To Selwyn Hubert Harrison
Towards an epitope specific vaccine:
murine immune responses to wildtype
and mutant HIV-1 gp120

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ABSTRACT

The envelope glycoproteins, gp120 and gp41 are the target of the main neutralising antibody response to HIV-1, however this tends to be type-specific and a high level of antigenic variation enables viral escape. The third variable loop (V3) of the external envelope glycoprotein (gp120) contains a dominant B cell epitope which may powerfully compete with other epitopes, in particular, with conserved epitopes, to enhance viral escape. The experiments described in this thesis are concerned with reducing the immunogenicity of V3 with the aim of testing this hypothesis of epitope competition and possibly to define a mutant gp120 construct which may have application as part of a subunit vaccine for HIV-1.

gp120 mutants were made with blocks of serine substitutions in one or both halves of V3. These constructs were used to establish stable protein expression in Chinese hamster ovary cell lines using glutamine synthetase as a selectable and amplifiable marker. Analysis of the secreted mutant proteins, using monoclonal antibodies, revealed that mutation of the C-terminal half of V3 produced detectable conformation changes.

Mice were immunised with the wildtype (unmutated) gp120 by conventional protein-plus-adjuvant immunisation and by nucleic acid immunisation (NAI). Protein immunisation generated ten-fold higher titres of gp120-specific IgG1 but NAI resulted in significantly higher levels of peptide reactivity in the immune sera.
Abstract

Following NAI of mice with the mutant gp120 genes it was found that certain mutations of V3 did result in reduction of V3 immunogenicity and that V3 is probably immunodominant in these mice. Wildtype DNA sequences were required on the N-terminal side of V3 if the resulting antisera were to recognise wildtype V3 peptides. No concomitant increase in immunogenicity of constant regions of the mutated gp120 was recorded in the experiments presented here but this issue is still open.
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<th>Full Form</th>
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<tr>
<td>ADP</td>
<td>MRC AIDS directed program</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
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<tr>
<td>Alk Phos</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette-Guerin</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CD4</td>
<td>cluster determinant 4</td>
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<td>CD4bs</td>
<td>CD4 binding site on gp120</td>
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<td>CD8</td>
<td>cluster determinant 8</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>CDR2</td>
<td>complementarity determining region 2</td>
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<tr>
<td>chloroform</td>
<td>trichloromethane</td>
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<td>CHO</td>
<td>chinese hamster ovary</td>
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<td>CIP</td>
<td>calf intestinal alkaline phosphatase</td>
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<td>CPZ</td>
<td>chimpanzee</td>
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<td>CT</td>
<td>cholera toxin</td>
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<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<td>DNA</td>
<td>deoxy ribonucleic acid</td>
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<td>DC</td>
<td>dendritic cell</td>
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<td>dNTP</td>
<td>deoxy nucleotide triphosphates</td>
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<td>dsDNA</td>
<td>double stranded DNA</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>dTTP</td>
<td>deoxy thymidine triphosphate</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EGTA</td>
<td>ethyleneglycol bis tetraacetic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>env</td>
<td>envelope proteins gene of retroviruses</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>gag</td>
<td>structural proteins gene of retroviruses</td>
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<tr>
<td>geneVIIIp</td>
<td>filamentous phage geneVIII protein</td>
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<td>GMCSF</td>
<td>granulocyte monocyte colony stimulating factor</td>
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<td>GMEM-S</td>
<td>medium used for growth of these CHO cells</td>
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<tr>
<td>GPGR</td>
<td>glycine proline glycine arginine</td>
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<tr>
<td>GS</td>
<td>glutamine synthetase</td>
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<td>HA</td>
<td>haemagglutinin</td>
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<td>HBcAg</td>
<td>hepatitis B core antigen</td>
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<td>HBsAg</td>
<td>hepatitis B surface antigen</td>
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<td>HIV-1</td>
<td>human immunodeficiency virus type 1</td>
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<td>ICAM-1</td>
<td>intracellular adhesion molecule-1</td>
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<td>IFNγ</td>
<td>interferon γ</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL2 etc.</td>
<td>interleukin 2 etc.</td>
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<tr>
<td>IPTG</td>
<td>isopropylthio-b-D-galactoside</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>ISCOMS</td>
<td>immunity stimulating complexes</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani growth medium for <em>E. coli</em></td>
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<td>LBAmpl</td>
<td>LB with ampicillin 100μg/ml</td>
</tr>
<tr>
<td>LFA3</td>
<td>leucocyte function antigen 3</td>
</tr>
<tr>
<td>LMP agarose</td>
<td>low melting point agarose</td>
</tr>
<tr>
<td>LT</td>
<td><em>E. coli</em> heat labile enterotoxin</td>
</tr>
<tr>
<td>M cells</td>
<td>microfold cells</td>
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<tr>
<td>Mab</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>Marvel</td>
<td>dried 99% non-fat milk powder</td>
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<tr>
<td>MCS</td>
<td>multicloning site</td>
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<td>major histocompatibility complex</td>
</tr>
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<td>MPL</td>
<td>monophosphoryl lipid A</td>
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<td>methionine sulfoximine</td>
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<td>natural killer cells</td>
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<td>NMR</td>
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<td>poly-DL-lactide coglycolide</td>
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<td>PND</td>
<td>principle neutralising determinant</td>
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<td>pNPP</td>
<td>para-Nitrophenylphosphate</td>
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<tr>
<td>pol</td>
<td>enzymatic proteins gene of retroviruses</td>
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<td>room temperature</td>
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<td>ribonucleic acid</td>
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<td>reverse transcriptase</td>
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<td>S.D.</td>
<td>standard deviation</td>
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<td>SCID</td>
<td>severe combined immunodeficiency</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SDS PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
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<td>ssDNA</td>
<td>single stranded DNA</td>
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<tr>
<td>TAE LMP gel</td>
<td>LMP agarose gel with TAE as buffer</td>
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<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminoethane</td>
</tr>
<tr>
<td>TT</td>
<td>tetanus toxoid</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>VLP</td>
<td>virus-like particle</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-b-D-galactoside</td>
</tr>
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CHAPTER 1

GENERAL INTRODUCTION

The ideas behind the work presented in this thesis have come from the overlap of several different disciplines. The main themes which are relevant are outlined in this introduction with emphasis on gp120 and its third variable loop. These are (i) a brief description of HIV-1 biology, (ii) the immune responses found in HIV-1 infection and an overview of the disease pathogenesis, (iii) vaccines, especially for HIV-1; and (iv) epitope competition.

HIV-1

The retrovirus HIV-1 was discovered separately by three groups in 1983-4 (Barre, et al. 1983: Gallo, et al. 1983: Levy, et al. 1984) and named LAV, HTLV III and ARV respectively. The virus, renamed HIV-1, is classified as a lentivirus, a subgroup which share molecular, biological and clinical characteristics which distinguish them from other retroviruses (Haase 1986). The lentiviruses include equine infectious anaemia virus (EIAV), caprine arthritis encephalitis virus (CAEV), visna-maedi virus, zwoegerziekte, progressive pneumonia virus (PPV), and the immunodeficiency viruses:- human immunodeficiency virus type 1 (HIV-1), HIV-2, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

The origin of HIV-1 is as yet unclear. Sequence comparisons and seroepidemiological data have been used to construct a putative HIV-1
evolutionary tree. It is possible that SIV, perhaps from the Sooty Mangeby (Cercocebus atys), was transmitted to humans and gave rise to HIV-2 which in turn gave rise to HIV-1 (Gardner and Luciw 1988). Alternatively, HIV-1, HIV-2 and SIV may each have arisen independently from a common ancestor which was present in the common ancestor of the old world primates and humans (Gardner and Luciw 1988). A strikingly different hypothesis is that HIV-1 is a relatively recent natural recombinant between two other lentiviruses; Visna virus (with which it shares extensive DNA sequence and morphological homology (Gonda, et al. 1985) and BIV, and that this recombinant has accidentally been introduced into humans (Siefkes 1993). Sequence analysis of HIV-1 isolates has shown extensive divergence especially in the envelope proteins gene (env).

Based on analysis of the sequences of the structural proteins gene (gag), HIV-1 is at present divided into two groups, O and M. Within the larger group M, nine subgroups or ‘clades’ are identified. The sequence variability within clades being 10-20% and between clades 30% or more (Sternberg 1992).

**Structure of HIV-1**

*Figure 1.1* Diagram of the structure of HIV-1 (from Peterlin and Luciw 1988)
HIV-1 is an enveloped icosahedral spherical particle, 90-130 nm in diameter, containing a bar shaped nuclear core (Gonda, et al. 1985). The core is composed of multiple copies of p24, a gag gene product, which enclose two copies of the single stranded RNA genome and the reverse transcriptase (RT) protein. HIV-1 has a typical retroviral genome with the three main genes gag, pol (polymerase) and env, and also several genes which regulate its life cycle. Surrounding the core is a shell made of myristoylated p17 which internally supports the lipid envelope taken from the host cell membrane (Peterlin and Luciw 1988). The complex interdependent functions of the regulatory proteins; rev, tat, nef, vpu, vpr and vif are not discussed here but some may contribute to the pathogenesis of the infection as described below. The envelope carries the HIV-env gene products gp41 and gp120 and also host-derived membrane proteins.

**Structure and function of the envelope proteins.**

The precursor of the mature envelope proteins is gp160 which is has a 36 amino acid signal sequence which directs its secretion. It is cotranslationally heavily glycosylated and forms stable homodimers within the lumen of the endoplasmic reticulum (ER). After carbohydrate processing (Merkle, et al. 1991), gp160 is inefficiently (10-12%) (McKeating and Willey 1989) cleaved by furin or a furin-like host cell protease into gp120 and gp41 (Morikawa, et al. 1993). gp41 is anchored in the membrane, having cytosolic, transmembrane and external domains while gp120 is external but remains associated with gp41 by non-covalent interactions. This conformation-dependent cleavage of gp160, which is essential for viral infectivity (McCune, et al. 1988), causes slight destabilisation of the former gp160 homodimer, (Earl, et al. 1990) but the dimeric complex is maintained by interactions between amino acids 550-561 in the external domains of the gp41 moieties (Poumbourios, et al. 1995). It is possible that other interactions contribute to oligomer maintenance but it has been found that the leucine zipper motif in the gp41 transmembrane domain (Chen, et al. 1993) and the carbohydrate structures (Haidar, et al. 1994) are probably not involved. At
the cell surface and in the viral envelope, the dimeric gp120/gp41 complex is present as higher order oligomers, probably tetramers (Earl, et al. 1990; Thomas, et al. 1991), however, some studies find evidence of trimers (Weiss, et al. 1990).

**gp120 structure**

 gp120 has approximately 450 amino acids giving it a protein mass of about 54 kDa and sustains a high level of amino acid variability, making it extremely polymorphic. This variability is due to substitutions, insertions and deletions and is concentrated in five regions of the primary sequence (Modrow, et al. 1987). These 'variable loops' have 25% or less amino acid conservation while the intervening constant regions are those with more than 25% (Simmonds, et al. 1990).1

The heavy glycosylation of gp120 almost doubles its mass to approximately 89 kDa (Thomas, et al. 1991) and causes it to appear even larger on reducing SDS-PAGE at approximately 120 kDa. The gp120 band is typically broad due to extensive microheterogeneity of the carbohydrates. There are usually 24 sequons for N-linked carbohydrate and, when expressed in Chinese hamster ovary (CHO) cells, all the sites are utilised, 13 bearing complex-type structures and 11 high-mannose (Leonard, et al. 1990). About 14% of the complex oligosaccharides may be sulphated (Shilatifard, et al. 1993). Although originally controversial, there may also be O-linked carbohydrate structures (Bernstein, et al. 1994; Hansen, et al. 1992). Prior glycosylation is necessary for correct folding in the ER as shown by synthesis in the presence of tunicamycin after which gp120 is expressed but fails to bind CD4, and the observation that the gp120-CD4 interaction is not inhibited if the carbohydrates are enzymatically

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1 Some discrepancy exists regarding the naming of the constant regions of gp120. Many authors describe 5 regions while others include the short segment between V1 and V2 and name this C2, increasing the number of constant regions to 6. Amino acid numbers are often quoted as clarification but the problem is compounded by differences in numbering from the start of the signal sequence or from the start of the processed N-terminus. This is further complicated by the length variability of gp120. In this thesis where amino acid numbers are quoted they are those used by the authors in their papers since certain individual amino acids are recognisable by a particular number, for example "Trp 427".
removed after folding has occurred (Li, et al. 1993). Mainly because of the extensive glycosylation, there is as yet no crystal structure for gp120.

Figure 1.2
Diagram (after Capon and Ward 1991) of gp120 from HIV-1 IIIB showing the amino acid sequence and the positions of the disulphide bonds and the N-linked glycosylation sites. Variable regions are shaded.
gp120 has nine conserved disulphide bonds (Leonard, et al. 1990) but these do not preclude a high degree of subtle conformational flexibility which is apparently crucial to its functions. An immunochemical approach to determining the structure of gp120 has been taken by Moore and colleagues and others. Analysis of gp120 and gp120 mutants with large numbers of monoclonal antibodies (Mabs) has revealed some of the interactions between different regions in the primary sequence of gp120. There is an association between C2 (constant region 2) and V3 (third variable loop; variable region 3) (Stamatatos and Cheng 1993: Willey and Martin 1993), between C3, C4 and V3 (Moore, et al. 1993b) and between C1, C2 and C5 regions (Moore, et al. 1994). It has become clear that mutations in one domain of gp120 can influence the conformation of other domains and are detectable by alteration in binding of Mabs to defined epitopes (Moore, et al. 1993a).

These interdomain interactions may be reciprocal (Pinter, et al. 1993: Stamatatos and Cheng 1993) and many of the conformational shifts detected are mediated by point mutations demonstrating a supreme sensitivity and lability to sequence changes. Since gp120 is so variable it indicates that many of the sequence changes must be reciprocated by others to enable conformationally functional molecules to exist in polymorphic forms. The tertiary structure of gp120 and the quaternary structure of gp120/gp41 are not yet known. A recently proposed model for the surface glycoproteins of all retroviruses delineates three domains with two conserved hinge regions between them. The disulphide bonding is only within domains and not between domains. This model does not directly confirm the possible regional interactions described by others (see above) but is consistent with these proposals (Gallaher, et al. 1995).

Structure of the V3 loop of gp120

The V3 loop is an extremely important region of gp120 and the focus of a great deal attention for vaccine development. V3 is thought to be the immunodominant B cell epitope and the principal neutralising determinant as
discussed below. Extensive analysis of V3 primary sequences has revealed its hypervariability but within this context there are regions which are relatively conserved. The loop spans approximately 35 amino acids and its base is formed by a disulphide bond between two invariant cysteines. The tip of the loop has the motif Glycine Proline Glycine Arginine (GPGR) which is approximately 93% conserved (LaRosa, et al. 1990) the parts of the loop nearest to the disulphide bond are also relatively conserved, confining the main hypervariability to regions immediately either side of GPGR.

Apart from the primary amino acid sequence there are at least two other levels in the repertoire of V3 variability, the first being its own conformational flexibility. On the basis of the "neural network analysis" technique (LaRosa, et al. 1990) which ascribes values for helix tendency and for sheet tendency to each amino acid, LaRosa et al have predicted the secondary structure for V3 to be β strand, β turn, β strand, α helix. They suggest that the relative conservation of parts of the loop reflect a selective pressure to maintain this conformation. The predicated type II β turn, with the preferred residues P G R at positions +1,+2 and +3 after the turn, has been partially confirmed by X-ray crystallographic analysis of V3 loops complexed with a monoclonal antibody (Ghiara, et al. 1994; Rini, et al. 1993) and by nuclear magnetic resonance (NMR) studies of cyclic V3 peptides (Tolman, et al. 1993) which show the loop is in this conformation for roughly 50% of the time. Catasti et al (Catasti, et al. 1995), also using NMR, have demonstrated that the flexibility of the loop is inherent and that its conformation can be profoundly affected, for example, by the polarity of the solvent.

The evolution of V3 sequences as the disease progresses is marked by the accumulation of an increasing number of basic amino acids. Okada et al (Okada, et al. 1994) have shown that these basic amino acids may maintain a particular structure of the loop. It is possible that V3 structure may also depend on the patency of the disulphide bond. This is 100% conserved and Mabs have been shown to react more strongly in solid-phase ELISAs with cyclised V3 loop
peptides than with linear peptides of the same sequence (Catasti, et al. 1995).

A final level of V3 variability is shown by its display of hinge bending and folding and the ability to alter its position relative to other regions of gp120, termed "conformational V3 hypervariability" (Nara, et al. 1990). Although the base of V3 is usually inaccessible to antibodies in native gp120 (Laman, et al. 1993; Moore, et al. 1994), other regions of V3 may become more or less available depending on the three dimensional context. Pinter et al (Pinter, et al. 1993) have demonstrated the presence of V3 neutralisation-epitopes which are exposed in virion bound gp120 but are partially sequestered in dissociated gp120 and in gp160, and that these are due to reciprocal allosteric interactions caused by CD4 binding or by conformation changes after cleavage of gp160 respectively. The variability of V3 may be an important functional attribute and is discussed below in the context of virus neutralisation.

**Viral entry to target cells:**

HIV-1 gains access to the cytosol after fusion of the viral envelope with the plasma membrane. The principal cell-surface receptor for HIV-1 is the conserved glycoprotein CD4 (Dalgleish, et al. 1984) which is present in high levels on helper T cells but also on macrophages, monocytes, dendritic cells, haematopoietic stem cells, certain rectal lining cells and microglia (Levy 1993).

**gp120-CD4 interactions**

**CD4 structure and the binding site for gp120**

CD4 has cytoplasmic, transmembrane and external domains and is a member of the immunoglobulin (Ig) superfamily. Crystal structures of the outer two domains (D1 and D2) of human CD4 have shown that they both resemble an immunoglobulin fold, but that one β strand is shared between them imposing some rigidity on this part of the protein (Ryu, et al. 1990; Ryu, et al. 1994:}
The crystal structure of the rat CD4 membrane-proximal domains D3 and D4 again indicates immunoglobulin-like folds with D3 lacking a disulphide bond (Brady, et al. 1993). These structures are confirmed for human CD4 (Bour, et al. 1995). The external part of CD4 is thought to be flexible between the outer two and inner two domains (Ankel, et al. 1994).

