M.J. (1991). Proliferative responses of T cells primed against human rhinovirus to other rhinovirus serotypes. *J. Gen. Virol.* 72, 2947-2952.

Hastings, G.Z., Chain, B.M., Campbell, R.O., Rowlands, D.J., and Francis, M.J. (1992). Identification of T-cell epitopes from human rhinovirus for use in synthetic peptide vaccines. In *Vaccines 92, - Modern approaches to new vaccines including prevention of AIDS.* pp 259-264. Cold Spring Harbor Symposium 1991, Cold Spring Harbor, New York.

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G. Z. Hastings, M. J. Francis, D.J. Rowlands, R.O. Campbell, and B. M. Chain (1992). Identification of functional Th cell epitopes of human rhinovirus. Manuscript in preparation.

Proliferative responses of T cells primed against human rhinovirus to other rhinovirus serotypes

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Lymphocytes from mice immunized with human rhinovirus (HRV) serotypes 1A or 15 proliferated *in vitro* in response to HRV and the activated cells were shown to be helper T (Th) cells. Lymphocytes from mice primed with HRV-1A responded to seven of eight heterologous virus serotypes, the responses to other minor cell receptor group viruses being greater than to those belonging to the major cell receptor group. A similar bias was seen with cells from mice primed with HRV-15 in that they responded preferentially to other major receptor group viruses. This pattern of cross-

serotype recognition was shown to be similar in three inbred mouse strains and was not dependent upon the major histocompatibility complex haplotype. These results have revealed that there are determinants within the viral proteins of a number of serotypes of HRV that are recognized by Th cells primed against a single HRV serotype. Thus, at the level of Th cell recognition of HRV, a cross-serotype reactivity is seen which is not reflected in the B cell antibody response to virus, which is generally highly serotype-specific.

Introduction

Human rhinoviruses (HRVs) are the major causative agents of the common cold (Stott & Killington, 1972) and possibly the most common cause of acute infections in man (Gwaltney, 1975). The virus capsid is made up of 60 protein trimers comprising three surface structural proteins, VP1, VP2 and VP3, with a fourth protein, VP4, located internally, and contains a single copy of positive-strand RNA (Mendappa *et al.*, 1971; Rueckert, 1976). HRVs exhibit a high degree of antigenic variation, with over 100 serotypes having been identified (Hamparian *et al.*, 1987). Although the presence of virus-neutralizing antibodies correlates with protection against disease (Doggett, 1965), anti-HRV antibodies are highly serotype-specific, demonstrating little or no cross-neutralization of other rhinovirus serotypes. The different serotypes of HRV can also be divided into two groups, major or minor, depending on the cellular receptor to which they bind (Abraham & Colonna, 1984).

HRVs cause a rapid lytic infection of the cells lining the nasal endothelium (Bynoe *et al.*, 1961), and it has been observed that during the course of such an infection the number of circulating T cells in the peripheral blood

decreases dramatically, possibly due to migration of the lymphocytes from the blood into infected tissues (Levandowski *et al.*, 1986). These *in situ* lymphocytes may then exhibit cytotoxic activity and/or mediate the activity of other cells involved in the cellular immune response. An investigation of peripheral blood mononucleocytes (PBMCs) from infected human volunteers has demonstrated both non-specific and virus-specific cellular activity (Hsia *et al.*, 1990), indicating that HRV infection activates a systemic immune response. The antigen-specific proliferation of PBMCs is indicative of the presence of virus-specific T cells, a subset of which would be T helper (Th) cells capable of stimulating antibody production by B cells. The generation of Th cells that recognize and proliferate with different HRV serotypes could be utilized in the design of synthetic human vaccines by incorporating the HRV Th cell epitopes (Francis & Clarke, 1989) with identified B cell epitopes from HRV (Francis *et al.*, 1987; Hastings *et al.*, 1990) or possibly other viruses.

In this study we have investigated the Th cell responses to HRV in mice of different genetic backgrounds with a view to characterizing further the T cell epitopes responsible for the cross-recognition of different serotypes of the virus.

Methods

Virus serotypes. HRV-3, -14, -15 and -49 were received from W. Barclay, MRC Common Cold Unit, Salisbury, U.K.; HRV-7 and -29

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were from V. V. Hamparian, Children's Hospital, Columbus, Ohio, U.S.A.; HRV-1A was from D. C. Pevear, Sterling Winthrop, Rensselaer, N.Y., U.S.A.; HRV-1B was from The Wellcome Foundation, Beckenham, U.K.; HRV-2 was received from D. Blaas, Vienna, Austria. The viruses were plaque-purified three times and their identities confirmed using virus serotype-specific antisera.

Preparation of virus stocks. Virus was grown in OH-5 cells, a cloned line of O-HeLa cells which is highly susceptible to rhinoviruses. Stocks were prepared by infecting confluent cell monolayers with 50 μ l of plaque-purified virus. After 1 h at room temperature to allow virus attachment, 20 ml of Eagle's MEM supplemented with 2% foetal calf serum (FCS) and 30 mM-magnesium chloride were added, and the flasks were incubated at 34 °C. When c.p.e. was apparent (36 to 48 h post-infection depending on virus serotype) virus was released by freezing and thawing twice, and the cell debris sedimented at 3000 r.p.m. in a bench centrifuge. The supernatant was stored in 0.5 ml aliquots at -70 °C. Large-scale virus preparations were produced by growing inoculum as above, using 0.5 ml of the stock virus to infect each flask, and then infecting roller bottle cultures containing approximately 10^9 cells with 5 ml of fresh inoculum. The bottles were stored at -20 °C when c.p.e. was complete.