The interaction between gp120 and CD4 is high affinity with a dissociation constant of approximately $4 \times 10^{-9}$ M (Lasky, et al. 1987). The gp120 binding site on CD4 is in the most membrane distal domain, D1. Crystal studies show a ridge on its CDR2-like region with the hydrophobic side chain of Phe 43 jutting out into the solvent (Choe and Sodroski 1992; Moebius, et al. 1992; Ryu, et al. 1990). Certain positively charged residues near Phe 43 in the primary sequence; Lys 29, Lys 35, Lys 46 and Arg 59, are thought to be important in the interaction with gp120 and reversal of the charge at position 46 (Lys to Asp) results in a fifteen-fold reduction in affinity (Choe and Sodroski 1995). It has also been suggested, however, that Phe 43 is enclosed in a hydrophobic pocket surrounded by these charged residues (Bour, et al. 1995).

**The CD4 binding site of gp120**

The CD4 binding site on gp120 is a discontinuous conformationally-dependent region. The most critical amino acids involved are Trp 427, Asp 368, Glu 370 and Asp 457 in the C3 and C4 regions of gp120 (Moore, et al. 1993b; Olshevsky, et al. 1990) with positions 368 and 370 being almost invariant between strains of HIV-1, HIV-2 and SIV. Trp 427 is at the base of a hydrophobic cleft formed by the disulphide bridge between Cys 378 and Cys 445. The negatively charged residues are in hydrophilic regions which also show a propensity to form β turns and may be exposed on the surface (Olshevsky, et al. 1990). It seems likely that an interaction occurs between the complementary charges of the crucial hydrophilic amino acids of gp120 and CD4 (Figure 1.3), however, using a truncated gp120 model, Gabriel and Mitchell (Gabriel and Mitchell 1993)
have shown that the main binding energy is between the hydrophobic side chains of Phe 43 of CD4 and Trp 427 of gp120 creating a thermodynamically stable interaction between the two aromatic groups.

**Figure 1.3**


**Above:** CD4:- The CDR2-like region of domain 1.

**Below:** gp120:- The critical amino acids in regions C3 and C4.

# Hydrophobic, + Positive charge, - Negative charge.
At physiological temperature (37°) the binding of gp120 to CD4 causes conformational changes in gp120 which allow gp120 to dissociate from gp41; following this the exposure of the hydrophobic N-terminus of gp41 leads to subsequent membrane fusion events (Moore, et al. 1990: Sattentau and Moore 1991: Sattentau, et al. 1993). At 4°, dissociation of gp120 from gp41 does not occur but conformational changes are still detected in gp120 by changes in Mab binding. The affinity of the interaction between gp120 and CD4 is fourfold lower at 4° (Moore and Klasse 1992) which suggests that this interaction may be dependent on conformation changes in gp120. Moore has also suggested that CD4 may undergo conformation changes on binding to gp120 (Moore, et al. 1992). It is therefore possible that the exact topographical shapes required for 'perfect' high affinity fit may be to some extent induced (Ptaszek, et al. 1994: Ryu, et al. 1994). Taken together, these findings may be the result of a global conformation change of gp120 which allows both CD4 binding and dissociation from gp41 and a simultaneous conformation change of CD4. Conceivably this may account for the two different models of the local conformation of Phe 43 in relation to the charged residues in CD4.

**Second receptors for HIV and fusion**

CD4 itself is not necessary for initiation of infection of some cells and is not sufficient for infection of some others and a putative second receptor for gp120 has been proposed. gp120 binds to the glycosphingolipid galactosyl ceramide (GalCer) or a derivative of this on neural cells (Bhat, et al. 1991). HIV-1 can also infect vaginal and colorectal epithelial cells via interaction with sulphated lactosyl ceramide (Furuta, et al. 1994). Recently Yahi et al (Yahi, et al. 1995) have demonstrated infection by HIV-1 of a CD4 negative mucosal epithelial cell line via interaction between GalCer and V3. The cell surface proteases Tryptase TL2 (Kido, et al. 1991) and dipeptidyl peptidase IV [CD26] (Callebaut, et al. 1993) have also been proposed as second receptors for HIV-1 via
interactions with V3 and this is supported by the observation that protease inhibitors such as trypstatin can inhibit HIV-1 induced syncytium formation and have sequence homology with the crown of the V3 loop (Hattori, et al. 1989).

It has been strongly suggested that V3 is cleaved by a cell surface protease and that this step is a prerequisite for infection (Wemer and Levy 1993). The undisputed conformational change in gp120 may cause a cis-trans isomerisation of Pro 313 (within GPGP) which enables the loop to assume substrate conformation for a protease (Johnson, et al. 1994). Others find no evidence of cleavage of V3 peptides when these peptides act to inhibit the protease activity (Avril, et al. 1995: Murakami, et al. 1991). These results do not resolve the controversy since the peptides were not cyclised, but they do support the involvement of a surface protease in gp120 binding.

The biochemical events leading to membrane fusion which occur subsequent to gp120-CD4 binding are not clear. The requirement for functionally correct conformation of gp120 and gp41 is supported by the observation that uncleaved gp160 supports viral assembly but not infectivity (McCune, et al. 1988). The V3 loop of gp120 is also thought to be intimately involved in post CD4-binding events since alterations in V3 sequence, even point mutations, can affect cellular tropism, syncytium formation /fusion /infectivity and replication capacity (Andeweg, et al. 1993: de Jong, et al. 1992: Fouchier, et al. 1992: Freed and Risser 1991: Ivanoff, et al. 1992: Ivanoff, et al. 1991: Milich, et al. 1993: Nehete, et al. 1993: Page, et al. 1992: Rusche, et al. 1988). Some of these effects have been linked with the conformation of V3 (Ebenbichler, et al. 1993: Harrowe and Cheng 1995) or to its interaction with other parts of gp120 (Stamatatos and Cheng 1993). The pattern of basic amino acid substitutions in V3 is also important, and an increase in basic substitutions increases the T cell tropism and the syncytium inducing activity of the isolate (Fouchier, et al. 1992). This can occur with a single additional basic residue in some cases (Shioda, et al. 1994). However, some phenotypic determinants of tropism are independent of V3

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**Immune responses to HIV-1 and pathogenesis**

**Specific immune responses to HIV-1**

![Temporal associations of HIV-1 specific immune responses with the observed decline in CD4 cell number (from Weiss 1993).](image)

**Figure 1.4**

Temporal associations of HIV-1 specific immune responses with the observed decline in CD4 cell number (from Weiss 1993).
Humoral Responses

HIV-1 infected individuals mount a specific antibody response to HIV-1 proteins soon after infection (Khalife, et al. 1988) which includes responses to the structural proteins p17 and p24, regulatory proteins vif, nef and RT and env proteins gp120 and gp41 (Boyd and James 1992) with the majority of the response being direct to env (Barin, et al. 1985). The response to env is mainly restricted to the IgG1 isotype and the proportion of IgG correlates positively with the CD4 cell numbers (Kozlowski, et al. 1994). A large array of epitopes have been identified and have been reviewed by Nixon (Nixon, et al. 1992). Tomiyama has shown that human serum contains natural antibodies to HIV which react with the carbohydrates of gp120 and gp41 (Tomiyama, et al. 1991). There is a subset of HIV 1 specific antibodies which are neutralising to HIV-1. This response is directed predominantly to the V3 loop but also to the CD4 binding site and other regions of gp120 (Ho, et al. 1987). The neutralisation of HIV 1 is described below in the context of vaccines.

Cellular responses

CD8 cytotoxic T lymphocytes (CTL) are generated with specificity for several HIV proteins including env, gag, RT and nef (Nixon, et al. 1992). In vitro, CD8 CTL prevent transmission of HIV, by a mechanism which is not clear but could be either their cytotoxic activity itself or a secreted lymphokine (Clerici, et al. 1992b). It is believed that CTL control the initial viraemia and this is based on the correlation between the first detection of CTL and the decline in viral load (Paul 1995). In vivo there is no direct evidence to show that CD8 cells can delay disease or be protective (Cease and Berzofsky 1994), however, there is strong evidence for the emergence of CTL escape mutants (McMichael 1993: Phillips and McMichael 1993) which argues that CTL do have antiviral activity in vivo. The effects may be unpredictable as a recent study has shown. HIV-specific CD8 cells were removed from an infected patient, expanded in vitro and then re-
infused, but instead of amelioration, this caused an acceleration of the disease progression (Koenig 1995). Recently, cytotoxic T lymphocytes from individuals at high risk of infection but apparently not infected with HIV-1 have been shown to recognise peptides shared between HIV-1 and HIV-2 (Rowland-Jones 1995). It has been suggested that previous infection with HIV-2 has generated a protective response to HIV-1 which prevents infection (Paul 1995). As well as CD8 CTL, CD4 T cells of the Th1 type have been shown to have cytotoxic activity. They kill B cells when effector to target ratios are greater than 1:1 (Del Prete, et al. 1991) and are also known to kill uninfected CD4 T cells. Therefore, the possible anti-HIV role of such CD4 cytotoxic cells is unclear.

**Mucosal responses**

Since HIV can enter the body at mucosal surfaces and infect lymphocytes and macrophages (Fleming, et al. 1992) and probably epithelial cells (Yahi, et al. 1994), an effective mucosal immune response would be a very important first line defence. The mucosal immune system is a dispersed group of functionally interconnected tissues composed of specialised inducer and effector sites (McGhee, et al. 1992). Inducer sites such as tonsils and Peyer’s patches have a unique microenvironment and are rich in microfold (M) cells which are specialised to transport antigen, with minimal processing, from the gut lumen to the local B cells, dendritic cells and macrophages. Dendritic cells subsequently migrate to draining lymph nodes and initiate a primary systemic immune response (Steinman 1991) while effector B cells traffic through the blood and home to multiple effector sites in the lamina propria and secrete specific IgA and IgG. The rectal and genital mucosae are thought to be the major mucosal sites of entry and a specific immune response to HIV-1 in these tissues is not prominent. To establish protection from primary infection by sexual transmission, an induced immune response to HIV-1 is now a major goal.
Immunopathology of HIV-1 infection

In the pathogenesis of most lentiviral infections there is a strong inflammatory component, with a relatively mild immunodeficiency, but HIV-1 differs from the other lentiviruses in that its major impact is on immune functions and this has been attributed to the more extensive infection of monocytes by HIV-1 (Haase 1986). Macrophages may be the first cells infected by HIV-1 and possibly require a slow-replicating non-syncytium-inducing phenotype (Milich, et al. 1993; Zhu, et al. 1993). There is evidence both for (Shpaer, et al. 1994), and against (Antonioli, et al. 1995) a conserved V3 loop sequence being required for the initial infection. Dendritic cells are also infected by HIV-1, support viral replication and transmit the virus to CD4 T cells as described below. Follicular dendritic cells can retain viral particles on their surfaces, possibly for extended periods (Fox and Cottler 1992). The lymph nodes are therefore thought to be a reservoir of virus during the clinically latent phase when viraemia may be low (Embretson, et al. 1993; Fox 1992; Pantaleo, et al. 1991; Pantaleo, et al. 1993; Patterson, et al. 1994). The viraemia may be reduced by efficient clearance of virions rather than low replication levels since studies of the dynamics of emergence of drug resistant variants indicates a rapid turnover of both virus and CD4 cells (Ho, et al. 1995; Wei, et al. 1995).

As a result of HIV-1 infection there is a stimulation of Ig secretion by peripheral blood B lymphocytes and an inhibition of differentiation of B cells (Pahwa, et al. 1985). In particular, raised levels of total polyclonal IgE and of IgA (serum IgA1 and A2 and secretory IgA) (Vincent, et al. 1992) are found. The IgE is non gp120-specific (Mazza, et al. 1995) and can be a predictor of the clinical course due to a strong inverse correlation with the numbers of CD4 cells (Israel, et al. 1992). The IgE also correlates positively with the CD8 cell count (Shor, et al. 1995). IgA committed B cells may be more susceptible to the polyclonal activation signal than are IgG B cells, (Kozlowski, et al. 1994) and those B cells bearing the VH3 family of Ig receptor are natural ligands for gp120 and respond
by secreting immunoglobulin, indicating that gp120 is a possible superantigen for this large (approximately 30% of all B cells) family (Berberian, et al. 1993).

Other putative causes of the polyclonal activation (Pahwa, et al. 1985) are the presence of cytomegalovirus or Epstein Barr virus coinfection, and the reduction of normal immune regulation.

**CD4 T cells**

In HIV infection helper T cells show functional defects (Shearer and Clerici 1991) and have reduced ability to respond to specific antigen (e.g., tetanus toxoid). This is thought to be due to the presence of CD4-specific antibodies (Weimer, et al. 1991); to gp120-CD4 interaction leading to blocking of CD4-mediated MHC-restricted activation; to dysregulation of the expression of costimulatory molecules (Chirmule, et al. 1995); by inhibition by tat (Viscidi, et al. 1989) or by a suppressor T cell factor (Clerici, et al. 1992b). gp120 causes a decline in IL2 levels and the cells also express lower levels of the IL2 receptor. This is due to an inhibition of IL2 production at the level of mRNA (Oyaizu, et al. 1990). Cellular responses can be reduced leading to an increased susceptibility to intracellular pathogens (e.g. Mycobacterium tuberculosis, Pneumocystis carinii), CD8 CTL have reduced ability to eliminate virus-infected cells and natural killer (NK) cells are less activated allowing tumours (e.g. Kaposi’s sarcoma) to occur (reviewed in (Levy 1993)). There can also be autoimmune reactions and these are thought to be a major cause of the morbidity of AIDS.

The major effect of HIV on the immune system is a fluctuating but overall continuing decline in the number of CD4 T cells, until critically low levels are reached. CD4 cells infected with HIV-1 are in the minority. These can be killed by various mechanisms including; membrane damage due to budding of virus, antibody-mediated complement lysis and HIV-specific cytotoxic T cell killing. HIV-1 cDNA can accumulate in infected cells, which could sometimes lead to arrest of cell division (Tang, et al. 1992) and could cause cell damage in vivo.
General Introduction

(Pang, et al. 1990) demonstrated that in AIDS dementia, there is a high level of accumulated unintegrated HIV DNA. Several HIV-1 proteins have been shown to be cytotoxic: Nef has structural and functional similarity to scorpion toxin (Werner, et al. 1991) and tat has sequence homology with a snake neurotoxin (Garry and Koch 1992; Garry, et al. 1991). Both of these mechanisms involve effects on membrane ion channels. The cytotoxicity of gp120 is seen clearly in neurones where it causes a rise in intracellular calcium and subsequently cell death (Dreyer, et al. 1990). This effect is dose dependent and cannot be prevented by antibodies to CD4 (Kaiser, et al. 1990). The mechanism for this induction of high intracellular calcium is not clear but in astrocytes it may be secondary to depolarisation following potassium channel activation by gp120 (Bubien, et al. 1995).

The majority of the CD4 cells which are lost are not infected by HIV-1 and several hypotheses have been proposed to account for this. These include gp120 binding to surface CD4 leading to killing by NK and CD8 CTL - the bystander effect - (Weinhold, et al. 1989); the presence of antilymphocyte autoantibodies and the induction of apoptosis which arises from a number of potential mechanisms. These apoptosis-linked mechanisms include inappropriate TCR stimulation by TCR β chain-specific superantigens (Akolkar, et al. 1995; Imberti, et al. 1991); TCR-mediated induced apoptosis of primed T cells caused by crosslinking of CD4 by gp120 (Banda, et al. 1992); induced apoptosis of primed T cells by gp120 and gp120-specific antibody in the absence of the TCR signal (Foster, et al. 1995); dysregulation of apoptosis control mechanisms (Ameisen, et al. 1994) and direct contact with infected CD4 cells (Nardelli, et al. 1995). T cells can be activated non-specifically by any activated B cell or antigen presenting cell (APC) which ligate T cell surface CD28 (Asjo, et al. 1993) making them susceptible to those depletion mechanisms which require prior activation.

Cease and Berzofsky have proposed a mechanism of CD4 cell depletion based on a chain reaction which they term 'CD4 fratricide' (Cease and Berzofsky
In this model, infected CD4 cells present antigen to uninfected CD4 cells (Lanzavecchia, et al. 1988) which become simultaneously activated and infected and pass the infection on to more uninfected CD4 cells (Cease and Berzofsky 1994). The CD4 cell decline may lead to an imbalance in expression of cytokines and following this to disturbed immune regulation and to cytokine modulated activation induced apoptosis (Clerici, et al. 1994). Similarly, dysregulation can lead to autoimmune responses through mechanisms relying on molecular mimicry between several HIV proteins and self components (Lake, et al. 1994: Silvestris, et al. 1995: Trujillo, et al. 1993).

The CD4 helper T cell subsets, Th1 and Th2, were originally defined according to the cytokines produced by murine T cell clones (Mosmann, et al. 1986: Mosmann and Coffman 1989). In the mouse, Th1 secrete mainly IFNγ and IL2, Th2 secrete IL4, 5 and 6 and both types secrete GM-CSF and IL3 and the cell populations are able to cross regulate each other. Clerici and Shearer have put forward a model of HIV-1 pathogenesis based on dominance of one set of cytokines over the other (Clerici and Shearer 1994b). Type 1 (IFNγ, IL2) cytokine dominance appears to be protective against the development of AIDS (Clerici and Shearer 1994a) and a switch to Type 2 dominance may be a critical event in the natural history of the disease progression (Clerici and Shearer 1993). This switch, with reduced IL2 levels and the secretion of the Type 2 cytokines IL4, 5 and 6 may contribute to the increased Ig production and to the overall increase in IgA and IgE. In support of this proposal are the observations of Type 1 and Type 2 cytokine profiles in other diseases. A predominance of Type 2 cytokines is involved in pathogenesis of other diseases e.g. schistosomiasis, leprosy and murine AIDS (reviewed in (Sher, et al. 1992)) and conversely it has been established that a Type 1 cytokine dominated response can be protective against schistosomiasis (Wynn, et al. 1995a), leprosy (Yamamura, et al. 1991) and leishmaniasis (Scott, et al. 1988).
**CD8 T cells**

The eventual decline in HIV-specific CD8 cells is not fully understood but again several mechanisms have been proposed. These include overstimulation by persistent antigen possibly leading to clonal exhaustion (Moskophidis, et al. 1993) and a lack of T helper cell function. There is evidence both for (Rowland-Jones 1993) and against (Bevan and Braciale 1995) the selective pressure of CTL leading to viral escape, and this coupled with lack of recruitment of resting T cells due to dendritic cell dysfunction may contribute to the lack of specific CTL. Specific functional defects in CD8 cells have been suggested which include failure to express the IL7 receptor and subsequent lack of response to IL7 growth signal (Carini, et al. 1994). Others suggest that the expanded CD8 population is the result of the overstimulation and does not represent a population of defective cells (Bofill, et al. 1995).