Virus purification. A modification of the method described by Appleyard *et al.* (1990) was used. Virus was harvested from 10 to 20 roller cultures by twice freezing at -20 °C and thawing, the supernatant was clarified by centrifugation and virus was precipitated by the addition of an equal volume of saturated ammonium sulphate in 0.04 M-phosphate buffer pH 7.0. After 1 h at 4 °C, the precipitate was sedimented at 3000 r.p.m. for 40 min and resuspended in 120 ml of ice-cold Eagle's MEM containing 1% Sarkosyl and 5 mM-EDTA. Virus was pelleted by centrifugation at 33000 r.p.m. for 90 min using a Beckman T35 rotor, resuspended in 1 ml of 10 mM-Tris-HCl, 1 mM-EDTA, 0.1% 2-mercaptoethanol buffer at pH 7.5 and fractionated on a linear 15 to 45% (w/v in PBS) sucrose gradient for 2 h at 40000 r.p.m. in an SW40 rotor. Pooled 0.5 ml fractions were taken from two gradients and the virus peak was detected by measuring the A_{260} in a spectrophotometer. Fractions representing the peak were pooled, sterile filtered through a 0.22 μ m filter and stored at -70 °C. The serotypes of the sucrose gradient-purified viruses were confirmed by cross-neutralization assays performed with HRV serotype-specific antisera (data not shown).

Measurement of protein concentration. The concentration of protein in the purified virus preparations was measured using a microplate assay. A bovine serum albumin standard (100 μ g/ml) and the samples were serially diluted twofold in 100 μ l of distilled water in eight wells of a microplate. Bio-Rad protein assay reagent (100 μ l) diluted fivefold in distilled water was added and the A_{600} read using an automated plate reader. The protein concentrations were calculated using linear regression analysis of the standard curve.

Preparation of primed lymph node cells. Purified virus was diluted in sterile PBS and emulsified 1:1 with Freund's complete adjuvant (Difco) to give a final virus concentration of 2 μ g/ml. Female, 8 to 10-week-old specific pathogen-free mice, either BALB/c, C3H.He or C57.BL (obtained from Charles River), were immunized in the base of the tail with 100 μ l of the experimental preparation. Seven to 10 days after immunization the draining inguinal and para-aortic lymph nodes were taken and passed through a stainless steel mesh to produce a single-cell suspension which was washed twice in ice-cold RPMI 1640 supplemented with penicillin [100 units(U)/ml], streptomycin (100 μ g/ml) and 20 mM-glutamine (RPMI'). Viable cells were counted and resuspended on ice at 5×10^6 cells/ml in RPMI' supplemented with 1% normal mouse serum and 1×10^{-4} 2-mercaptoethanol.

Cell proliferation assay. Control antigens, concanavalin A (Con A;

500 μ g/ml; Sigma) and purified protein derivative (PPD; 1000 U/ml; Evans) and purified viruses (2 μ g/ml, 2×10^{-4} μ g/ml) were diluted in RPMI' and 100 μ l was added in triplicate to 96-well flat-bottomed tissue culture plates; controls containing medium alone were also included. Cell suspension (100 μ l) was then added and incubated at 37 °C in a 5% CO₂ humidified atmosphere for 4 days. The cultures were then pulse-labelled with 1 μ Ci [³H]thymidine (Amersham) for 16 h prior to harvesting onto filter mats using an automated cell harvester (Skatron), and measuring the amount of thymidine incorporated into the cells by liquid scintillation counting (LKB Betaplate). The results were expressed as the mean of three determinations minus the background reading obtained using cells incubated with medium alone.

Antibody and complement depletion assay. Tissue culture supernatants containing monoclonal antibodies (MAbs) against CD4 (GK1.5), CD8 (3.168) and Thyl (H.0139), from B. Chain, University College, London, U.K., were used in conjunction with guinea-pig complement (Seralab) to deplete different populations of primed lymph node cells. Cells (1 ml, 2×10^7 /ml) were incubated with 1 ml of MAb at 4 °C for 45 min, washed twice with ice-cold RPMI', and then incubated at 37 °C with complement at a final dilution of 1:20 for 30 min. Cells not treated with MAb were also included as a control. After washing twice, viable cells were counted, and the preparations were resuspended at 5×10^6 cells/ml and assayed in triplicate for proliferation with control or specific antigens, as described above. Cells were also cultured with antigens in the presence of TIB 120, a major histocompatibility complex (MHC)-binding agent supplied by B. Chain, added to a final concentration of 20 μ g/ml.