**Dendritic Cells**

Dendritic cells (DC) appear to be crucial participants in HIV-1 induced pathology. They are exceptionally efficient constitutive antigen presenting cells, have a large surface area rich in major histocompatibility molecules (MHC) Class I and especially MHC II and express the adhesion molecules, intracellular adhesion molecule-1 (ICAM-1) and B7 (Steinman 1991). They have reduced surface charge due to hyposialisation of surface glycoproteins which may reduce charge repulsion between cells. DC are susceptible to HIV infection (Patterson, et al. 1994), support HIV replication (Langhoff, et al. 1991) and are efficient transmitters of HIV-1 to T cells (Cameron, et al. 1992), especially in the lymph node paracortex where this contact is promoted (Fox 1992).

As well as ongoing depletion during disease progression, DC show impaired function early in infection before the reduction in T cell response becomes apparent (Macatonia, et al. 1990). Stimulation of humoral responses by DC continues longer than stimulation of T cell proliferation (Roberts, et al. 1994) and it is proposed that DC failure leads to lack of recruitment of naive T cells.
General Introduction

(Blauvelt, et al. 1995: Macatonia, et al. 1992). This may contribute to the observed failure to generate new CTL and to the increase in virus load in late stage infection and may cause a cumulative loss of T cell memory leading to progressive loss of immune responses.

Induced responses to HIV-1 and vaccines

An ideal vaccine for a naive animal would protect the individual completely from infection by all strains of the pathogen without causing harm or disease, this is however a distant goal. Other more achievable aims of vaccination may be to assist the immune system to eliminate the pathogen after infection, to reduce transmission within the host population and to reduce the morbidity and mortality of the disease caused by the pathogen.

Live attenuated viruses can be extremely efficient as vaccines (e.g. the Sabin attenuated polio virus). By naturally infecting and replicating within host cells, the attenuated virus uses the processing and presentation pathways normally encountered by the wild-type virus leading to presentation of appropriate epitopes and to a protective response. Killed virus vaccines can also be efficient (e.g. the Salk killed polio virus). These different whole virus immunogens have different risk factors (Table 1.1) but have innate adjuvanticity. Small protein subunits or peptides, however, may require both adjuvant and carrier. Established vaccine immunogens such as vaccinia and BCG have been used as carriers with innate adjuvanticity for subunits of other pathogens ("piggy-back" vaccines).
An immunological parallel has been drawn by Cease and Berzofsky (Cease and Berzofsky 1994) between HIV-1 and other chronic viral infections in which a carrier state is generated. Chronic infections have been a challenge for vaccine development but the steadily increasing understanding of the biology of HIV-1 and of its interaction with the human immune system has allowed various strategies for action to be developed. Some mechanisms of HIV-1 pathogenesis involve self destructive activity by the HIV-1 stimulated immune system. It may be possible to design a vaccine to avoid inducing these self destructive responses by omitting potentially harmful epitopes, such as the immunosuppressive epitope in gp41 (Jin, et al. 1993). This may necessitate the use of subunit or partial subunit immunogens. Table 1.1 shows the wide variety of different approaches being taken to develop suitable vaccine immunogens. This table is not intended to be exhaustive, but to reflect the breadth of the present research effort.

**A vaccine for HIV-1. Requirements and strategies**

A vaccine which prevents infection by HIV-1 would ultimately be the most desirable, however, one that could prime a naive immune system to deal effectively with the virus and be able to eliminate it would be protective. Alternatively, a vaccine which will maintain a status of low virus load during the clinically latent period may reduce the risk of transmission between infected/non-infected sexual partners who could be unaware of the existence of the infection. Equally important is the amelioration of AIDS and/or elongation of the clinically latent period to promote a raised quality and length of life for the huge numbers of infected individuals. However, the development of a therapeutic HIV-1 vaccine for late stage disease may be hampered by the ravages of HIV-1 itself, since any intervention may be unable to stimulate or elicit appropriate responses from a severely compromised immune system.
Table 1.1

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<tr>
<th>Construct</th>
<th>Possible Advantages</th>
<th>Possible Disadvantages</th>
<th>Use in animal models</th>
<th>Other points</th>
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<tr>
<td>whole killed virus</td>
<td>All proteins present</td>
<td>gp120 may be dissociated (1)</td>
<td>SIV-protection from challenge (2,3)</td>
<td>Protection achieved equivocal - may be due to xenoresponse to human producer cell lines (6,7) or not (8). New methods of inactivation available for HIV (9).</td>
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<td></td>
<td>Does not replicate</td>
<td>Does not replicate. Possible incomplete killing</td>
<td>Non - protective (4)</td>
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<td></td>
<td>Can give good protection e.g. Salk polio.</td>
<td>Possible denatured proteins</td>
<td>Microencapsulated gives protection from SIV challenge (5)</td>
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<td>whole subunit monomers</td>
<td>May be able to avoid undesired epitope responses.</td>
<td>Epitope presentation cannot mimic supermolecular quaternary structure.</td>
<td>Protection of chimpanzee from HIV-1 using gp120 (14)</td>
<td>gp160 may not present gp120 moiety in its native conformation (16) May elicit stronger T cell help than natural infection (17) Humans gp120 Nabs (18) Clinical trials of gp120 passed phases I and II</td>
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<td>May be efficiently produced in native conformation (10)</td>
<td>Expression system can have profound effects on epitope exposure (13)</td>
<td>Cross reactive Nabs in baboons (15)</td>
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<td></td>
<td>Chimeric proteins can efficiently present specific epitopes (11,12)</td>
<td></td>
<td>Nabs and CTL in mice (11)</td>
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<tr>
<td>peptides</td>
<td>Further avoid harmful epitopes. Immunogenicity may be enhanced by many methods.</td>
<td>Usually poorly immunogenic. Usually do not present conformational epitopes. T cell epitope included may not be presented. May depend on structural context and flanking sequences (20,21,22) or not (23,24,25). Carbohydrates may influence T cell epitope (26) therefore testing each construct for T help essential</td>
<td>V3 peptides induces powerful Nabs: goats - type specific (27,29) primates - type specific (29) rabbits - cross reactive (30) Nabs in multiple haplotypes from single administration in mice (23) which can be cross reactive (31). CTL (31) and cross reactive CTL (32,33) can be generated.</td>
<td>V3 loop of gp120 is an unusually potent peptide epitope. Lipid tails on peptides more immunogenic (25) and can induce mucosal immunity (34). More immunogenic in ISCOMS (35)</td>
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<td>multimers of subunits</td>
<td>Epitope displayed at high density - very immunogenic - may need no adjuvant.</td>
<td>More complex to produce</td>
<td>SIV gag-Ty Virus-like particles (VLP) produces mucosal and systemic responses (37, 38). HIV V3-Ty VLP induce CTL (39) and Nabs in mice (40). V3-gag VLP induce Abs and CTL in mice (41). HIV env VLP induce Nabs in guinea pigs (37). CTL and Abs in cats (42). Macromolecular assemblies induce Nabs to HIV-1 gp120 in mice (43) and in guinea pigs which can be long term and high titre and cross reactive (44)</td>
<td>T cell epitope can be incorporated into very immunogenic fusion partner. V3 on HBsAg (45) and on HBcAg (46) very immunogenic. gp120-CD4 construct elicits conformation dependent Abs (47,48)</td>
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<td>and viral cores</td>
<td>Can mimic native conformation and higher order structure. Self assembly possible.</td>
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<td></td>
<td>Mucosal immunity can be generated. Cellular and antibody responses. No genome and non replicating. Responses can be cross-reactive (36)</td>
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<td>live attenuated virus</td>
<td>Presented to immune system as in natural infection. Replicates.</td>
<td>Dangers of reversion to virulence or transformation involving oncogenes. Demonstrated prolonged persistence</td>
<td>SIV attenuated protects against high dose challenge (49). Protects against infected cells (38)</td>
<td>Originally discounted as too dangerous for HIV. Possible deletion mutant of HIV for a vaccine (50,51,52)</td>
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<td>vaccinia</td>
<td>(?) Potent immunogen in its own right (53). Large genome can accommodate several extra genes (54)</td>
<td>(?) Potent immunogen in its own right (53). Weak responses to recombinant protein in vaccinia primed individuals and may be hazard in immune compromised (55).</td>
<td>CTL and Abs in macaques (56) Abs in mice (57,58) SCID-Hu mice can be protected from infection (59)</td>
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<td>adenovirus</td>
<td>Mucosal immunity Cellular and antibodies</td>
<td>Limited cloning capacity and risk of reversion to virulence by trans complementation (60)</td>
<td>High titre type specific Nabs to HIV in dogs (61) Nabs to HIV in chimpanzee (62)</td>
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<td>avipox viruses</td>
<td>Do not replicate in mammalian cells.</td>
<td>May have less than optimal immunogenicity (ref in 63)</td>
<td>Measles virus protein expression (64)</td>
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<tr>
<td>poliovirus</td>
<td>Well studied, heat stable, mucosal and systemic immunity. Cellular and Ab responses, Early age immunisation possible (65)</td>
<td>Limited cloning capacity (54)</td>
<td>Broadly reactive Nabs to HIV in rabbits (66)</td>
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<td>minireplicons</td>
<td>Do not replicate in mammalian cells. Safer in immune compromised. May have some of the same advantages as poliovirus (65)</td>
<td>Immunogenicity not known</td>
<td>HIV gag and pol protein expression (67,68)</td>
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<td>BCG</td>
<td>Innate adjuvanticity of BCG (69) Can induce mucosal immunity (70) Inexpensive</td>
<td>May be inefficient in BCG primed or TB infected (71)</td>
<td>Cellular and antibody responses to HIV-BCG in mice (72)</td>
<td>May be best if antigen is a fusion protein with outer surface protein A (73)</td>
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<td>salmonella</td>
<td>Oral administration is possible Mucosal immunity possible</td>
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<td>S. typhimurium protective in mouse malaria model (74)</td>
<td>Attenuated S. typhi may be possible vector for HIV-1 (13,75)</td>
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<tr>
<td>DNA immunisation</td>
<td>(?) Persistent antigen. Non replicating Fast, inexpensive CTL and Abs Can be delivered by biolistic process (76,77,78)</td>
<td>(?) Persistent antigen (?) Persistent DNA Possible insertional activation of oncogenes. Unknown safety</td>
<td>Heterologous protection from influenza challenge in mice (79) and chickens (80).</td>
<td>Already in trials in humans (81).</td>
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Abbreviated references are on the reverse of this page.
### References for Table 1.1

Full citations are given in the References section.

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T cell epitopes

For any subunit vaccine, especially synthetic peptides, the incorporation of effective T cell epitopes is vital for recruiting T cell help (Hart, et al. 1990; Haynes, et al. 1993). T cell epitopes which can be presented by different MHC molecules of both class I and II are sometimes clustered together overlapping each other forming a ‘multideterminant region’, which occasionally have so great an overlap that a ‘promiscuous epitope’ is created which is presentable by several Class II alleles (Panina, et al. 1989) or Class I alleles (Shirai, et al. 1992). Both multideterminant regions and promiscuous epitopes have been described in HIV-1 envelope proteins (Hale, et al. 1989) and many other HIV proteins also contain T cell epitopes (Nixon, et al. 1992). The existence of clusters of T cell epitopes in HIV-1 envelope and even of promiscuous epitopes may be of value in the design of strongly immunogenic peptide vaccines for the outbred human population (Berzofsky, et al. 1991a; Berzofsky, et al. 1991b). In support of this, only two immunisations with a V3 peptide linked to a multideterminant helper T cell epitope has induced high titres of neutralising antibodies in four different haplotypes of mice (Ahlers, et al. 1993). T cell epitopes can also be manipulated to increase cross reactivity. Takahashi et al (Takahashi, et al. 1989) show that the CTL response can also be manipulated by making a single amino acid change from Tyr to Val or Leu in a T cell epitope. This abolished the ability of CTL to distinguish between IIIB and MN strains of HIV-1 and the altered peptide was also recognised by CTL specific for other additional strains.

Cytokine profiles and protection

Th1 cells are important for the induction of HIV-specific CTL (Shirai, et al. 1994) but also important in giving help for HIV envelope-specific antibody production (Hosmalin, et al. 1991). It has been found that some individuals have remained seronegative for several years during repeated exposure to HIV-1 and that cells from these individuals produce IL2 in vitro (Clerici, et al. 1992a). Salk
(Salk, et al. 1993) proposed that a prolongation or re-establishing of Type 1 cytokine dominance may be an important goal for a prophylactic vaccine. There are several possible mechanisms which might be evoked to achieve this. These include the use of adjuvants to direct the response towards Type 1 cytokine induction including the addition of IL12 to the vaccine mixture (Wynn, et al. 1995a: Wynn, et al. 1995b); polymerisation of protein immunogens with glutaraldehyde (Yang, et al. 1993) and the use of very low doses of antigen (Bretscher, et al. 1992: Parish and Liew 1972) which has also been shown to skew the response towards IFNγ and IL2 production (Type 1) and away from antibody production (Type 2) in human volunteers immunised with gp160 (Clerici, et al. 1991). HIV-1 furthermore replicates less efficiently in the Type 1 cytokine environment (Maggi, et al. 1994: Vyakarnam 1994: Vyakarnam 1995). Paradoxically it is Type 1 cytokines which increase HIV expression through IFNγ synergising with TNFα (Kinter, et al. 1995: Poli and Fauci 1993) and Type 2 which suppress it through IL4 and IL10. This, together with the cytotoxic activity of Th1 cells, may make it important to limit the induction of Th1 to safe effector to target ratios (Del Prete, et al. 1991). This limitation may make the task of developing a safe Type 1 cytokine based vaccine strategy very problematic.

**B cell epitopes and neutralising antibodies**

Neutralising antibodies (Nabs) are those which prevent infection, either by sterically or otherwise blocking receptor binding or by inhibiting a post-binding event. The V3 loop has been widely described as immunodominant since it elicits a large proportion of the gp120 specific antibodies in humans, mice, rabbits, goats, horses and chimpanzees and contains both T and B cell epitopes (Goudsmit 1988). It is also described as the principal neutralising determinant (PND), but while its power as a B cell epitope is undisputed and corroborated by many (Goudsmit 1988: Palker, et al. 1988: Rusche, et al. 1988), it is not clear that V3 is in fact the PND in primary isolates (Moore 1995) which...

Despite the observation that sera with neutralising capacity for laboratory HIV-1 often fail to neutralise primary field isolates described above, there is a strong body of evidence in favour of Nabs having an antiviral effect *in vivo*. Nabs can prevent infection in challenge studies (Table 1.1) and high titre neutralising antibodies can influence positively the clinical course (Weiss, et al. 1985). The antiviral activity of neutralising antibodies is also supported by the emergence of neutralisation escape mutants (Nara, et al. 1990). The V3 loop has been the target of studies to develop a vaccine based on V3 Nabs (see below). Other sites in gp120 also serve as neutralising epitopes, namely V1/V2 loops (McKeating, et al. 1993b) and constant regions (Ho, et al. 1987; Nakamura, et al. 1993). Importantly, Nabs to the functionally conserved CD4 binding site (CD4bs), are also generated *in vivo* (Hariharan, et al. 1993; Moore and Ho 1993; Thali, et al. 1991). These Nabs are broadly reactive and not dependent on V3 sequences *per se*. but CD4bs Nabs and V3 Nabs are able to synergise in neutralisation (Cavacini, et al. 1993; Montefiori, et al. 1993; Thali, et al. 1992; Tilley, et al. 1992). Nabs can also block vertical transmission to the fetus. In this case, antibodies to the CD4bs of gp120 (Khouri, et al. 1995) or Nabs to the mother's own isolate of gp120 (Scarlatti, et al. 1993) both correlate with non-transmission.

**V3 as a vaccine candidate**

This attractive epitope has been tested as the target for a protective vaccine. Passive Nabs (Emini, et al. 1992) and active immunisation with gp120 (Berman, et al. 1990) can be protective in challenge studies in chimpanzees (*Pan troglodytes*), but protection was only afforded to the homologous isolate. In
addition, active immunisation with gp160 failed to produce strong responses to V3 (Berman, et al. 1990) which may indicate a conformational difference in gp160 of the gp120 moiety before cleavage from gp41. Another study (Pinter, et al. 1993) showed that certain V3-specific monoclonal antibodies raised to gp160 could only recognise V3 in gp160 and not in gp120 supporting the idea of a conformational change in gp120 as a result of cleavage. The hypervariability of V3 is an obstacle to vaccine development (Goudsmit, et al. 1991) but not an insurmountable one, since analysis of 243 V3 sequences (LaRosa, et al. 1990) revealed partial conservation of amino acids. Another analysis (Holley, et al. 1991) of 76 V3 sequences showed that a limited number of hexapeptides could be used to represent the vast majority of the V3 loop crown sequences (incorporating GPGR) found in vivo.

As described above, neutralising antibodies may be able to provide sterilising immunity (complete protection from infection). However, due to the conformational effects mediated by point mutations (Klasse, et al. 1993; McKeating, et al. 1993a), the reactivity of Nabs with the continually evolving gp120 protein will be unpredictable and they may represent a powerful but unreliable defence. Therefore a strategy to induce only Nabs and not cellular responses in addition may be insufficient as a vaccine.

**Mucosal Immunity**

Strong mucosal immunity is vital for prevention of sexual transmission HIV-1 naive individuals and may be able to prevent or limit transmission from those infected (Marx, et al. 1993). The induction of mucosal immunity has been the focus of extensive recent research not only for HIV-1 but for many other infectious agents. The particular route of administration and the delivery system/adjuvant have a major impact on the characteristics of the immune response induced, affecting induction of cellular and/or antibody responses and the isotype of the antibody. The induction of a mucosal response does not

Successful induction of mucosal immunity can result from the delivery of the same variety of vectors which can induce systemic responses, and can also support long lived memory (London, et al. 1987: Morrow, et al. 1994). Attenuated bacterial pathogens, Salmonella typhimurium in mice (Chatfield, et al. 1992) and S. typhi and Vibrio cholerae in humans (Cryz, et al. 1993: Staats, et al. 1994) have been proved safe for use as oral vectors and Bacille Calmette Guerin (BCG) has also been shown to be safe and effective as an oral vector carrying HIV-1 nef (Lagranderie, et al. 1993). Viral vectors; adenovirus, poliovirus and vaccinia virus (see Table 1.1) including those carrying the antigen as a fusion protein with a viral protein (Evans, et al. 1989) can result in Nabs and naked DNA vectors delivered to the mucosae in liposomes have generated protective immunity to influenza in mice (Fynan, et al. 1993).

Delivery of the antigen to a mucosal surface is far more potent in inducing mucosal responses than is systemic delivery and this appears to be especially true if the antigen is particulate rather than soluble or is in some way encapsulated. Biodegradable microspheres composed of poly-DL-lactide-coglycolide (PLG); a copolymer of lactic and glycolic acids, can be used to encapsulate antigen and can induce systemic and mucosal responses, the rate of release of antigen being controlled by the particular PLG architecture of the microsphere (Yan, et al. 1995). After oral administration SIV microspheres induced protective responses against SIV (Marx, et al. 1993). Virus-like particles formed by self assembly of Ty P1-foreign antigen fusion proteins are effective in inducing both IgG and IgA in serum and mucosal fluids (Lehner, et al. 1992). Similarly encapsidated poliovirus minireplicons can elicit antibodies in serum and secretions and have been used to induce responses to HIV-1 proteins (Moldoveanu 1995: Morrow, et al. 1994). In the context of particulate antigens,
liposomes containing the antigen can be taken up by M cells in the mucosae (Childers, et al. 1990) and can induce both systemic and mucosal IgG and IgA (Vadolas, et al. 1995). Liposomes, as well as being delivery vehicles, also have innate adjuvanticity and, if given intranasally simultaneously with or up to 48 hours before the antigen, stimulate IgG and IgA production (de Haan, et al. 1995a). Some enteric toxins such as cholera toxin (CT) and E coli heat labile enterotoxin (LT) are powerful mucosal adjuvants. The adjuvanticity of CT may be associated with the toxic subunit A while the potentiating effect of the non-toxic subunit B may be mediated by its carrier-molecule activity (Staats, et al. 1994). LT can be coadministered orally with the antigen without the need for conjugates or sophisticated vehicles. Its toxicity is not as severe as that of CT its safety is being assessed in phase I trials (Johnson 1994). Cytokines such as IFNγ can be administered in liposomes and have been shown to augment the response to HIV-1 subunit vaccines (Lachman 1995).

Many mucosal routes of administration can be used and the resulting mucosal response is usually not restricted to the immunisation site. Intranasal administration of antigen can elicit IgG and IgA in vaginal, nasal and salivary (Lubeck, et al. 1994) and lung secretions (Abraham 1992). Immunisation of the lungs can result in urogenital secretion of IgA (de Haan, et al. 1995b) and work by Lehner et al (Lehner, et al. 1994a), in which particulate antigen (Ty VLP-SIVp27) was injected into the region of the internal iliac lymph nodes, has produced a broad mucosal distribution of secreted antibody in rectal, vaginal, urethral and seminal fluids and in the serum and the urine. A similar broad distribution was attained using topical urethral immunisation followed by oral boosting (Lehner, et al. 1994b). Different cytokine profiles can be induced by a single route of mucosal immunisation depending on the conditions. For example S. typhimurium displaying tetanus toxoid (TT).can induce cellular responses and some IgA secretion whereas soluble TT with adjuvant predisposes to high secretory IgA production (Staats, et al. 1994). The isotype of the antibody
produced can also be influenced by the relative hydrophilicity and lipophilicity of the formulation (Gizurarson, et al. 1995). There are therefore precedents for the control of the different qualities of the induced immune response when it becomes clearer which form(s) of immunity may be most beneficial against HIV-1.

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**Epitope competition**

**Introduction**

Central to the aim of the work to be presented in this thesis is the phenomenon of 'intramolecular' or 'epitope' competition. Since this is an important concept for this work, the mechanisms thought to be operating are outlined below with a very brief description of the work of Taussig in the 1970’s and the more recent work of Kilbourne and others which has contributed to the understanding of this phenomenon. Epitope competition is observed when the immune response to an antigen is directed mainly to one or more of its epitopes with a concomitant reduction in the response to its other epitopes. Taussig demonstrated intramolecular B cell competition using Ig as the model antigen (Taussig 1971). The Fc part of Ig competes with the Fab part: the resulting polyclonal response is skewed in favour of Fc which is said to be immunodominant. It is demonstrable that if the epitopes are physically separated, then the observed differences in the level of response disappear. This confirms that an active competition is occurring which depends on a physical link between the competing epitopes. Taussig showed furthermore, that this competition could be abolished by physically separating the epitopes or by
blocking the dominant epitope with Fc-specific antibodies at the time of immunisation (Taussig and Lachmann 1972).

**B cell epitope competition**

In the first response of an immune system which is naive to a particular antigen, some epitopes will elicit a 'better' response than others due to the intrinsic immunogenicity of the individual epitopes and the repertoire of reactive B cells. The epitopes may have greater or lesser immunogenic power due to their density, accessibility or affinity with the B cell's surface Ig, and repertoire holes can exist which cause an individual to be genetically unresponsive to a particular epitope or antigen. In the anamnestic response, epitope competition may also take place, often due to events which occurred in the primary response.

One of the major mechanisms is clonal dominance which depends on antigen presentation by specific B cells rather than by non-specific antigen presenting cells (APC) such as macrophages. It has been shown that B cells can present antigen to T cells between $10^3$ and $10^4$ times more efficiently than other APC due to the specific uptake of antigen via their surface Ig (Lanzavecchia 1988: Rock, et al. 1984). B cells are only efficient APC during the secondary response as demonstrated by Ju et al (Ju, et al. 1993) using random polymers of Glu Ala Tyr. In this system an affinity matured and expanded population of B cells specific for the dominant epitope is generated in the first response. During the second response B cells specific for other epitopes are literally robbed of antigen due to its sequestration by the dominant clone. The secondary response is skewed even more in the direction of the dominant epitope. Affinity differences may also contribute to B cell epitope competition. If two epitopes are equally accessible but one has a higher affinity for the reactive B cell's immunoglobulin, this epitope may be dominant due to its preferential uptake. This may occur especially if the difference in affinity was due to a difference in the rate of association rather than in the rate of dissociation. A classical example of clonal
dominance is original antigenic sin in which a primary response to a new antigen fails in favour of a secondary response to a previous antigen. This is due to B cell cross-reactivity between the two antigens which allows B cells specific for the previous antigen to sequester the new antigen and prevent the primary response to it being initiated.

T cell epitope competition

A hierarchy can exist within the available T cell epitopes of an antigen. Ria et al have shown that the hierarchical position is correlated with the peptides' affinity for the presenting class II MHC molecule (Ria, et al. 1990). They used two peptides which showed equal affinity for MHC and equal T cell stimulatory capacity when supplied separately. They then demonstrated an equal stimulatory capacity of each when the two peptides were synthesised as one covalently joined molecule indicating either a state of equal competition or of no competition. When a point substitution was introduced into one of the peptides in the joined molecule which weakened that peptide’s affinity for the MHC but did not affect its affinity for the TCR, then the other peptide in the joined molecule became dominant. The same hierarchy was found in experiments with the induction of T cell tolerance to these same epitopes. Their study avoids the potential effects of differential processing on T cell epitope hierarchy and of holes in the T cell repertoire. It shows that the observed phenomenon of T cell epitope hierarchy can be due to competition between T cell epitopes and that peptide:MHC affinity is a crucial factor. Tussey (Tussey, et al. 1995) has also demonstrated this effect.

Intravirionic competition

Kilbourne, Johansson and colleagues have been analysing the immune response to the influenza virus surface glycoproteins haemagglutinin (HA) and neuraminidase (NA) in their research into protective vaccines. In natural
infection HA competes with NA and suppresses the antibody response to NA. This has been a major stumbling block in the development of a protective vaccine as HA shows a high degree of antigenic drift and allows influenza virus escape from previous protective immunity. NA is much less polymorphic and a more suitable vaccine epitope. In vaccination experiments, Kilbourne et al (Kilbourne, et al. 1987) have shown that with a weakly immunogenic variant of HA, the competition is abolished and a strong response to NA is mounted. When HA and NA are physically separated, these authors have established that, as individual glycoproteins, HA and NA are equally immunogenic even without adjuvant, which indicates that NA as well as HA can be a powerful immunogen in its own right (Johansson and Kilbourne 1993). This is on a weight for weight basis which is important since HA is present on the viral surface in greater amounts than NA. They have also demonstrated that there is no hole in the B or T cell repertoire for either HA or NA and that equal antibody responses in both primary and secondary immunisations can be obtained showing that B and T memory for NA is as good as for HA (Johansson, et al. 1987). In summary, they showed abolition of the competitive effects by physically separating the antigens. Furthermore, using isolated spleen cells, it was shown that B and T cells are equally involved in the competitive effects seen when HA and NA are presented on a virus particle.

They describe this competition as ‘intravironic antigenic competition’ and even though the protagonist epitopes on HA and NA are not linked covalently as one primary sequence it appears to be directly relevant to intramolecular/epitope competition. This follows from the fact that HA and NA are physically linked via the viral envelope, enabling clonally dominant HA-specific B cells to effectively sequester the whole virion and deprive NA-specific B cells of antigen. HA and NA are therefore competing like two epitopes on one antigen. It might be predicted that NA could dominate over HA if a naive animal were immunised with NA alone. Such priming could allow the
expansion and affinity maturation of a clone(s) of NA-specific B cells which could effectively dominate over naive HA-specific B cells. In fact this does occur but only if the subsequent NA/HA virus carries the homologous NA (Johansson and Kilbourne 1994).

Thus, intramolecular competition can occur for both B and T cell epitopes. The mechanisms operating include clonal dominance, repertoire holes and affinity differences, and might be grouped under the term "selective molecular capture".

**Epitope competition hypothesis**

Based on these concepts there is a working hypothesis for the experiments described in this thesis (Roitt 1989). If it is true that intramolecular B cell competition is operating to maintain one or more epitopes as immunodominant, then other epitopes may exist which are intrinsically immunogenic but are suppressed due to this competition. A corollary to this to this may be that if the immunogenicity of the dominant epitope is reduced or abrogated then other epitopes may become more functionally immunogenic.

**Application of the epitope competition hypothesis to V3 and conserved regions of gp120**

As described earlier, in humans V3 has been shown to be immunodominant, but due to its hypervariability, neutralising antibodies to V3 tend to be virus-type specific, although the response may become more cross reactive with time (Wang, et al. 1991). It may be possible to design subunit or peptide vaccine constructs which are able to induce protective, strain-crossreactive neutralising antibodies to V3 and such constructs might even be tailored to different geographical locations using knowledge of the locally dominant clades of HIV-1. However, the proven ability of HIV-1 to escape neutralisation by mutation, together with high mobility of individuals between
geographical regions, would make this strategy unreliable on its own. It has been suggested (Coffin 1986: Ho, et al. 1987) that directing the neutralising antibody response towards conserved regions of gp120 would reduce viral escape. This response would also need to be cross reactive but to a far lesser extent than for variable region epitopes. Therefore, if epitope competition is occurring between V3 and other regions it may be advantageous to reduce this competition and this might possibly be achieved by reducing the immunogenicity of V3.

Another approach to augmenting the response to constant regions of gp120 might be to immunise with partial subunits or peptides displaying constant region epitopes. It is known that the CD4bs is discontinuous and likely, therefore, to be highly conformation dependent and it would be formidable to attempt to make a partial subunit or peptide construct to represent its three dimensional structure. However it may not be impossible, as some peptide constructs have been made which are able, to some degree, to mimic the CD4bs (Morrow, et al. 1992: Ramsdale, et al. 1993). Since the tertiary and quaternary structure of gp120 is not yet known it is not clear exactly which constant regions, other than the CD4bs, are available as B cell epitopes. A benefit of using the whole gp120 molecule as an immunogen is that even in the absence of precise structural knowledge, a conformationally correct gp120 will per se display its normal constant and variable epitopes. The masking of some of these by oligomerisation on the virion surface is a very important consideration and ideally an oligomerised gp120/41 might be used as the immunogen since this may also present those epitopes which are only present on the oligomer.
The aim is to test the hypothesis that reduction in immunogenicity of a powerful B cell epitope may allow the immunogenicity of other epitopes to increase by being, to some extent, released from epitope competition. To this end, gp120 is used as a model protein.

We have taken up the suggestion that a strong response to the conserved regions of gp120 could reduce viral escape by mutation. With the aim of increasing the immunogenicity of the CD4bs and other accessible conserved epitopes we have attempted to reduce the immunogenicity of V3. Due to its immunodominance V3 is the logical epitope with which to begin, but later V1/V2 and possibly of other regions might also be similarly mutated. The aim is also to maintain the native conformation of gp120 so that other potentially beneficial epitopes are not disturbed. Such constructs may have application in a subunit vaccine for HIV-1.
CHAPTER 2

MATERIALS AND METHODS

Solutions

**Acridine orange/Ethidium bromide:** Acridine Orange 0.001 %/Ethidium Bromide 0.002 % in PBS. An equal volume mixed with an aliquot of the CHO cells to be counted.

**Adjuvant components:**

*Freund's complete and incomplete adjuvants.* Mineral oil with, in addition, killed mycobacteria in the 'complete' formulation. It forms a water in oil emulsion.

*TitreMax.* Squalene (a metabolisable oil) with a 'unique microparticulate stabiliser' and block copolymer CRL87-41. It forms a water in oil emulsion.

*AF.* Squalane (saturated squalene) with Tween 80 and block copolymer pluronic L121. It forms an oil in water emulsion.

*Ribi.* Squalene with monophosphoryl Lipid A and synthetic trehalose dicorynomycolate. It forms an oil in water emulsion.

*Quil A.* A heterogeneous mixture of saponins extracted from *Quillaia saponaria* Molina.

**Agarose gels loading buffer:** Ficoll (400) 20 % in TE (v.i.) plus a few grains of Orange G.

**Amido black:** Naphthol blue black 1 g in 100 ml 2.5 % acetic acid.

**Ampicillin:** Stock 100 mg/ml in deionised water. 0.2 μm filter sterilised. Stored in aliquots at -20°.

**Coomassie blue stain:** Coomassie brilliant blue 1 g in 500 ml of methanol 50 %, acetic acid 10 %.
**Denhardt's solution x 50**: BSA 1%, Ficoll (400) 1%, Polyvinylpyrrolidone 1% in deionised water. Stored at -20°C.

**Destains**: For 0.4 mm gels: Methanol 5% Acetic acid 7%. For 1.5 mm gels: Methanol 45%, Acetic acid 10%.

**dNTP 200 µM**: dATP, dCTP, dGTP and dTTP at 200 µM each.

**Ethidium bromide**: 5 mg/ml (12.7 mM) in deionised water.

**Franz I**: MES 10 mM, RbCl 100 mM, CaCl₂ 10 mM, MnCl₂ 50 mM adjusted to pH 5.8 and autoclaved.

**Franz II**: PIPES 10 mM, RbCl 10 mM, CaCl₂ 75 mM, Glycerol 15%, adjusted to pH 6.5. 0.2 µm filter sterilised.

**Freezing medium for E.coli (Hoegnes's)**: K₂HPO₄ 26.5 mM, KH₂PO₄ 2.6 mM, Na₃C₆H₅O 4 mM, MgSO₄ 3 mM, 8.8% glycerol in deionised water. 0.2 µm filter sterilised. Stored at 4°C.

**GMEM-S stock solutions**:
1) **Deionised water** autoclaved in 400 ml aliquots.
2) **10 x Glasgow MEM without glutamine**. Stored at 4°C.
3) **7.5% sodium bicarbonate**. Stored at RmT.
4) **100 x MEM non-essential amino acids**. (L-alanine, L-asparagine, L-aspartate, L-glutamate, glycine, L-proline, L-serine 10 mM each). Stored at 4°C.
5) **100 x glutamate + asparagine**. 50 mM L-glutamate, 50 mM L-asparagine in deionised water. 0.2 µm filter sterilised. Stored at 4°C.
6) **100 mM sodium pyruvate**. Stored at 4°C.
7) **50 x nucleosides**. Adenosine, guanosine, cytidine, uridine 1.5 mM each, thymidine 500 µM in deionised water, 0.2 µm filter sterilised. Stored at -20°C.
8) **Dialysed fetal calf serum** with complement components heat inactivated at 56°C for 30 minutes. Stored at -20°C.
9) **Penicillin-Streptomycin** Penicillin 5000 units/ml: Streptomycin 5000 µg/ml. Stored at -20°C.
10) **100 mM L-methionine sulfoximine (MSX)**. 0.2 µm filter sterilised. Stored at -20°C.
11) **200 µM L-glutamine**. Stored at -20°C.

The stock solutions are combined in the following order: Water 400 ml, 10 X GMEM 50 ml, Sodium bicarbonate 18.1 ml, non-essential amino acids 5 ml (100 µM each), glutamate and asparagine 5 ml (500 µM each), sodium pyruvate 5 ml, nucleosides 10 ml (A, G, C, U: 30 µM each; T: 10 µM), dialysed FCS 50 ml, penicillin-streptomycin 5 ml.
**Materials**

**Herring sperm DNA:** DNA autoclaved in water, phenol-chloroform extracted, precipitated with alcohol and adjusted to 10 mg/ml. Stored at 4\(^\circ\).

**IPTG:** 0.1 M in deionised water. 0.2 \(\mu\)m filter sterilised. Stored at -20\(^\circ\).

**LB:** Bactotryptone 10 g, Yeast extract 5 g and NaCl 10 g per litre. Autoclaved.

**LB-agar plates:** LB with 1.5 % bactoagar. Autoclaved. Plates stored at 4\(^\circ\).

**LB-ampicillin plates:** LB agar plates with 100 \(\mu\)g/ml ampicillin added after autoclaving.

**NaOH/SDS:** NaOH 200 mM, SDS 1 \%.

**PBS:** NaCl 137 mM, KCl 2.7 mM, Na\(_2\)HPO\(_4\) 8 mM, KH\(_2\)PO\(_4\) 1.5 mM adjusted to pH 7.4.

**PBS-T:** PBS with 0.05% Tween 20.

**PBS-T-B:** PBS-T with 1 % BSA.

**Phenol-chloroform:** Phenol:Chloroform:Isoamyl alcohol in the ratio 25:24:1 with 0.1 % 8-hydroxyquinoline. Equilibrated to pH 8.0 with TE. Frozen in aliquots at -20\(^\circ\).