Results

Lymphocytes from BALB/c mice immunized with HRV-1A proliferate in response to HRV-1A and heterologous HRV serotypes

Initial experiments with BALB/c mice indicated that a proliferative response to HRV-1A was obtained after immunization with 0.2 μ g of purified virus and that the peak response occurred 7 to 10 days post-immunization, at *in vitro* antigen concentrations of 2×10^{-1} to 2×10^{-3} μ g/ml (data not shown). The proliferative response of HRV-1A-primed cells to eight other HRV serotypes was investigated over a range of antigen concentrations from 2 to 2×10^{-5} μ g/ml. Cell proliferation was observed with homologous virus and with seven other HRV serotypes; only one, HRV-15, gave little or no proliferation (Fig. 1). The responses to the different serotypes were varied; HRV-2 and -29 stimulated cells as well as HRV-1A, with high levels of [³H]thymidine incorporation at low concentrations of virus. HRV-1B and -49 also gave good incorporation, but at higher virus concentrations, whereas HRV-3, -7 and -14 stimulated proliferation to a lesser extent and only at higher antigen concentrations.

Proliferative responses to HRV-1A and other virus serotypes are observed in different mouse haplotypes

To determine whether the response to heterologous HRV serotypes and the pattern of cross-reactivity seen with BALB/c (H-2^d) mice was unique to that haplotype,

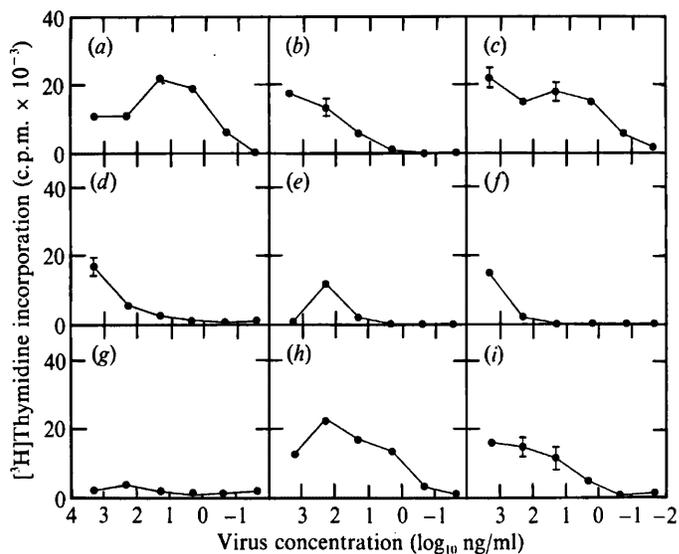


Fig. 1. Proliferation of HRV-1A-primed BALB/c (H-2^d) lymph node cells in response to nine serotypes of HRV (HRV-1A, -1B, -2, -3, -7, -14, -15, -29 and -49; a to i). [³H]Thymidine incorporation was determined in triplicate for each concentration of virus tested, the mean taken and the background c.p.m., obtained from cells incubated with medium alone, was subtracted. The results represent the mean of three experiments and error bars represent the S.E.M.

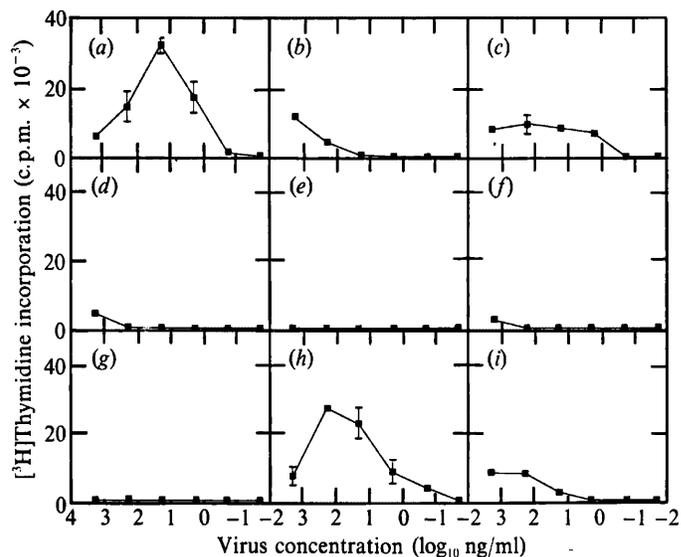


Fig. 2. Proliferation of HRV-1A-primed C57.BL (H-2^b) cells with different HRV serotypes (a to i, see legend to Fig. 1). Results represent the mean of two experiments and error bars the S.E.M.

C57.BL (H-2^b) and C3H.He (H-2^k) mice were immunized with HRV-1A and assayed for lymphocyte proliferation with the same panel of viruses. H-2^b mice (Fig. 2) responded well to homologous virus and the heterologous serotype, HRV-29, as did H-2^k mice (Fig. 3). Lymphocytes of both haplotypes also proliferated with HRV-1B, -2 and -49, but gave lower levels of

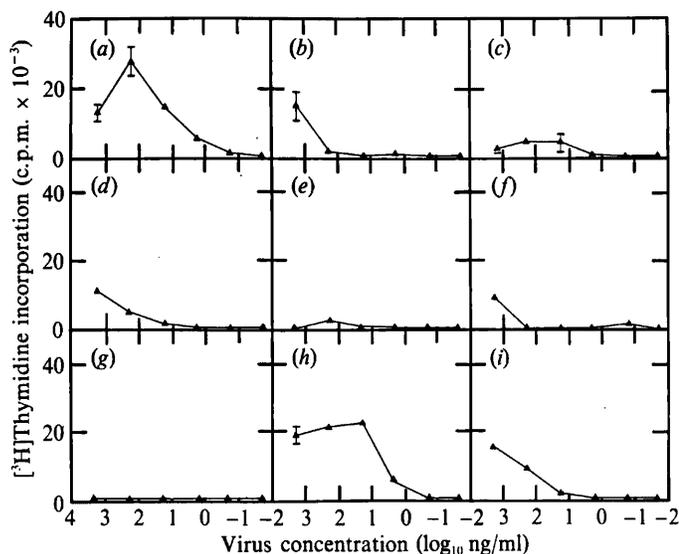


Fig. 3. Proliferation of HRV-1A-primed C3H.He (H-2^k) mouse lymphocytes with HRV serotypes (a to i, see legend to Fig. 1). The results represent the mean of two experiments and error bars the S.E.M.

[³H]thymidine incorporation at higher antigen concentrations than in the response to HRV-1A. The responses to HRV-3 and -14 were lower still (Fig. 2 and 3). Lymphocytes from neither mouse strain gave a proliferative response with HRV-7 or HRV-15.

HRV-15-primed lymph node cells exhibited different proliferative responses to those observed with HRV-1A-primed cells

Since little or no proliferative response to the major receptor group virus HRV-15 was demonstrated using lymphocytes from minor receptor group HRV-1A-primed animals, BALB/c mice were immunized with HRV-15, and tested for their response to the HRV panel (Fig. 4). The level of the response to the homologous HRV-15 was much lower than that of HRV-1A-immunized mice to HRV-1A, and occurred only at the two highest virus concentrations. Cells proliferated in response to HRV-3 to a similar degree, but responses to the other major receptor group viruses, HRV-7 and -14, were observed only at the highest antigen concentration, 2 µg/ml. Little or no response was seen to the minor group viruses, HRV-1A, -1B, -2, -29 and -49.

Proliferation in vitro is due to HRV and not non-specific antigens

Lymph node cells from unimmunized mice were tested for non-specific proliferation with each of the purified viruses. There was no proliferation in response to the three highest antigen concentrations used in the previous

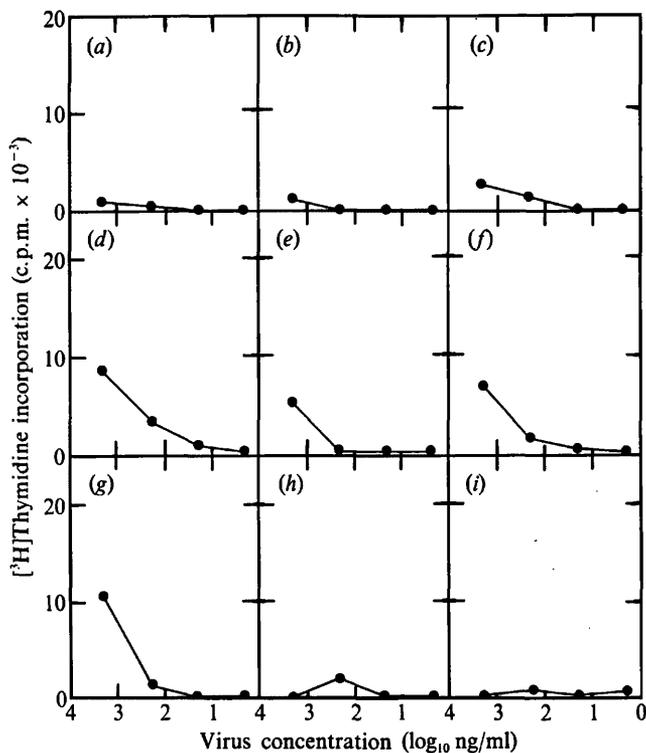


Fig. 4. Proliferation of HRV-15-primed BALB/c cells with HRV serotypes (a to i, see legend to Fig. 1). The results were obtained from a single experiment; S.E.M. values were <10% of the mean.

assays, with the exception that HRV-2 gave low [^3H]thymidine incorporation at the highest antigen concentration, 2 $\mu\text{g}/\text{ml}$. To demonstrate further that proliferation was antigen-specific and not due to other components in the virus preparations, HRV-1A-primed cells were cultured with a range of dilutions of sucrose and HeLa cell extract, containing FCS and cell-associated antigens, and no proliferation was observed (data not shown). In all proliferation experiments the cell controls in medium alone gave low backgrounds of <1000 c.p.m., but responded well to the positive control stimulants Con A and PPD (data not shown).