**Ponceau S:** Ponceau S 0.1 % in 5 % acetic acid.

**Potassium acetate pH 4.9:** Potassium acetate 3 M in deionised water. Adjusted to pH 4.9 with acetic acid.

**Prehybridisation solution:** SSPE (v.i.) x 6, SDS 1 %, Denhardt's solution x 5, 100 \(\mu\)g/ml herring sperm DNA.

**RNAse (free of DNAs):** 10 mg/ml in Tris (pH 7.5) 10 mM, NaCl 15 mM. Boiled for 15 minutes. Stored at -20 \(^\circ\).

**SDS PAGE stacking gels:** Acrylamide monomer 5.0 %, bisacrylamide 0.013 %, Tris (pH 6.8) 125 mM, SDS 0.1 %, Ammonium persulphate 0.05 %, Temed 1 %, Riboflavin-5-phosphate 0.0005 %.

**SDS PAGE resolving gels:** Acrylamide monomer 7.5 %, bisacrylamide 0.02 %, Tris (pH 8.8) 375 mM, SDS 0.1 %, Ammonium persulphate 0.05 %, Temed 0.5 %.

**SDS PAGE loading buffer:** Tris (pH 6.8) 100 mM, SDS 2 %, Bromophenol blue - a few grains, Glycerol 20 %. DTT 20 mg/ml (130 mM) added just before use if required.

**SDS PAGE running buffer:** Tris (pH 6.8) 0.025 mM, Glycine 192 mM, SDS 0.1 %. Adjusted to between pH 8 and 9.

**Sequencing gels:** Acrylamide monomer 6 %, bisacrylamide monomer 0.32 % Urea 8.3 M, in 0.5X TBE (v.i.), Ammonium persulphate 0.05 %, Temed 0.5 %.

**Sequencing gel fix:** Methanol 10 %, Acetic acid 10 %.
SSC x 20: NaCl 3 M, Na$_3$C$_6$H$_5$O 300 mM adjusted to pH 7.4 and autoclaved.

SSPE x 20: NaCl 3 M, NaH$_2$PO$_4$, 200 mM, EDTA 0.02 mM adjusted to pH 7.4 and autoclaved.

TAE: Tris 40 mM, EDTA 1 mM, acetate 1 M, adjusted to above pH 8.0.

TBE: Tris 90 mM, EDTA 2 mM, borate 90 mM adjusted to above pH 8.0.

TBS-T: TBS with 0.1% Tween 20

TBS: Tris 20 mM, NaCl 137 mM, adjusted to pH 7.6.

TE pH 7.5 or 8.0: Tris (pH 7.5 or 8.0) 10 mM, EDTA (pH 8.0) 1 mM.

Tris/glucose/EDTA: Glucose 50 mM, Tris (pH 8.0) 25 mM, EDTA (pH 8.0) 10 mM.

Western blot transfer buffer: Tris 25 mM, Glycine 192 mM, Methanol 20 %

X-gal: 4 % in dimethylformamide. Stored at -20°
Suppliers

1.5 ml ‘eppendorf’ tubes  Greiner Laborteknich Ltd.,
Acrylamide/Bisacrylamide ‘Protogel’ National Diagnostics, Aylesbury,
Acrylamide / Bisacrylamide ‘Sequagel’ Buckinghamshire.
Agarose National Diagnostics.
AIMV serum free medium FMC Bio Products, Rockland,
Ampicillin ME, USA.

Antibodies
Sheep anti gp120 (D7324) Sigma Directed Program.
Rabbit antiserum to gp120 (ADP421) Aalto Bioreagents Ltd., Dublin.
Mouse anti Rat κ and λ-Alk Phos (A1062) Sigma.
Goat anti Mouse IgG-Alk Phos(A2429) Sigma.
Mouse anti Rabbit IgG-Alk Phos(A2556) Sigma.
Goat anti Mouse IgG,A,M-Alk Phos (A0162) Sigma.
Rabbit anti Sheep IgG-Alk Phos (61-8622) Zymed Ltd. Cambridge
Sheep anti Mouse IgG1 (AA273) Bioscience
Sheep anti Mouse IgG2a (AA274) The Binding Site Ltd.,
Sheep anti Mouse IgG2b (AA275) Birmingham.
Sheep anti Mouse IgG3 (AA276) The Binding Site.

Bactoagar
Bactotryptone The Binding Site.
Bgl II linkers The Binding Site.

Caesium chloride
Calf intestinal alkaline phosphatase

Cardiotoxin
CHO cells
Cryotubes
Dimethyl sulfoxide
Dithiothreitol
DNA binding resin Wizard Minipreps

Greiner Laborteknich Ltd.,
Dursley, Gloucestershire.
National Diagnostics, Aylesbury,
Buckinghamshire.
National Diagnostics.
Gibco. Life Technologies Ltd.,
Paisley.
Sigma Chemical, Poole, Dorset.
Aids Directed Program.
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DNA binding resin Wizard PCR preps
DNA binding resin Wizard PCR preps
Promega.
dNTPs
Promega.
Enhanced chemiluminescence kit
Amersham International plc., Little Chalfont, Buckinghamshire.

Ethidium bromide
Sigma.
Fetal calf serum (dialysed)
Gibco
Hibond C extra
Amersham.
Hibond N
Amersham.
Isopropylthiogalactoside
Gibco.
Isotopes
Amersham.
Klenow polymerase
New England Biolabs.
Low melting point agarose
Sigma.
Magnesium chloride for PCR
Promega.
Marcaine
UCH Pharmacy, London
Marvel
Premier Beverages, Adbaston, Stafford.

Maxisorp C Immunoassay plates
Nunc.
Methionine Sulphoximine
Sigma.
Mineral oil for PCR
Sigma.
Molecular size markers for agarose gels
New England Biolabs and Pharmacia Biotech, St. Albans, Hertfordshire.

Molecular size markers for SDS-PAGE
Biorad Laboratories Ltd., Hemel Hempstead, Hertfordshire.
Oligonucleotides
Oswel Research Products Ltd. University of Edinburgh; and Genosys Biotechnologies, Cambridge.
pBluescript
Stratagene (UK) Ltd., Cambridge
Phenol
Appligene Oncor, Chester-le-Street, Co. Durham.

Pipette tips
Greiner.
Plastic ware
Sterilin Ltd., Middlesex.
pNPP
Sigma.
Polynucleotide kinase
New England Biolabs.
Qiagen 500 columns
Qiagen Ltd., Dorking, Surrey.
Recombinant (baculovirus) gp120
Aids Directed Program
Restriction endonucleases
New England Biolabs.

Ribi R-700
Promega.
Riboflavin-5-phosphate
Universal Biologicals Ltd. London.
RNase
BDH, Merke.
Sequenase Version 2.0 sequence kit
Sigma.
Standard chemicals 'AnalaR'
Amersham.
T4 DNA ligase
BDH, Merke.

New England Biolabs.
Materials

T4 Polynucleotide kinase
T7Gen mutagenesis kit
Taq Polymerase
TEMED
Tissue culture flasks
Tissue culture pipettes
Titre Max
Tris
Tween 20
X-gal
Kodak X-OMat R film
Fugi film
Yeast extract

New England Biolabs.
United States Biochemical,
Amersham Ltd.
Promega.
Biorad.
Nunc.
Phillip Harris Scientific, London.
Sigma.
Sigma.
Sigma.
Gibco.
Scientific Image Centre Ltd.
Genetic Research Instrumentation
Ltd.
Difco.

Equipment

Centrifuges
Beckman J-6B
Beckman ultracentrifuge L7-65
Heraeus bench top Biofuge 15
MSE Microcentaur
IEC Centra-3RS
Sorvall Superspeed RC-5B
Perkin Elmer Cetus
Tekne Dry Block DB 2A
Flowgen Instruments, MH 1051
and Life Technologies
Dynatech MR 5000
Flow Laboratories Titretek
Edwards Modulyo
Hoefer Slab Gel Dryer SE1160
Luckham R100
Hybaid Maxi Oven
Leec MkII
Nikon TMS
Pharmacia GPS 200/400
Biorad 200
Hoefer PS 52500

DNA thermal cycler
Dry heat block
Electrophoresis tanks
ELISA reader
ELISA washer
Freeze drier
Gel drier
Horizontal shaking platform
Hybridisation oven
Incubator
Microscope for cell culture
Power packs
Materials

SDS PAGE large format  Biorad Protean II
SDS PAGE minigel format  Hoefer 2050
Sequencing tanks  Flowgen Instruments, VM 4133
Spectrophotometer  LKB Biochron Ultrospec 4050
Vortexer  Jencons Scientific, Miximatic
Water baths  Grant Instruments Ltd.
Western blotting transfer tank  Biorad Laboratories Ltd.
METHODS

General DNA methodology

Restriction Endonuclease DIGests

1-5 units of restriction endonuclease(s) per 1 µg of plasmid DNA were incubated at 37° (or individual optimal temperature) at a DNA concentration of 100 ng/µl. Reaction buffer and bovine serum albumin (BSA) if required were supplied with the enzyme.

Phenol-chloroform extractions

The reaction volume was increased to 300 µl with TE and the EDTA concentration increased to 10 mM. An equal volume of phenol-chloroform was added, shaken vigorously for 2 minutes and then microfuged at 15,000 g for 5 minutes at room temperature (RmT). After recovery of the aqueous phase traces of phenol were removed with an extraction using an equal volume of water-saturated chloroform. The DNA was then precipitated with alcohol in the presence of added salts.

Precipitation of DNA

DNA was precipitated as a complex with cations. 2-2.5 M ammonium acetate or alternatively 0.2-0.3 M sodium acetate pH 5.3 and either 2.5 volumes of ethanol (70%) or 0.6 volumes of propan-2-ol (38%) were added and mixed well. The precipitated DNA was pelleted by centrifugation either immediately or
following cooling at -20° or -70° (see individual texts). The DNA pellet was washed with 70% ethanol, air dried and dissolved in buffer for the next step or in TE for storage at -20° (not stated in subsequent methods).

**Agarose gel purification of DNA**

DNA was electrophoresed in the presence of ethidium bromide in 1-2.5% Tris acetate EDTA (TAE) agarose gels. DNA was recovered from normal melting point agarose gel slices by centrifugation at 4,500 g through a wad of siliconised glass wool for 5 minutes at RmT. The DNA solution recovered was phenol-chloroform extracted until no material remained at the interface, then precipitated. When low melting point (LMP) agarose gels were used, the DNA was recovered using DNA binding resin (Promega Wizard PCR preps).

**Plasmid Preparation**

Plasmid DNA was extracted from *E. coli* by alkaline lysis (Birnboim and Doly 1979). All glassware was rigorously cleaned to remove detergent and plasmid DNA by soaking for 15 minutes in 0.5 M HCl then 0.5 M NaOH. Growth medium was sterilised in the container by autoclaving.

**MiniPrep.** A single colony of transformed *E. coli* was added to 5 ml of LB with 100 µg/ml ampicillin and grown overnight at 37° with shaking. The culture was pelleted at 2,000 g, resuspended in 300 µl of Glucose/Tris/EDTA, lysed with 600 µl NaOH/SDS and mixed gently with 300 µl of Potassium acetate pH 4.9 to precipitate cellular debris and *E. coli* chromosomal DNA. After centrifugation at 15,000 g at RmT for 5 minutes, 1 ml of the supernatant was incubated with RNase A at 50 µg/ml for 90 minutes at 37°. The mixture was extracted once with phenol-chloroform (v.i.) and the DNA precipitated (v.i.) with 600 µl propan-2-ol at RmT and then centrifuged at 15,000 g for 5 minutes.
**MiniPrep - Promega. (DNA binding resin purification).** For sequencing, plasmid DNA was prepared using Promega Wizard Mini Prep protocol which employs alkaline lysis as above followed by plasmid purification using column centrifugation with a DNA binding resin.

**Medium scale plasmid preparation.** The *E. coli* culture volume was increased to 120 ml and all other volumes increased proportionally. After alkaline lysis, nucleic acids were precipitated with propan-2-ol and resuspended in 2 ml for RNase digestion and phenol-chloroform extractions. After a second propan-2-ol precipitation, the DNA was dissolved in 500 µl of TE/1M NaCl and mixed with an equal volume of autoclaved 13% polyethylene glycol (PEG) and kept on ice overnight. The precipitated plasmid was pelleted at 30,000 g at 4°C for 20 minutes, washed and resuspended then reprecipitated with ethanol.

**Maxiprep - CsCl gradient purification.** A 1 litre culture was grown to saturation overnight in a 2.5 litre baffled flask. After alkaline lysis as above, using proportionally increased volumes, and propan-2-ol precipitation, the nucleic acids were dissolved in TE and adjusted to 9.0 g. 10.3 g of caesium chloride and 1 ml of ethidium bromide (5 mg/ml) were added and the mixture sealed in an ultracentrifuge tube. After ultracentrifugation for 60 hours at 20°C at 85,000 g the supercoiled lower plasmid band was collected and diluted in 5 ml of sterile water. The DNA was precipitated with ethanol. Excess ethidium bromide was removed by phenol-chloroform extractions and the DNA was precipitated in the presence of sodium acetate once with propan-2-ol and once with ethanol.

**Maxiprep - Qiagen (DNA binding resin purification).** Qiagen-500 columns were used to prepare plasmid DNA for injection into mice. The manufacturer's instructions were followed exactly but the volumes of solutions for alkaline lysis were increased, from the suggested 20 ml per litre of culture to 70 ml per litre. Empirically, it was found that the reduced viscosity obtained increased the final plasmid yield per litre of culture. The plasmids were
Methods

Dissolved in sterile 0.9 % NaCl (154 mM) at 100 μg/100 μl and stored in aliquots at -20°.

Optical Density (OD) measurement for purity of DNA. After final resuspension in 100 μl TE, the ratio of OD (absorbance) at 260/230 nm greater than 2:1 was taken as an indication of low levels of contaminating phenol and the ratio at 260/280 nm greater than 2:1 as an indication of low levels of contaminating proteins.

Preparation of Competent E. coli

400 ml LB containing 2.5 M KCl, 10 mM MgCl₂, 10 mM MgSO₄ in a 2.5 litre baffled flask was inoculated with 1 ml of a fresh overnight culture from a single colony of E. coli JS5 and grown at 37° with shaking at 200 rpm. The OD (absorbance at 600 nm) was monitored to confirm exponential growth. At OD 0.4 the flask was cooled in an ice-water slurry for 15 minutes and the culture was then harvested by centrifugation in pre-chilled sterile 50 ml tubes at 2,000 g at 4° for 15 minutes. The pellet was gently resuspended in 0.4 of the growth volume of Franz I solution at 0° and kept on ice for 15 minutes. The bacteria were pelleted again as above and gently resuspended in 0.02 of the growth volume of Franz II solution at 0° and kept on ice for another 5 minutes. 200 μl aliquots were transferred to pre-chilled 1.5 ml tubes and used immediately or stored at -70°.

Transformation of Competent E. coli

E. coli competent cells were removed from -70° and thawed on ice. 10 μl (= 50 ng DNA) of the ligation and control reactions or 1 μl (≥ 100 ng) of supercoiled plasmid DNA was added to the cells, very gently mixed and kept on ice for 30 minutes. A negative untransformed control (no added DNA) and a positive transformation control (addition of 1 ng of purified DNA) were always included. The cells were heat-shocked at 42° for 2 minutes then chilled on ice for 5 minutes prior to incubation at 37° with 800 μl LB for 45 minutes with shaking to
allow expression of antibiotic resistance gene(s). 100 µl of cells were spread on an LB agar plate containing appropriate antibiotic, the remaining 900 µl of cells gently pelleted and spread on a second plate. Plates were incubated at 37° overnight. Colonies on positive and control plates were counted to determine the level of background transformation caused by (i) contaminating (non-transformed) antibiotic resistant cells, (ii) cells transformed with uncut vector (native plasmid only) and (iii) cells transformed with self-ligated vector (no inserted fragment). Levels of background were used to estimate the number of colonies to be screened for the presence of the desired plasmid containing the cloned fragment.

Colour selection of transformed E. coli  Wherever possible, colour selection of transformed colonies was used. pBluescript contains the gene for the α fragment of β-galactosidase (β-gal) with the multicloning site positioned within it. In host strains of E.coli which lack the gene for this α fragment “α complementation” can yield a functional β-gal following expression of the α fragment from pBluescript. The colonies are stained blue if the growth plate contains IPTG (an inducer for β-gal) and X-gal (the chromogenic substrate). With cloned inserts in pBluescript the α fragment expression is disrupted and the colonies remain 'white'.

Cloning Vector Preparation

10 µg of purified DNA was digested with 10-20 units of the required restriction endonuclease(s) in a volume of 100 µl. If possible, directional cloning vectors were made using both enzymes simultaneously if their buffer requirements were compatible, or sequentially if not. The 5’ phosphate groups were removed by addition of 9 units of calf intestinal alkaline phosphatase (CIP) and 11 µl of CIP buffer to the reaction and continuing the incubation at 37° for a further 30 minutes. CIP was inactivated at 75° for 10 minutes following addition of EGTA to 10 mM. The digested and phosphatased DNA was agarose gel purified.
**Methods**

*Vector for the Bgl II fragment of gp120.* pBluescript SK+ was digested with EcoR V as above. 2 µg of phosphorylated Bgl II linkers were ligated with 0.2 µg of the linearised pBluescript overnight, the ligase was then heat inactivated at 65° for 10 minutes and the DNA digested with 10 units of Bgl II. The linearised vector DNA was purified from Bgl II linker fragments by 1 % TAE LMP agarose gel electrophoresis. Following phenol-chloroform extractions, the plasmid was self ligated and used to transform competent *E. coli* JS5. The resulting plasmid was digested with Bgl II and phosphatased as normal.