The proliferating lymph node cells are CD4^+ Th cells

The phenotype of the proliferating cells was determined by pretreating HRV-1A-primed lymph node cells from BALB/c mice with MAbs to cellular antigens and complement to deplete different populations of cells. The viable cell count obtained after treatment indicated that depletion had occurred (data not shown). Incorporation of [^3H]thymidine was eliminated after treatment with the anti-Thy1 MAb (H.0139), indicating that the cells proliferating in response to HRV-1A were T cells (Table 1). After treatment with the anti-CD4 MAb (GK1.5), [^3H]thymidine incorporation was reduced by approxima-

Table 1. Antibody and complement depletion of HRV-1A-primed lymphocyte populations

HRV-1A ($\mu\text{g}/\text{ml}$)	Antibody used to treat cells				
	None	CD4	CD8	Thy1	TIB 120
2×10^{-2}	21555*	ND†	21339	ND	112
2×10^{-3}	22781	1961	27248	6	0

* [^3H]Thymidine incorporation (c.p.m.).

† ND, Not determined.

tely 90% of that observed with control cells treated with complement alone, whereas the anti-CD8 MAb (3.168) had no effect. Moreover, cells cultured with HRV-1A in the presence of TIB 120 did not proliferate after virus challenge, demonstrating their I-A/I-E restriction. All cells responded to Con A and PPD, with the PPD response affected by antibody treatment in a manner similar to that observed with virus (data not shown). Background levels of proliferation of cells in medium alone were <400 c.p.m.

Discussion

In this study we have investigated the ability of different serotypes of HRV to cause proliferation of murine lymph node cells primed with specific serotypes, one from the minor receptor group and the other from the major receptor group.

Cellular proliferation, measured by incorporation of radiolabelled thymidine into the DNA of the dividing cells, was shown to be antigen-specific and the proliferating cells were identified as Th cells, being Thy1 $^+$, CD4 $^+$ and CD8 $^-$ and restricted in their recognition of antigen by MHC class II I-A/I-E molecules.

The observation that a single inoculation of 0.2 μg of purified HRV-1A emulsified with Freund's complete adjuvant was capable of priming Th cells specific for virus indicated that the virus was a potent immunogen. Moreover, optimal proliferation occurred *in vitro* with 2 to 20 ng of virus, amounts which are 10- to 100-fold lower than those reported for another picornavirus, poliovirus (Wang *et al.*, 1989), for mice which had been primed with 2 μg of virus. Higher concentrations of HRV-1A appeared to have an inhibitory effect on cell proliferation *in vitro*.

HRVs are highly immunogenic in experimental animals, with low doses of virus raising high titres of antibodies directed against the surface epitopes of the virus in mice (M. J. Francis & G. Z. Hastings, unpublished data). Neutralizing antibodies to most virus serotypes have been shown to be highly serotype-specific (Cooney *et al.*, 1982), with only a small number of the 90

serotypes tested exhibiting any cross-reactivity with heterologous viruses. Similarly, in humans immunity to infection by a specific serotype of HRV relies upon the presence of pre-existing antibody to that serotype (Dogget, 1965), with antibodies to other serotypes giving little or no cross-protection. In humans infected experimentally with an unspecified serotype of HRV, lymphoblastic cells given prior to infection have been shown to proliferate to the virus when no virus-neutralizing antibodies had been detected, implying that cells previously primed by infection with different virus serotype(s) recognize determinants present on the challenge virus (Hsia *et al.*, 1990).

Although there is an element of serotype-specific responsiveness to HRV-1A similar to that described for poliovirus by Leclerc *et al.* (1991), this study has shown that cross-serotype recognition of HRVs does occur at the level of Th cells, in agreement with the findings of Wang *et al.* (1989) and Katrak *et al.* (1991) which demonstrated cross-serotype Th cell responses to poliovirus in mice. Cross-serotype Th cell responses have also been seen with foot-and-mouth disease virus in lymphoblastic bovine cells (Collen & Doel, 1990).

HRVs can be divided into two groups depending on the cellular receptor to which they bind (Abraham & Colonna, 1984). Most serotypes bind to the major receptor (Uncapher *et al.*, 1991), now identified as the ICAM-I molecule (Greve *et al.*, 1989; Staunton *et al.*, 1989), with a lesser number binding to the uncharacterized minor receptor (Mishak *et al.*, 1988). These two groups of viruses gave different proliferation profiles with HRV-1A-primed cells, which proliferated preferentially with other minor receptor group viruses, whereas major receptor group-primed cells respond almost exclusively to other major receptor group HRV serotypes.

These results indicate that there may be some difference between the abilities of the two receptor groups of virus to exploit the Th cell response. Major group viruses bind only to higher primate cells (Colonna *et al.*, 1986), whereas minor group viruses have also been shown to bind to mouse L cells (Yin & Lomax, 1983). In the assays described in this paper it is possible that HRV-1A becomes bound to mouse cells *in vivo* and/or *in vitro*, and that the uptake of the antigen into the cells was thus facilitated. This binding could then affect the processing of the virus and its subsequent presentation by the cells. This may explain the finding that low doses of HRV-1A prime and induce proliferation of murine Th cells, but that higher concentrations of major group virus are required *in vitro*, and probably *in vivo*, to produce similar levels of proliferation to those obtained with minor group viruses. Receptors for both groups of virus are expressed on human cells, and a similar mechanism of virus uptake

could occur in individuals infected with HRV.