*Directional T Vectors.* (Marchuk, et al. 1991) EcoR V linearised pBluescript KS+ was incubated with 10 units of Taq polymerase and 2 mM dTTP at 70° for 2 hours before phenol-chloroform extraction and precipitation. The resulting T vector which contained a single overhanging T on each 3’ end was digested with Sac I followed by phenol-chloroform extraction and ethanol precipitation, to generate a directional vector for the PCR product. A similar Kpn I directional T vector was also made.

**Ligations**

A 5-10 molar excess of fragment was mixed with 100 ng linearised vector and 5x reaction buffer (supplied by the manufacturer) in 25 µl volume with 2 units of T4 DNA ligase. The reactants were usually incubated at 16° overnight. For blunt ended ligation, the ligase was increased to 10 units, 4% PEG was added and the incubation temperature reduced to 4°. Control reactions were always included (i) without fragment and (ii) without fragment and without ligase, to aid in interpretation of the results of the subsequent transformation. Occasionally ligations were made using DNA in melted LMP agarose.

**Phosphorylation of oligonucleotides**

Phosphate groups were transferred from ATP to the 5’end of oligonucleotides by T4 Polynucleotide Kinase in the absence of ammonium ions.
Methods

40 pmoles oligonucleotide in 70 mM Tris pH 7.6, 10 mM MgCl₂ and 5 mM DTT with 1 mM ATP were incubated with 5 units T4 Polynucleotide Kinase, in a 25 µl volume at 37° for 30 minutes. The kinase was then heat inactivated at 70° for 10 minutes.

**Preparation of single stranded DNA by phagemid rescue**

100 ml of LB in a 2.5 litre baffled flask was inoculated with enough of a fresh overnight LB Ampicillin culture of *E. coli* containing the desired plasmid to give an OD₆₀₀ of approximately 0.5. R408 phages were added at a multiplicity of infection of 100:1 and shaken at 200 rpm for 4 hours at 37°. The cells were pelleted by centrifugation at 12,500 g at 4° for 20 minutes and the supernatant mixed well with 1/10 volume of autoclaved 20% PEG (6000)/2.5M NaCl. The mixture was kept on ice overnight, then centrifuged at 30,000 g at 4° for 30 minutes. The discrete phage pellets were resuspended in TE in 1/100 of the culture volume and microfuged twice at 15,000 g at RT for 10 minutes. The phage coat proteins were stripped by four phenol-chloroform extractions and the single stranded DNA (ssDNA) precipitated with ethanol at -20° overnight. The DNA was pelleted at 10,000 g at 4° for 20 minutes and washed with prechilled 70% ethanol. The ssDNA was dissolved in 50 µl TE and the concentration and purity were estimated by agarose gel electrophoresis with ethidium bromide.

**T 7 Gen site-directed mutagenesis**

The T 7 Gen kit employs oligonucleotide directed site specific mutagenesis with modifications to increase the yield of the mutated plasmid relative to the 'parental' plasmid in order to reduce the extent of colony screening required. Oligonucleotides coding for the mutant sequence plus flanking regions were annealed to parental ssDNA. The complementary strand was synthesised by DNA polymerase, using methylated nucleotides and self ligated by T4 DNA ligase. The double stranded DNA was digested with a methylation-sensitive
restriction endonucleases (Msp I, Hha I or Hae III depending on the sequence of the oligonucleotide) to nick the non-methylated parental strand only which was then removed with exonuclease III. Competent *E. coli* SDM which are permissive for methylated DNA were then transformed with the methylated ssDNA containing the specific mutation (for diagram, see Figure 4.5). The colonies were then screened for the presence of the mutated plasmid.

**Screening of colonies with labelled oligonucleotide probes**

Following the method of Grunstein and Hogness (Grunstein and Hogness 1975), colonies were replicated with sterile toothpicks to a master plate, and to nylon membrane (Hibond N) marked with a 98 square grid on a second plate and incubated at 37° overnight. The colonies growing on the membranes were lysed with 0.5 M NaOH/1.5 M NaCl for 5 minutes, neutralised with 1 M Tris pH 7.5 for 5 minutes and washed by immersion in 100 ml of 0.5 M Tris pH 7.5/1.5 M NaCl with shaking for 5 minutes. Excess bacterial debris was removed from the membrane with 2 x SSC/0.1 % SDS. The DNA was covalently linked to the nylon membranes soaked in 10 x SSC by 1200 kJ UV exposure (Stratalinker) then rinsed with 2 x SSC. The membranes were prehybridised with 50 μg/ml boiled herring sperm DNA in 15 ml of preheated prehybridising solution for 90 minutes. The oligonucleotide probe was phosphorylated with 40 mCi ^{32}PγATP in 25 μl phosphorylation reaction volume as described above and then diluted to 500 μl, added directly to the prehybridised membranes and hybridised for 2 hours at 42°. The membranes were washed exhaustively with 50 ml of preheated 2 x SSC/0.1 % SDS for 15 minutes each time until the wash solution reached background levels of radiation. The washing temperature was 2-5 degrees below the calculated probe melting temperature. The membranes were blotted dry and orientation marks were made using ink mixed with ^{35}S ATP before being covered with cling film.
and exposed to X-Ray film (Kodak X-OMAT AR) at -70° overnight. Positive spots were used to identify the colonies on the master plate.

**PCR**

The Polymerase Chain Reaction (PCR) was used to construct mutants C, D, E, 3 and 4 using the following conditions: 1 ng template DNA; 1 μM primers; 200 μM dNTPs; 2.5 units Taq Polymerase; 2 mM MgCl₂; covered with mineral oil and 30 cycles of: 94° 1 minute, 55° 1 minute, 72° 2 minutes. For subcloning mutant V3 loops into pKfdH: The same component concentrations and 35 cycles of: 94° 1 minute, 65° 1 minute, 72° 1 minute.

**Screening of colonies by PCR**

Colonies were transferred with sterile toothpicks to a master plate. Broadly following the method of Gussow and Clackson (Gussow and Clackson 1989) the remaining cells on the toothpick were suspended in 50 μl of sterile water and boiled for 7 minutes. Bacterial debris was pelleted at 15,000 g for 3 minutes. 5 μl of the supernatant was used as template in a PCR with the following components: 0.5 μM primers; 200 μM dNTPs; 0.5 units Taq Polymerase, 1.5 mM MgCl₂; covered with oil and 30 cycles of 94° 15 seconds, 50° 30 seconds, 72° 30 seconds. 10 μl of the PCR product were electrophoresed in a 2 % agarose gel with 1 μg ØX174HaeIII molecular size markers.

**Sequencing**

Following the chain-termination method of Sanger (Sanger, et al. 1977), at least 5 μg of double stranded miniprep DNA was denatured with 2M NaOH and heated to 65° for 5 minutes. After neutralisation with 3M sodium acetate, 2.5 pmoles of primer were added and the DNA mixture precipitated with ethanol at -70°. All subsequent steps were performed according to the Sequenase Version 2.0 protocol (USB) using the dGTP labelling mix diluted 1:5, the
Sequenase diluted 1:8 and 0.5 µl (0.19 MBq) of $^{35}$S αATP. Both labelling (at RmT) and termination (at 37°) reactions were allowed to proceed for 5 minutes. 6 % polyacrylamide gels were cast in 33 x 42 cm glass plates with 0.4 mm spacers. The gels were pre-run overnight at 300 V. Sequence reaction products were heated to 75° for 2 minutes and 3 µl aliquots were loaded on the gel. The DNA was electrophoresed at a constant 1,500 V for various durations depending on the primer position relative to the sequence to be read. Gels were fixed for 15 minutes, dried for 15 minutes at 75° with vacuum and exposed to X-ray film at RmT overnight.

**Immunisations**

**Housing and care of animals**

Mice were individually identified with ear marks and housed 5 to a cage under the care of Mr. P. Levy and all procedures were carried out according to his instructions and Home Office regulations. Mr. P Levy and myself performed the protein plus adjuvant immunisations and bleeds. The nucleic acid immunisations and bleeds were performed by Dr. B. de Souza. At the end of the experiment mice were killed by a schedule 1 method and exsanguinated by cardiac puncture by myself. Clotted blood was centrifuged at 15,000 g for 5 minutes and all the liquid collected. This was recentrifuged at 15,000 g for 5 minutes and the serum removed and stored in aliquots at -20°.

**Preparation of adjuvants**

All adjuvants were mixed with gp120 according to the manufacturers’ instructions or published methods to reach a final dose per mouse of 1 µg gp120 in 100 µl volume. **Freund’s** Freund’s complete (for the first immunisation only) or incomplete adjuvant was emulsified with an equal volume of gp120 in phosphate buffered saline (PBS). **TitreMax** Hunter’s TitreMax was emulsified
with an equal volume of gp120 in PBS. **AF** 1 volume of triple strength AF-A was mixed with 2 volumes of gp120 in PBS and vortexed for 1 minute. **Ribi R-700**

The anhydrous preparation was vortexed in the manufacturers’ container with gp120 in PBS. **Quil A** 1 mg/ml in PBS was mixed with an equal volume of gp120 in PBS. (Each dose contained 50 µg Quil A). **Alum** gp120 was dialysed against 0.9 % NaCl (154 mM) to remove phosphate ions and precipitated with aluminium potassium sulphate (AlKSO₄.12H₂O). To gp120 in solution, 0.45 volumes of fresh 1 M NaHCO₃ were added slowly then 1 volume of 10 % AlKSO₄.12H₂O added dropwise, with stirring. The precipitate was both washed and resuspended in 154 mM NaCl at 1 µg/100µl.

**Protein immunisation**

Two F1 crosses of male mice CBA/Ca x BALB/c [k x d haplotype] and BALB/c x C57/BL6 [d x b haplotype] were immunised according to the schedule used by Bomford et al (1992). Mice were anaesthetised with halothane and immunisations were made subcutaneously at two sites. Serum samples were stored at -20° and subsequently to thawing at 4° with 0.01 % sodium azide.

<table>
<thead>
<tr>
<th>Immunisation</th>
<th>Serum sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preimmune</td>
<td>Day 0</td>
</tr>
<tr>
<td>First</td>
<td>Day 1</td>
</tr>
<tr>
<td>Second</td>
<td>Day 29</td>
</tr>
<tr>
<td>Third</td>
<td>Day 90</td>
</tr>
</tbody>
</table>

**Nucleic Acid immunisation - first experiment**

Qiagen purified supercoiled plasmid DNA was dissolved at 100 µg/100 µl in 0.9% NaCl. 8-week old female CBA/Ca mice were injected with the plasmid pEE14tPAGp120.3 (n=15) or with the control plasmid pCDM8 (n=3). The fur was shaved above the right gastrocnemius and the injection site marked. The facilitator bupivacaine (marcaine) (100 µg) was injected the day preceding the
first two DNA injections. DNA was injected at 2-week intervals and 50 µl heparinised blood samples were taken as shown in the table below. Groups of mice (n=3) were killed at 1 month intervals after the last DNA injection. The plasma was frozen at -20° until the start of the analysis and subsequently to thawing at 4° with 0.01 % sodium azide.

<table>
<thead>
<tr>
<th>Immunisation</th>
<th>Serum sample</th>
</tr>
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<tbody>
<tr>
<td>Preimmune</td>
<td>Day 0</td>
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<tr>
<td>First</td>
<td>Day 1</td>
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<td>Day 14</td>
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<td>Second</td>
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<td>Day 28</td>
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<td>Third</td>
<td>Day 29</td>
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<td>Day 42</td>
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<tr>
<td>Fourth</td>
<td>Day 43</td>
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<td>Day 56</td>
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<td></td>
<td>Monthly intervals after day 56</td>
</tr>
</tbody>
</table>

**Nucleic Acid immunisation - subsequent experiments**

The conditions were kept as close as possible to those of the first experiment which had produced positive results. The wild type gp120 gene and all mutant genes were in plasmid pEE6HCMVgp120GS. The control plasmid was pEE14 (similar to pEE6HCMVgp120GS but without the gp120 gene). An extra group was included of 8 female CBA/Ca immunised with wildtype gp120 but pretreated with a single 6.8 µg injection of cardiotoxin one week before the first DNA injection to compare cardiotoxin's facilitatory action against that of marcaine. For all other groups the dose of marcaine was reduced from 100 µg to 25 µg because in the first experiment several mice died due to severe marcaine-induced fits. The protocol of injections and bleeds was the same as the first experiment. Heparin was avoided because by binding to the V3 loop of gp120 (Javaherian, et al. 1994) it might have interfered with subsequent assays.
**Methods**

**Tissue culture**

**CHO cell transfection**

Mycoplasma free chinese hamster ovary (CHO) cells were grown in GMEM-S medium supplemented with L-Glutamine, Penicilllin and Streptomycin. Cells were maintained in exponential growth by splitting every 2-3 days. On the day before transfection, three 80 cm² flasks were seeded with $1.3 \times 10^6$ cells/flask in GMEM-S without glutamine. The cells were transfected with 30 µg of plasmid DNA per flask using calcium phosphate coprecipitation. After removal of the precipitate, the cells were incubated overnight in GMEM-S without glutamine. The day after transfection, the medium was replaced with fresh GMEM-S containing 25 µM methionine sulphoximine (MSX). The cells were incubated for 7-10 days until discrete foci appeared. The medium and MSX were replaced once during this period.

**Gene Amplification**

The glutamine synthetase gene copy number of individual foci was amplified by increasing the MSX concentration. Foci resistant to 25 µM MSX were picked individually into 25 cm² flasks or 24 well plates, expanded to approximately 80 % confluence and incubated with 100 µM-250 µM MSX. GS gene amplified foci (see Chapter 4) were grown individually at this increased MSX concentration then tested by ELISA for gp120 production (Wild Type, 4E and 4C cell lines), or allowed to become confluent then tested by ELISA for the population gp120 production (4A and control cell lines). Chosen cell lines were expanded to 80 % confluence in 80 cm² flasks then stored in liquid nitrogen.

**Cell storage**

Cells were released from the flask with Ca²⁺ and Mg²⁺ free 0.02 % EDTA solution and washed once with PBS. They were resuspended in FCS with 10 %
dimethylsulphoxide and aliquoted into cryotubes. The tubes were placed at -20° for one hour then transferred to -70° for one hour, and finally placed in liquid nitrogen.

**Analysis of proteins and immune responses**

**Freeze drying and reconstitution of serum free CHO supernatants**

The harvested supernatants were centrifuged at 2,000 g for 20 minutes, pipetted into a new tube and shell frozen in dry ice/ethanol. The water was evaporated overnight at -40° and reduced pressure of 10⁻¹ mbar. The remaining powder was redissolved in 1/20 volume of PBS.

**Denaturation of gp120**

gp120 was denatured as described by Moore and Ho (Moore and Ho 1993). 10 μg/ml baculovirus rgp120 (ADP 607) was boiled in PBS containing 10 % FCS, 1 % SDS, 50 mM DTT for 5 minutes and then diluted into 9 volumes of PBS containing 10 % FCS, 1 % Nonidet P40 to bring the final gp120 concentration to 1 μg/ml.

**Reducing SDS PAGE of gp120**

7.5% polyacrylamide resolving gels and 5% polyacrylamide stacking gels were made. Samples and high molecular weight size markers were mixed with 2 x SDS PAGE loading buffer containing DTT and immediately boiled for 5 minutes. Empty wells were filled with an equal volume of 1 x loading buffer. The gel was run at constant 100 V until the bromophenol blue had run out of the bottom of the resolving gel.
**Western Blotting**

The gels were equilibrated in transfer solution for 15 minutes and proteins electrophoretically transferred to nitrocellulose membranes (Hibond C extra) overnight at a constant 30 V or for 3 hours at a constant 70 V with cooling. The proteins were temporarily stained with ponceau S and the molecular size marker lane removed for reference and permanently stained with amido black. The membranes were blocked in 100 ml of 4 % Marvel in tris buffered saline with 0.1 % Tween 20 (TBS-T) for 1 hour with shaking. Antibodies were diluted in blocking solution and incubated with the membranes in sealed bags with shaking for 1 hour. 4 washes were made in 100 ml TBS-T for 5 minutes each time, with an extra 2 washes before the final detection step using an enhanced chemiluminescence (ECL) protocol (Figure 2.1).

**ELISAS**

All samples were assayed in duplicate using 100 µl volumes and Maxisorp C immunoassay plates (Figure 2.2). gp120 was captured using Sheep anti-gp120 (D7324). This antibody is raised to a peptide from the extreme C-terminus of gp120 (APTKAKRRVQRREKR). The plates were coated overnight at 4°, with D7324 or peptides, diluted to 3 µg/ml in bicarbonate/carbonate buffer at pH 9.6 (B/C). Blocking was with 10 % FCS in PBS for at least 30 minutes at RmT. When the rabbit gp120 antiserum was used, 4 % Marvel in PBS was used in place of FCS. Three washes were made between incubations using PBS with 0.05 % Tween 20 (PBS-T). Antigen and antibodies were diluted in PBS-T containing 1 % BSA (PBS-T-B). In assays with denatured gp120, the antibodies were diluted in PBS with 1 % BSA and 0.5 % Tween 20 (a 10 X increase in Tween 20) as described by Moore and Ho (1993). In assays of gp120 in CHO supernatants, the standard gp120 used for reference was baculovirus recombinant gp120 (ADP 607 (Lot MY27R)) diluted in medium harvested from confluent untransfected CHO cells. Incubations were at 37° for 2-4 hours or overnight at 4°. The plates were
covered with cling film and then damp tissues during all incubations. The substrate for alkaline phosphatase was *para*-nitrophenylphosphate at 1 mg/ml in B/C with 2 mM MgCl₂. The alkaline phosphatase reaction was not stopped. The OD₄₁₀ was read at intervals until a set of readings was obtained where a reasonable signal had been recorded (approximately 1.0 or above) and the negative control wells were still in the range 0.03 - 0.08.

**Statistics**

Statistical analysis was made using Minitab software. A one-way analysis of variance was used to compare the mean of two or more groups. Values of F and p are given in the text. In certain cases only non-parametric tests were possible and values of χ² and p are given.
Figure 2.1
Diagramatic representation of Western blot used for detection of gp120

Figure 2.2
Diagramatic representation of various ELISA formats used for detection of gp120 or for detection of gp120-specific antibodies.
CHAPTER 3

IMMUNOGENICITY OF WILDLTYPE gp120 IN THE MOUSE

Introduction

The characteristics of an immune response may be tailored towards the stimulating pathogen. However many pathogens, HIV-1 being a quintessential example, can survive by manipulating the immune response into ineffectiveness and in the case of HIV-1 this usually causes death of the host. In HIV-1 infection one of the major targets of the immune response is gp120 and as described in Chapter 1, the response to the V3 loop may dominate. Some qualities of an humoral response may be indicative of the character of the total immune response induced. The IgG subclass may reflect the type of cytokines which have dominated and the epitope specificity may reflect the potency of the B cell epitopes of the immunogen. In a vaccine formulation it may be possible to control the quality of the induced response by the choice of adjuvant, the dose of immunogen, and perhaps by the mode (protein plus adjuvant or nucleic acid) or route of immunisation.

Adjuvants stimulate general or specific immune mechanisms and are often required to augment the response to subunit protein or peptide antigens. During adjuvant activity, one or more mechanisms may operate which affect the presentation of the antigen, the duration of this presentation, the stimulation of cytokine expression or other possible mechanisms and which result in increased stimulation to the immune system. However, as well as their adjuvant activity,
many compounds can be too toxic, non-metabolisable, unstable or potentially pathological. At present, for use in vaccine formulations, only aluminium compounds are generally regarded as safe for humans but modified molecules retaining adjuvanticity but reduced in toxicity are being investigated.


Both bupivacaine (marcaine) (Wang, et al. 1993b) and cardiotoxin (Davis, et al. 1993a) can be used as facilitators for the uptake of the DNA to cause local necrosis at the injection site, since regenerating muscle (differentiating myoblasts and myotubes) has been found to take up the DNA with greater efficiency that mature skeletal muscle fibres. Hypertonic sucrose has also been used (Davis, et al. 1994: Davis, et al. 1993c) with good effect as a facilitator but the mechanism for this is not clear. The mechanisms involved in DNA uptake, protein expression and the processing and presentation of the antigen are similarly
unresolved, but it is known that myoblasts and myotubes are able to express MHC class I and II, ICAM-1 and LFA-3 following stimulation by IFNγ and TNFα and can function as antigen presenting cells (Hohlfeld and Engel 1994).

Before assessing the immune response to gp120 carrying mutated V3 loops, it was necessary to characterise the murine response to wildtype gp120 to see if strong V3 responses were detectable and to chose the method of immunisation for the subsequent experiments. Owing to the robust finding that mice are among those animals which respond to NAI, this mode of immunisation was compared with protein plus adjuvant in experiments to find that mode of immunisation most appropriate for the experiments with mutant gp120. Two cohorts of mice were immunised with protein and adjuvant and another two cohorts with nucleic acid. Assays were made of the kinetics of the development of the response, the IgG endpoint titre, subclasses of IgG and reactivity with denatured antigen. The reactivity to the CD4 binding site, V3 and some other individual epitopes was also measured.
Results

Response to whole gp120

Protein immunisation and IgG endpoint titre

In these experiments a selection of experimental adjuvants have been used with different properties. Freund's adjuvant was chosen as a reference adjuvant and TitreMax because it is similar to Freund's but includes surfactants. AF was included as it has been reported to induce strong cellular as well as humoral responses (Raychaudhuri, et al. 1992). Based on the results from the first cohort, AF was included in the second cohort as a reference, Ribi as an adjuvant with bacterial components and Quil A and Alum so as to be able to compare our results with those of Bomford (Bomford, et al. 1992) and others. The components in each of these adjuvants are listed in Chapter 2. Two F1 crosses of mice were used to increase the number of haplotypes investigated. The k x d haplotype was strain CBA/Ca x BALB/c and the d x b haplotype was strain BALB/c x C57BL/6. The immunisation schedule was that used by Bomford et al (Bomford, et al. 1992) who investigated the effect of adjuvant on the isotype of antibody produced in mice.

Ribi and Freund's were the only adjuvants which demonstrated a haplotype dependence. Ribi being apparently inefficient at inducing an IgG response in k x d mice and Freund's in d x b (Figure 3.1) With Ribi as adjuvant (Figure 3.2) the IgG endpoint titre was significantly reduced (F=14.84, p=0.005) compared with d x b haplotype in this small sample of 5. AF was used in both cohorts of mice and produced high titres of IgG in both groups. Of the other adjuvants, only Quil A was similarly effective. In all adjuvants other than Ribi, there were no statistically significant differences in the measured IgG titre between haplotypes k x d and d x b.
IgG response to native gp120 in Protein immunised F1 mice

Figure 3.1
Tertiary response serum 1:1000 IgG reactivity with baculovirus recombinant gp120 in F1 mice immunised with protein in different adjuvants. Sera in A and B were assayed in separate experiments. A: First cohort B: Second cohort. Bars represent individual mice.
Figure 3.2
Log 10 endpoint dilution of gp120-specific IgG reactivity with baculovirus recombinant gp120. The endpoint was taken to be that serum dilution which gave a signal equal to the mean + 2 S.D. of a cohort (n=9) of unimmunised control mice.
The IgG response after the third protein immunisation was clearly greater than after the second as measured for the AF adjuvant (Figure 3.3), but it is not clear from the data whether the maximum achievable response had been attained at this point.

**Nucleic acid immunisation and IgG endpoint titres**

In two separate experiments, k haplotype mice and the same two F1 hybrids (k x d and d x b) were immunised with the wildtype gp120 gene. The serum from nucleic acid immunised mice was assayed at 1:100 (10 X more concentrated than sera from protein immunised mice) with an anti-IgG,A,M conjugate to determine if any antibody response had occurred Figure 3.4 (top). The assay was repeated with an IgG specific conjugate. These assays were performed on subsequent days and are therefore only generally and not directly comparable. Figure 3.4 (top and bottom) indicates that the humoral response may be substantially IgG in nature. Following this finding in k haplotype mice, the sera from the nucleic acid immunised F1 mice was similarly assayed at 1:100 but only the anti-IgG conjugate.

As with protein immunisation, in these nucleic acid immunised mice there were similarly no differences observed in IgG endpoint titre (Figure 3.2) between the three different haplotypes, however protein immunisation resulted in a 10 times higher total IgG endpoint titre than nucleic acid immunisation (F=12.67, p=0.001). This is despite the fact that the low responding Ribi k x d mice are included in the protein group for statistical analysis.

The k x d haplotype nucleic acid immunised mice show an earlier development of the IgG response than d x b or the k haplotypes (Figures 3.4 and 3.5). Figure 3.4 also shows that in k haplotype mice immunised with nucleic acid, the level of antigen specific IgG stabilises at or slightly below the maximum measured level and shows no tendency to decline over 5 months.
IgG response kinetics with AF adjuvant

Figure 3.3
Preimmune, primary, secondary and tertiary response serum 1:1000 IgG reactivity with baculovirus recombinant gp120 in mice immunised with rgp120 and AF adjuvant (1st cohort).
IgG response kinetics to wild type recombinant gp120 in Nucleic acid immunised k haplotype mice

Figure 3.4
Serum 1:100 reactivity with baculovirus recombinant gp120. k haplotype mice immunised with wild type gp120 plasmid (n=14) or control plasmid (n=1). Groups of 3 mice were killed at monthly intervals after the fourth plasmid injection.
IgG response kinetics to wild type recombinant gp120 in Nucleic acid immunised F1 mice

**Figure 3.5**
Serum 1:100 IgG reactivity with baculovirus recombinant gp120. F1 haplotype mice were immunised with wild type gp120 plasmid (n = 10 each haplotype) or control plasmid (n = 10 each haplotype). Results are from a single assay.
Effect of different facilitators and signal sequences.

For nucleic acid immunised mice, a comparison of the effectiveness of two different facilitators was also made. Two groups of mice were immunised with wildtype gp120 DNA to test cardiotoxin against marcarene as the facilitator (Figure 3.6). There was no significant difference (F=0.36  p=0.52) in the endpoint titre of total gp120-specific IgG and the kinetics of the response were likewise not significantly different. There were two groups of k haplotype mice both immunised with the wildtype gp120 gene but utilising different signal sequences. The tPA signal sequence is from tissue plasminogen activator and has been found to be more efficient than the gp120 signal sequence for expression of recombinant gp120 (P. Stephens, personal communication to T. Lund). However, in these experiments there was no significant difference in endpoint titre of the IgG induced by DNA with these two signal sequences (Figure 3.7).

Variability is intrinsic

Consistently throughout the immunisations described here, there is great variability between individuals within groups. AF produced consistency of response in k x d haplotype in the 1st cohort (see Figure 3.1) and the shape of the titration curves (Figure 3.8) confirm this impression. However, this was not repeated in the second cohort with AF and the variability was especially noticeable in the titration curves. The combined results from all cohorts of mice demonstrate that this variability is intrinsic. It is independent of mode of immunisation; within protein immunised mice it is independent of haplotype and adjuvant; and within nucleic acid immunised mice, it is independent of both facilitator and signal sequence.
A comparison between the effects of different facilitators on the kinetics of IgG response and IgG endpoint titre.

**Kinetics of IgG response**

![Graph showing kinetics of IgG response for Marcaine and Cardiotoxin.]

**IgG endpoint titre**

![Graph showing IgG endpoint titre for Marcaine and Cardiotoxin.]

**Figure 3.6**
Endpoint titre of gp120-specific IgG from mice immunised with gp120 DNA. Both cohorts of mice were immunised with the gp120 gene utilising the gp120 signal sequence, but with two different facilitators, Marcaine and Cardiotoxin.
A comparison between the effects of different signal sequences on IgG endpoint titre.

Figure 3.7
Endpoint titre of gp120-specific IgG from mice immunised with gp120 DNA. Both cohorts of mice were immunised using marcarine as the facilitator but with gp120 genes utilising different signal sequences. The tPA signal sequence is from tissue plasminogen activator.
Figure 3.8
Individual mouse serum IgG (dilutions, from left to right in each block, 1:1,000 1:3,000 1:9,000 1:27,000 1:81,000) reactivity with baculovirus recombinant gp120 as an example of variability. The curves were continued in a second titration to find the endpoints.
Subclasses of IgG

All four subclasses of IgG were assayed in an initial experiment using the individuals from each group with the highest overall IgG. Antibodies of single isotypes IgG1, IgG2a, IgG2b and IgG3 were used as controls for each alkaline phosphatase conjugate. No IgG2b or IgG3 were detected at all in this experiment (n = 7, results not shown). In each of the 3 subsequent assays (Figures 3.9 and 3.10) only those mice whose IgG reactivity was above O.D. 0.4 at 1:100 (or 1:1000 for protein immunised mice) were included. The single isotype controls were included on all plates and were maximally reactive with the relevant conjugate and gave no signal with the other conjugate (not shown on the figures). Protein and nucleic acid immunised sera were distributed randomly between the plates. The main IgG subclass induced by both protein and nucleic acid immunisation was IgG1. The only relatively strong IgG2a reactivity detected was in mice immunised with protein in Quil A adjuvant.

Denatured gp120

All the available gp120 specific rat monoclonal antibodies (rat Mabs) from Dr. J. McKeating (Figure 3.11) were tested for reactivity with native and denatured gp120 (Figure 3.12). The gp120 was denatured according to Moore and Ho (Moore and Ho 1993). The rat Mabs were tested for reactivity with native and denatured thyroglobulin as an irrelevant antigen and were unreactive with either form (results not shown). Serum from all mice was assayed on both native and denatured gp120. Rat Mabs A1, B1, C1, and G1 against both mapped linear and conformation sensitive epitopes of gp120 (Figure 3.11) were used on every plate to confirm that the gp120 had been successfully denatured and subsequently captured by D7324.
IgG1 and IgG2a subclasses of gp120-specific serum antibody in Protein immunised mice.

Figure 3.9
gp120-specific IgG1 and IgG2a. Serum reactivity with baculovirus recombinant gp120 at two serum dilutions (1:300 and 1:900). IgG1 and IgG2a were measured in the same ELISA with control single isotypes for each conjugate. Reactivity with the relevant isotype control was maximal and cross reactivity with the irrelevant isotype was zero on all plates (not shown).
IgG1 and IgG2a subclasses of gp120-specific serum antibody in Nucleic acid immunised mice.

**Figure 3.10**

gp120-specific IgG1 and IgG2a as in Figure 3.9
### Rat monoclonal antibodies to gp120

<table>
<thead>
<tr>
<th>Code</th>
<th>Region/Peptide</th>
<th>Isotype</th>
<th>Full identity</th>
</tr>
</thead>
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<tr>
<td>A1</td>
<td>C1 aa 102-121</td>
<td>IgG2b</td>
<td>RAID/11/65a/5h</td>
</tr>
<tr>
<td>B1</td>
<td>C5 aa 471-491</td>
<td>IgG2a</td>
<td>RAID/11/41a/2f/4f</td>
</tr>
<tr>
<td>C1</td>
<td>V1/V2 aa 152-181</td>
<td>IgG2a</td>
<td>RAID/11/4c/7/3j</td>
</tr>
<tr>
<td>D1</td>
<td>V3 aa 311-321</td>
<td>IgG2a</td>
<td>RAID/10/36e/2j/5a</td>
</tr>
<tr>
<td>D2</td>
<td>V3 aa 311-321</td>
<td>IgG1</td>
<td>RAID/10/54ow/6i/6i</td>
</tr>
<tr>
<td>D3</td>
<td>V3 aa 311-321</td>
<td>IgG2b</td>
<td>RAID/11/85b/4l/4l</td>
</tr>
<tr>
<td>D4</td>
<td>V3 conformation sensitive</td>
<td>IgG2a</td>
<td>RAID/11/5a/2f/4l</td>
</tr>
<tr>
<td>F1</td>
<td>C4 + aa 427-436</td>
<td>IgG2b</td>
<td>RAID/10/35c/2h/1a</td>
</tr>
<tr>
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<td>RAID/11/16b/11/3b</td>
</tr>
<tr>
<td>G1</td>
<td>conformation sensitive</td>
<td>IgA</td>
<td>RAID/8/19b/3c/4i</td>
</tr>
<tr>
<td>G2</td>
<td>V1/V2 + C4 conformation sensitive</td>
<td>IgG1</td>
<td>RAID/11/68b/2a</td>
</tr>
</tbody>
</table>

**Figure 3.11**
Rat monoclonal antibodies, made by Dr. J. McKeating and C. Shotton, raised to HIV-1 IIIIB rgp120 expressed in CHO cells.
Rat gp120-specific monoclonal antibody reactivity with native and denatured gp120

Figure 3.12
Reactivity of Rat monoclonal antibodies with native and denatured baculovirus gp120. gp120 was denatured then captured in ELISA with Sheep anti-gp120 D7324. Rat monoclonals were used at approximately 80% of their saturating concentration from titration curves on native baculovirus gp120.

A1, B1, C1 ..... Identity of Rat monoclonal given in Figure 3.11.
* Used as controls in subsequent ELISAs with denatured gp120.
In Figure 3.13 for protein immunisation, the data from a single ELISA where both native and denatured gp120 were captured on the same plate are shown. In Figure 3.14 for nucleic acid immunisation, serum reactivity with denatured gp120 was assayed and the results of the assays with native gp120 is presented from Figures 3.4 and 3.5 for comparison. There is minimal serum IgG reactivity with denatured gp120 in all individuals and in no case is the reactivity with denatured antigen greater than that with the native gp120.

The process of denaturation of gp120 by boiling in the presence of other proteins (FCS), SDS and a reducing agent has severely disrupted recognition of gp120 by the murine antisera. It appears that there may be a slight difference in reactivity with denatured gp120 between d x b and k x d haplotype mice. This is indicated both in protein immunisation and in nucleic acid immunisation (Figures 3.13 and 3.14), but the numbers and the differences are too small for any general conclusions to be made about haplotype dependence.

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**Responses to individual epitopes of gp120**

**Conformational epitopes**

*CD4 binding site (CD4bs).*

Several attempts to measure the CD4bs specific response were made using a competition assay between mouse serum and soluble CD4. An example of the results is given in Figure 3.15 which shows that there is some competitive effect, and therefore a measurable response to the CD4bs in the mouse. This assay gave conflicting results in several repeats - and no competition was observed with sera from the first AF cohort. During the time available it was unfortunately not
Comparison of serum reactivity with native and denatured gp120 in Protein immunised mice

Figure 3.13
Reactivity of serum 1:1000 IgG from Protein immunised mice with native and denatured baculovirus recombinant gp120. gp120 was first denatured then captured in ELISA with Sheep anti-gp120 D7324. Rat monoclonal antibodies A1, B1, C1, and G1 were used as controls on each plate to confirm successful denaturation and capture of the gp120. The pattern and degree of reactivity of these antibodies were consistently the same as in Figure 3.12. Only monoclonals B1 and G1 are shown. Native and denatured data in this figure are from assays carried out at the same time.
Comparison of serum reactivity with native and denatured gp120 in Nucleic acid immunised mice

Figure 3.14
Reactivity of serum 1:100 IgG from Nucleic acid immunised mice with native and denatured gp120. Native and denatured data are from separate ELISAs. Rat monoclonal control antibodies were used during the denatured gp120 assay as described for the Protein immunised mice (Figure 3.13).
Competition assay to detect mouse response to the CD4 binding site

Figure 3.15
Competitive effect of individual mouse sera (dilutions, left to right: 1:100 1:500 1:2,500 1:12,500 1:62,500 1:3,125,000) on the binding of recombinant soluble CD4 to captured gp120. Diluted mouse serum was incubated with captured gp120 for half an hour before the soluble CD4 was added and incubated overnight. Bound CD4 was detected the next day.
possible to optimise the assay, but comparing Figures 3.3 and 3.14 it appears that, within individuals, the response induced to the CD4bs as measured by percent maximum inhibition may be roughly proportional to the total IgG response as measured by IgG endpoint titre (correlation \( r = 0.741 \)).

**V3 loop**

Many of these assays were performed by Bruno Ramos under my direction. In an attempt to measure the response to native epitopes a competition was made between the immune mouse sera and rat monoclonal antibodies (rat Mabs) to V1/2 and V3. The results in Figure 3.16 show that the mouse serum could effectively inhibit rat Mab C1 which is specific for the V1/2 region of gp120 indicating a measurable V1/2 region response. However, the mouse serum was unable to compete with rat Mab D2, specific to V3. Figure 3.17 shows the results of an investigation into the V3 epitopes recognised by a mouse antiserum and rat Mab D2. The mouse antiserum recognises the peptide after this peptide had been captured by the rat Mab. This demonstrates that the two antibodies recognise different epitopes and do not sterically interfere with each other for binding to the V3 peptide. Competition between mouse sera and the available rat Mabs to V3 was therefore an inappropriate method of measuring the mouse anti-V3 response.