This difference in proliferative response to the two receptor groups of virus was also observed in two other mouse strains with different genetic backgrounds, demonstrating that the T cell recognition of HRV is not restricted by MHC haplotype. Differences in the T cell proliferative responses of inbred mouse strains have been reported with poliovirus (Wang *et al.*, 1989) and Theiler's mouse encephalitis virus (Clatch *et al.*, 1987). In contrast, comparison of the stimulation indices for the three mouse strains used in this study showed that they gave similar proliferative responses to most of the virus serotypes.

In a previous study with HRV-2 we used a predictive algorithm (Rothbard, 1986) to identify five potential T cell epitopes from the amino acid sequence of the virus structural proteins (Skern *et al.*, 1985). These were synthesized in conjunction with an identified HRV-2 VP2 B cell epitope (Francis *et al.*, 1987), which was unable to elicit an antibody response in the absence of Th cell determinants, and used to elicit antibodies in mice. One was found not to be restricted by MHC haplotype in both outbred mice and six inbred mouse strains (Francis *et al.*, 1989), implying that universally recognized T cell epitopes may exist on HRV. It is of interest that this particular epitope (VP1 amino acids 251 to 260) is not highly conserved between the HRVs for which amino acid sequences are available (Palmenberg, 1989). HRV-1A has been used in this study due to the availability of structural coordinates (Kim *et al.*, 1989), which enables the relationships of different epitopes, whether T or B cell, present in the virus structural proteins to be examined at the molecular level.

Having demonstrated that cross-reactive Th cells to HRVs can be generated in mice of different genetic backgrounds, further studies will attempt to identify the specific regions of the structural proteins of HRV-1A which are involved in T cell recognition of virus.

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Identification of T-cell Epitopes from Human Rhinovirus for Use in Synthetic Peptide Vaccines

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Human rhinovirus (HRV) is the major causative agent of the common cold in humans (Scott and Killington 1972). More than 100 virus serotypes exist, against which antibody responses are weakly cross-reactive. Intact virus is thus unlikely to be an effective vaccine for eliciting broad protection against natural rhinovirus infection.

Synthetic peptides comprising either conserved antigenic features of the virus, should they exist, or mixtures of peptides representing dominant antigenic sites of a range of virus serotypes may be useful in the development of broadly protective vaccines. Such peptides would, ideally, contain both T- and B-cell viral epitopes. A previous study explored T-cell responses to HRV type 2 (Francis et al. 1989) using epitopes identified by a predictive algorithm from the amino acid sequences of the structural proteins of HRV-2. These sequences were synthesized contiguously with a linear sequence from VP2 of HRV-2, previously identified as a B-cell epitope (Francis et al. 1987). These chimeric peptides were effective in raising virus-specific antibodies in a range of mouse haplotypes. More recently, we have also demonstrated that helper-T (T_H) cell responses to HRV-1A are heterotypic for other HRV serotypes in mice of different genetic backgrounds (Hastings et al. 1992).

To stimulate a long-lasting immunity, peptide vaccines should be based on those T-cell epitopes that cross-react with "natural" epitopes generated by antigen processing and presentation in the host. In this study, we have used synthetic peptides to identify the T-cell epitopes generated by immunization with HRV-1A in a variety of mouse haplotypes and have examined the ability of these peptides to prime for virus-reactive T_H cells.

Immunogenicity of HRV

HRV is a powerful immunogen, capable of inducing high-titer antisera in animals when inoculated at low doses. HRVs fall into two classes; the majority of serotypes belong to the major receptor group that bind to the cell-surface receptor ICAM-1. This interaction is species-specific, and major group viruses do not bind to mouse cells. In contrast, minor

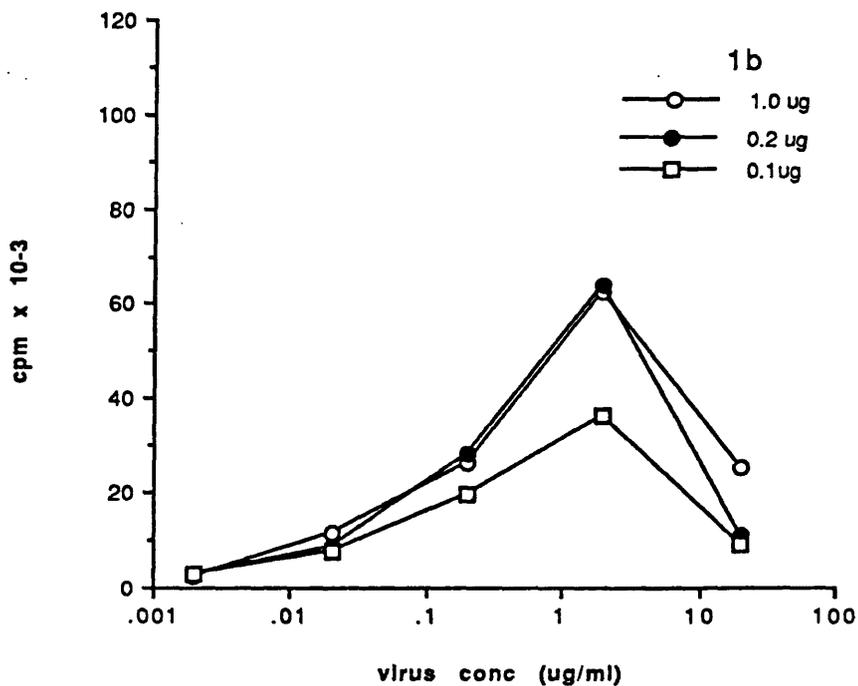
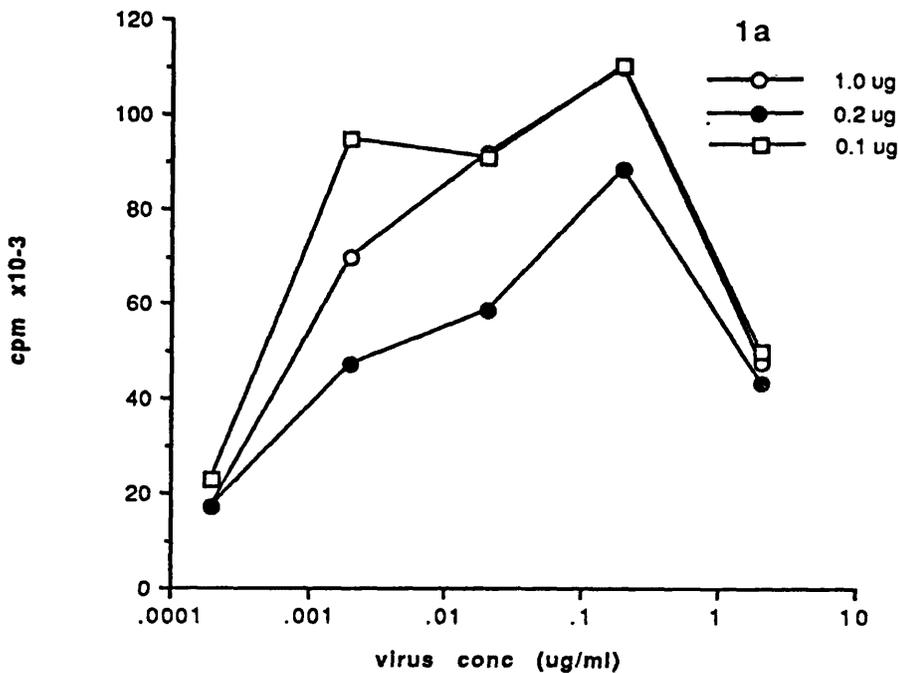


Figure 1
 Dose response curves of HRV-primed lymph node cells from H-2^d mice. (a) Response of cells primed with 1, 0.2, and 0.1 μg of HRV-1A to HRV-1A; (b) response of HRV-15-primed cells to HRV-15. Proliferation is expressed as the mean cpm of triplicate determinations of [³H]thymidine incorporation at each virus concentration minus the mean cpm of cells cultured with medium alone.

group viruses bind to an unknown receptor, and this interaction can be demonstrated in many species including mice. Immunization of mice with HRV-1A, a minor receptor group virus, stimulates a strong cellular proliferative response produced by virus-specific

T_H cells. Low doses of virus (0.1 μg per mouse) are sufficient to prime cells in vivo, which respond optimally in vitro to even lower virus concentrations. In contrast, the T-cell response to major group viruses (e.g., HRV-15; Fig. 1b) requires much higher doses and exhibits a narrower response range than that of HRV-1A-immunized animals (Fig. 1a). The differences between the T-cell responses to the two viruses could reflect the ability of minor group viruses to bind to mouse cells, thus facilitating the uptake of virus into antigen-processing cells or enhancing processing at the cell surface. In humans, such a facilitated uptake would explain the strong immunogenicity of rhinoviruses, which stimulate an effective protective antibody response against reinfection with the same virus serotype.

Identification of T-cell Epitopes of HRV-1A

To identify the specific regions of HRV-1A acting as T_H -cell epitopes, a series of peptides 20 amino acids long, overlapping by 10 amino acids, was synthesized spanning the sequences of the four virus structural proteins. The peptides were used to screen HRV-1A-primed lymph node cells (taken from BALB/c [H-2^d], CBA [H-2^k], and C57BL/10 [H-2^b] mice, primed 8–10 days earlier with 0.2 μg of purified HRV-1A emulsified with complete Freund's adjuvant [CFA]) for in vitro proliferation. The results are summarized in Table 1. A number of interesting points arise from these data. The most striking feature is the oligoclonal nature of the response to intact virus. Despite the previous demonstration that an HRV-2 VP4 peptide was capable of functioning as a T-cell epitope (Francis et al. 1989), none of the six VP4 peptides were recognized by any of the mouse strains. H-2^b mice failed to recognize any peptide from the VP1 series and only one from VP3. With the exception of one VP2 peptide, 2101–2120, peptides identified as positive were specific to one haplotype. These findings indicate the strength of dominance hierarchies set up by immunization with intact virus, which could result from differential processing or differences in T-cell repertoire between the different major histocompatibility complex (MHC) backgrounds. Such a restricted response clearly presents a potential problem in the design of broadly effective peptide vaccines.

Mechanisms of Hierarchy Formation

The nine strong/dominant peptides were investigated for their ability to prime in vivo for a T-cell response to native HRV-1A. Mice from three strains were immunized in the tail base with 50 μg of peptide in CFA and assayed in vitro for both peptide and virus proliferative responses (Table 2). All of the peptides primed peptide-specific T cells in the strains in which they were initially identified as positive. In addition, six of the nine peptides primed T cells recognizing peptide in a haplotype different from the one in which they were originally identified, and two peptides, 2101–2120 and 3121–3140, were effective immunogens in all three mouse strains. These results suggest a certain amount of degeneracy at the level of class II binding, an observation that has been reported for the binding of other viral antigens to both mouse and human MHC molecules (Partidos and Steward 1990). In contrast to peptide/peptide responses, the majority of peptides stimulate T cells that recognize whole virus only in the mouse strain in which the immunizing peptide was first identified as positive. This observation suggests that much of the dominance effect is due to differences in antigen processing/presentation, rather than to limitations within the T-cell repertoire. There are, however, exceptions to this observation. First, there are peptides that prime virus-responsive cells both in the haplotype in which they were initially identified and in another haplotype. The ability of peptide 1111–1130 to produce virus-reactive T cells in H-2^b mice as well

Table 1
Proliferative Responses of HRV-1A-primed Cells to Synthetic Peptides

Peptide	Mouse strain		
	BALB/c H-2 ^d	<i>cba</i> H-2 ^k	C57BL H-2 ^b
1051-1070	-	+	-
1111-1130	-	+++	-
1121-1140	-	+	-
1131-1150	-	+++	-
1211-1230	+*	-	-
1241-1260	-	+	-
2021-2140	-	+	-
2031-2050	-	-	++
2091-2110	-	-	+
2101-2120	++	++	-
2121-2140	-	++	-
2201-2220	-	-	+++
2211-2230	-	-	+
2221-2240	-	-	+
3041-3060	-	+	-
3121-3140	-	-	+*
3161-3180	+	-	-
3181-3200	++	-	-
3201-3220	-	+	-

Results are expressed as stimulation index (S.I.). (+) Weak response S.I. 2-5; (++) strong response 5-10; (+++) >10; (+*) weak but dominant response.

Table 2
Proliferative Responses of Peptide-primed Mouse Cells to Peptide and HRV-1A

Immunizing peptide	Mouse strain					
	BALB/c H-2 ^d		<i>cba</i> H-2 ^k		C57BL H-2 ^b	
	peptide	virus	peptide	virus	peptide	virus
1111-1130	-	-	++	+	+++	+++
1131-1150	-	-	++	++	++	-
1211-1230	+++	+	+++	-	-	-
2031-2050	+	-	-	-	+++	+
2101-2120	+++	+	++	+	+++	-
2121-2140	+++	+++	+++	-	-	-
2201-2220	+	-	+++	+	+++	+++
3121-3140	++	-	+++	-	++	++
3181-3200	+++	+++	-	-	-	-

Results are expressed as stimulation index. (-) <2.5; (+) 2.5-5; (++) 5-10; (+++) >10.

as in H-2^k mice suggests that virus processing can result in effective presentation of an epitope contained within this peptide in both strains but that priming for this epitope in vivo in H-2^k mice is prevented at some other level (e.g., clonal competition). Such differences would affect the generation of the correct T-cell epitope and the subsequent class II MHC binding. The other exception is peptide 2121–2140, which does not prime virus-responsive cells in H-2^k mice, although virus immunization does lead to a peptide response. In this case, the explanation may lie in differences of the processing of virus in vitro and the processing of virus in vivo. This peptide also primes for virus-responsive cells in H-2^d mice, again indicating that different mechanisms of processing occur in different genetic backgrounds.

Structural Analysis of the Virus Epitopes

The sequences of the identified B-cell epitopes of the minor receptor group virus HRV-2 (Appleyard et al. 1990) and the identified T-cell epitopes of HRV-1A were compared. Only one peptide, 2221–2240, overlapped with a B-cell epitope, namely, HRV-2 site C (amino acids 2236 and 2238). This finding differs from those reported for other viral antigens in which the T-cell epitopes have been located within or adjacent to B-cell epitopes (Leclerc et al. 1991) and may reflect differences between immunization with nonreplicating virus and natural infection. Plotting the coordinates of the peptides given in Table 2 within the three-dimensional crystallographic structure of HRV-1A (Kim et al. 1990) revealed that the epitopes were buried within the structure and were remote from the antibody-binding sites of the virus. Processing thus plays a crucial role in determining the protein sequences that act as viral T-cell epitopes.

CONCLUSION

In this study, no one peptide has been identified that primes for both peptide and virus-reactive T cells in all haplotypes, as has been found with other viral proteins (Tindle et al. 1991). Such a peptide, or cocktail of peptides, is an important prerequisite for the development of an effective peptide-based T-cell vaccine. We are currently testing extended analogs of one peptide, 2101–2120, for such activity. The incorporation of such an epitope into composite T/B-cell vaccines could result in T-cell priming for B-cell responses, which would be boosted by the frequent clinical and subclinical infections with HRV to which humans are exposed. In the meantime, T-cell immunity to HRV remains an elegant model system to probe the complex interactions that determine the nature of the T-cell response in vivo.

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