**Peptide epitopes**

Peptides were obtained which included mapped linear epitopes within V3, C1, C4, C5 and the extreme C-terminus of gp120 for which individual rat Mabs could be used as positive controls (their positions are shown on Figure 3.18). The sheep capture antibody D7324 was used as a positive control for the extreme C terminus peptide. Before making IgG endpoint titrations, all the sera was tested first at 1:100. The reactivity at this concentration was either
Competition between rat monoclonal antibodies and mouse serum for binding to gp120

Figure 3.16
A: gp120 at 100 ng/ml  B: gp120 at 25 ng/ml  Mouse serum (dilutions, left to right: 1:400 1:800 1:1,600 1:3,200 1:6,400) was incubated in liquid phase with gp120 overnight, then Rat monoclonal antibody added for 1 hour. The complex was then captured and detected with anti-rat conjugate. Monoclonal C1 is directed to the V1/V2 region of gp120; D2 is directed to V3.
V3 peptide epitope analysis for Rat monoclonal D2 and Mouse serum 1.13

Figure 3.17
The long V3 peptide (37-mer) was coated directly at 3 μg/ml (A) or captured at 10 μg/ml with Rat monoclonal antibody to V3 (B). Mouse sera (dilutions, left to right: 1:250 1:500 1:1,000) and Mouse monoclonal antibody to V3 (dilutions, left to right: 1:20,000 1:40,000 1:80,000) (ADP 3047) were then added to detect the peptide followed by anti-Mouse conjugate.
Figure 3.18
The amino acid sequence of gp120 from IIIB. N linked glycosylation sites mapped epitopes of rat monoclonals (-----) and the amino acid sequences represented by the peptides which showed reactivity with the mouse sera (-----------------------------) are shown. The single amino acid mismatch in the short V3 peptide (S to R) is marked (-----)
clearly positive or clearly negative. There were rat monoclonal antibodies which could act as positive controls for parts of the C5 or C4 regions of gp120 but there was no reactivity to these in any of the sera at 1:100.

**V3 peptides:**

Two V3 peptides were available; a ‘long’ 37-mer (ADP 792.3) spanning the whole of V3 including both cysteines and one extra asparagine, and a ‘short’ 20-mer (ADP 740.28) which spans the middle of the loop including GPGR and sequences either side. This short peptide had one amino acid mismatched with the immunogen. The number of sera containing IgG recognising these V3 peptides was higher than for any other peptides tested. Given the known immunodominance of V3 *in vivo* in many animals the number of reactive murine sera seemed low and several attempts were made to determine if the V3 peptide assay was sub-optimally sensitive but this did not appear to be the case.

**C1 peptides:**

The C1 peptide (ADP740.8) reactivity was a robust finding. Another C1 region peptide (ADP740.7) which overlapped the N-terminal half of the reactive peptide by 10 amino acids was negative in all assays.

**C terminus peptide:**

C-terminal peptide reactivity was strikingly different depending on the mode of immunisation (Figures 3.19 and 3.20) ranking second after V3 reactivity in nucleic acid immunised mice, but showing no reactivity with protein immunised mouse sera.

**V1/V2 peptides:**

The rat Mab to the V1/V2 region was unreactive with the corresponding peptides and none of the murine sera reacted with these peptides.

Figures 3.19 and 3.20 show that there was no pattern of reactivity to different epitopes within individuals except for the long and short V3 peptides. The data show that nucleic acid immunisation induces significantly more peptide
reactive antibodies than protein immunisation. Although there is no difference in peptide IgG endpoint titre between protein and nucleic acid immunisation (F=0.02, p=0.89), bearing in mind that the endpoint IgG reactivity with the whole gp120 molecule is 10 times higher in protein immunised sera than in nucleic acid sera, the relative peptide reactivity of nucleic acid serum is increased over that of protein. There is no difference between d x b and k x d haplotypes but overall, the number of peptide reactive sera from F1 mice is significantly greater than from k haplotype mice ($\chi^2=4.874$, p<0.05). It is possible that this is a manifestation of hybrid vigour in the immune response but these experiments would need to be repeated with increased group sizes and using additional inbred and F1 hybrid strains of mice.
Figure 3.19
Log 10 endpoint dilution of peptide-reactive IgG. The V3 long peptide is a 37-mer, the V3 short, C1 and C terminus peptides are 20-mers. The endpoint criteria were the same as those used for the whole gp120 IgG endpoint (Figure 3.2).
Endpoint dilution of peptide-reactive IgG in Nucleic acid immunised mice

Figure 3.20
Log 10 endpoint dilution of peptide-reactive IgG as in Figure 3.19.
Chapter 3

Discussion

Variability was seen in the antibody responses within individual groups of mice following all immunisations despite the fact that these mice were inbred. However, this degree of variability is commonly observed in inbred strains (B. De Souza, personal communication). For protein immunised mice this variability was not due to variation in proportion of adjuvant to antigen because the doses for all mice were made up in one batch. However, it may have been due to inaccuracy in dose volume, since in some mice a small amount of the dose leaked out through the injection site immediately after the needle was withdrawn. In nucleic acid immunisation, the injection volume is designed to be high in relation to the muscle volume in order to create tension in the tissue and partially separate the muscle fibres. Even though a smaller calibre needle was used for these plasmid injections (which was not possible for the protein in adjuvant injections), the same ‘dose-leakage’ occurred. The IgG titration curves (Figure 3.8) indicate that the contributions of both affinity and concentration to the overall reactivity of the polyclonal serum can also be variable between individuals. The protein immunisation cohort numbers are extremely small (n=5) considering the variability between individuals and since a clear (p=0.005) haplotype difference has been shown for at least one adjuvant (Ribi), it is possible that there may be haplotype differences with other adjuvants. Only two F1 hybrids have been used here and a broader experiment is necessary before any firm conclusions can be made.

The oil-in-water emulsions, AF and Ribi, and the natural mixture of saponins, Quil A, had good adjuvant activity with gp120 compared with the water-in-oil emulsions, Freund’s and TitreMax, and with Alum. This suggests a conformation dependency of many epitopes of gp120 as Hunter et al have recorded that oil-in-water emulsions induce more antibody against such labile epitopes than do water-in-oil emulsions (Hunter et. al. 1994). These results
showing a stronger adjuvant effect of Quil A over Alum are supported by the experiments of Bomford (Bomford, et al. 1992) and Powell (Powell, et al. 1995) who also found poor responses with Freund's as an adjuvant for gp120. Powell used QS-21 a purified component of Quil A. In the experiments presented here TitreMax performed equally poorly as Freund's, but in those of Bennett (Bennett, et al. 1992) TitreMax proved superior to Ribi in immunisations with hen egg albumin and trinitrophenol. It is known that the adjuvant can have strong effect on the immunogenicity of an antigen (Bomford 1980) and this has been recently demonstrated (Stieneker, et al. 1995) using gp120 from HIV-2. The present results indicate that gp120 antigen with TitreMax as adjuvant is a poor combination and suggests a significant adjuvant dependency of the antibody response to gp120.

The reason for the lack of a strong IgG response with Ribi in the k x d mice is unclear. This formulation of Ribi (R-700) includes bacterial components MPL and TDM. Unless there is a capacity to react specifically with MPL or TDM in haplotype b, then since these bacterial components are absent from the other 'effective' formulations (AF and Quil A) they may be irrelevant to the induction of a gp120-specific IgG response. It is unlikely that Ribi R-700 'failed' to induce any response at all. Using Ribi R-700 may have preferentially directed the response towards cell mediated immunity in k x d haplotype.

Following injection of naked DNA, the plasmid is persistent (Wolff, et al. 1992) but it is not known if the antigen is expressed persistently. Although antigen is detectable in the serum at 10 days after DNA injection (Davis, et al. 1993b) antigen levels then fall coinciding with the rise in levels of antigen-specific antibody. It would be important to establish whether the observed stability of serum IgG for 5 months continues for longer periods and if it is due to persistent antigen or not. Protein immunisation differed from NAI in inducing substantially higher titres of specific IgG but the mechanism for this is not clear
at present and a non-optimum immunisation protocol for NAI cannot be excluded.

It is possible that the relatively low dose of antigen (1 µg per immunisation, 3 µg in total) used in the protein immunisation contributed to the higher endpoint titre of the IgG with antibody competition for antigen favouring the evolution of higher affinity antibodies. This might not have occurred during immunisation with nucleic acid if there was a sufficient level of antigen expression. In the experiments presented here the dose of DNA was high compared with some protocols used recently by others as described above and this possibly influenced antigen expression levels.

For the NAI experiments, the control serum was from mice immunised with the control plasmid pEE14 which contained all the elements of the plasmid used to carry gp120 except for the gp120 gene itself. This plasmid was designed for transfection of CHO cells (and had been used for this purpose in these experiments) and was therefore carrying the glutamine synthetase (GS) mini gene. No experiments were made to determine if the GS gene product was expressed in the mouse, or if an immune response to this product was mounted. Although, a priori, there is no reason to expect antigenic competition between gp120 and glutamine synthetase, the possibility cannot be ruled out. If it did occur this may have important implications in these experiments, relating to the comparability of the nucleic acid and protein immunisations. The presence of the GS gene was consistent between experimental and control groups but an even better control for the gp120 expression plasmid used here might have been a plasmid carrying GS together with a gene for an expressible protein which was unrelated to gp120.

Four plasmid injections were given and the level of gp120 specific IgG apparently increased after the second, third and possibly the fourth injections. However, the mechanisms operating during the time between the injection of plasmid and detection of the humoral response are not elucidated. Wang (Wang,
et al. 1993a) showed that two injections of nucleic acid are usually necessary to cause seroconversion in mice and macaques. If substantial time is required for expression and presentation of antigen, then the rise in IgG detected after the second injection might actually be the primary rather than the secondary IgG response.

With respect to facilitators, cardiotoxin was far better tolerated by the mice, causing no deaths and no distress. Marcaine caused the death of two mice from severe fits and caused intense agitation in all of the others. The dose of marcaine may need to be carefully adjusted for each mouse probably based on individual body weight so that muscle-tissue necrotising (facilitating) levels can be attained without systemic toxicity. To avoid the morbidity and potential mortality found with marcaine, cardiotoxin may be the facilitator of choice for mice if larger groups confirm no differences in the immune responses obtained. Control immunisations with nucleic acid without the prior injection of facilitators were not made as part of these experiments and these will be an important control during future analysis of the effect of facilitators.

Assays of individual epitope reactivity using linear peptides are not ideal since the peptide may or may not assume a conformation matching the conformation of the sequence in the native protein which it represents. Moore has shown that solid-phase peptide assays do not reflect the reactivity of the same peptides with antibodies in solution (Moore 1993) and that solid-phase V3 assays can falsely identify the immunising strain of virus (Moore, et al. 1994). Moreover, the assay will be even less likely to detect those antibodies whose binding site is only partially represented by the peptide and this includes those antibodies whose binding sites overlap V3 and regions adjacent to it in native gp120. Likewise V3 specific antibodies whose epitopes are composed partially of carbohydrate may not be detected, as V3 has an N-linked glycosylation site within it and two more just outside. It is entirely possible that carbohydrates attached to asparagines distant from V3 in the primary sequence may lie adjacent
to V3 in folded gp120. However, in view of the failure of more sophisticated methods, assays of individual epitope reactivity were made with peptides.

Although in native gp120, the V3 disulphide bond and parts of the loop near to it are not thought to be exposed, the longer (37-mer) V3 peptide which includes these residues gave increased reactivity compared with the short (20-mer) peptide. It is possible that the incorrect amino acid in the short peptide is at a crucial position in a V3 epitope and that the non-conservative change from serine to arginine has a strong effect on antibody affinity. However it has been shown by NMR that some peptides do assume a secondary structure in solution with quantifiable populations of β turn and helical structures (Dyson and Wright 1995). Even a relatively short 16-mer V3 peptide has been shown by NMR to assume a loop structure stabilised by amino acid interactions either side of GPGR (Zvi, et al. 1995). It may therefore be that these peptides assume a partially native shape in solution and that the secondary structure of the 37-mer is potentially more stable than that of the 20-mer allowing it to adhere to the plate in this form and for antibodies to bind to it with greater affinity than to the shorter peptide. The conformation dependence of many antibodies to V3 may be the reason that the number of sera which showed reactivity with the V3 peptide was limited (even though it showed the best reactivity of all the peptides) and why the long V3 peptide was a superior target antigen in ELISA.

The CD4 binding site (CD4bs) is an important region for specific antibody recognition in humans able to neutralise across strains of HIV-1. In order to ascertain if the hypothetical reduction in V3 immunogenicity, caused by V3 mutation, results in a corollary increase in the immunogenicity of the CD4bs (and/or other epitopes), it is necessary to have an accurate measure for CD4 reactivity in immune sera. Although the assays were not reliable due to non-optimisation, the one in which the clearest inhibitory effect was seen (Figure 3.15) does indicate a possible relationship between CD4bs specific antibody and total
IgG. This may have important implications for the quality of immune response induced by any candidate gp120 vaccine.

Both protein and nucleic acid immunised mouse sera reacted equally poorly with denatured gp120 indicating that this denatured form does not present relevant epitopes. The conformation sensitivity of both the mouse sera and the rat Mabs indicates that most of the epitopes on gp120 may be conformation sensitive. In Dr. McKeating’s laboratory a folded construct of V1/V2 was expressed in E. coli and assays done by J McKeating with these mouse sera showed broad reactivity with this V1/V2 construct. In view of the fact that no reactivity was detected with the V1/V2 region peptides this confirms that epitopes in this particular region of gp120 are sensitive to conformation. The observation that NAI generates a broader peptide reactivity suggests that the immunogen in NAI may present these particular epitopes better than baculovirus gp120. Even though V3 epitopes are often described as ‘linear’ ie. continuous, they are subject to loss of reactivity following conformational changes induced in gp120 by point mutations elsewhere in the protein, showing that these linear V3 epitopes are also conformation sensitive, as described in Chapter 1. After denaturation of gp120 it is not known whether continuous epitopes of gp120 are still available or have been obscured by altered folding. The only conclusion that can be drawn from this group of experiments is that the B cells which recognise native gp120 react with epitopes which are conformation sensitive, these epitopes may be either discontinuous or continuous.

Although the peptides used in these experiments are very limited in number and represent only a minority of possible epitopes, the pattern of peptide reactivity obtained is in agreement with the pattern found by Bristow et al (Bristow, et al. 1994). The data do show consistency in the recognition of the C1 region and C-terminus peptides. Bristow et al also used baculovirus gp120 as the immunogen (40 μg intraperitoneally with Freund’s adjuvant) and found strong reactivity with the same C1 region 20-mer peptide as was used here. They also
found reactivity with a C-terminus peptide. Palker (Palker, et al. 1987) has described an immunodominant epitope in the C-terminus of gp120. Since V3 is known to be exposed, and V3-specific antibodies often react with peptides, the reactivity of the sera with the C1 and C-terminus peptides indicates that the primary sequences represented by these peptides are also accessible in native gp120. The consistency of the reactivities to these two regions identifies them as potential constant region target epitopes in a vaccine immunogen.

Nucleic acid immunisation produces a relative increase in the percentage of peptide reactive sera. Since gp120 is normally expressed from a viral gene, whose DNA has been made within the host cell, and not from bacterial DNA, it might be postulated that there could be a degree of aberrant expression of the antigen in NAI. The proportion of such putative atypical expression may be small but immunologically important. It is possible that the foreign nature of the injected DNA may affect transcription and/or translation in undefined ways and as a result truncated, partially processed, unfolded or otherwise imperfect forms of the antigen might be expressed. Since gp120 is known to be cytotoxic, there may also be degradation products of this antigen. On these postulated "subforms" some epitopes may be more accessible than on the native gp120 and this may be reflected in the increased peptide reactivity. The qualitatively different epitope responses observed with protein and nucleic acid immunisation may equally well be due differences in the processing and presentation pathways used as suggested by Coney (Coney, et al. 1994).

It is known that antibodies to gp120 can recognise carbohydrates but the relative proportion of such antibodies in the total IgG response is not yet known. Cell culture conditions (Andersen and Goochee 1994) and cellular expression systems can produce different patterns of glycosylation. As well as the glycosylation pattern Fouts et al. have shown that the epitopes exposed on an antigen can be profoundly influenced by the expression system (Fouts 1995). Baculovirus recombinant gp120 is produced in insect cells, lacks O-linked
carbohydrates and does not have the same N-linked glycosylation pattern as mammalian cell produced gp120 (Yeh, et al. 1993). Both of these antigens may differ significantly from the mouse-cell produced gp120 which acts as the antigen in the nucleic acid immunisations. The peptides used in the assays were unmodified by carbohydrates. Although the data presented do not address the question of how intimately the carbohydrates of gp120 are involved in its antigenicity, this is potentially extremely important, given the extent of the glycosylation. Carbohydrate is necessary for correct folding and may need to be taken into account when producing a mutant gp120 subunit vaccine.

Our findings of IgG2a antibodies in mice immunised with protein and Quil A adjuvant is in agreement with the results of Bomford et al (Bomford, et al. 1992). Quil A is a heterogeneous mixture of saponins of which the QS-21 purified component is able to elicit IgG2a in mice (Hancock, et al. 1995). During a Type 1 cytokine dominated response in the mouse, there is production of IgG2a antibody and substantial T cell and macrophage responses (Kemeny, et al. 1994). The expression of mainly IgG1 in the experiments presented here indicates that a Type 2 cytokine directed response may have been dominating in contrast to the Type 1 cytokine dominated response found by others following NAI (Manickan, et al. 1995). If the large amount of injected DNA, with the strong HCMV promotor, produced relatively large amounts of antigen, this may have favoured the development of Type 2 cytokine dominated responses and the production of IgG1 (Bretscher, et al. 1992: Parish and Liew 1972). For a therapeutic vaccine it still needs to be established which quality of immunity is most effective in order to choose the immunisation protocol which would be the most beneficial. In the case of AIDS the evidence for CTL being protective in the long term is at present equivocal as described in Chapter 1. For a prophylactic vaccine, it may be necessary to induce a different quality of response. For these different purposes, it may be possible to choose between protein and nucleic acid immunisation and between high and low doses of protein or DNA.
The difference in the response of the two cohorts of k x d mice immunised with AF (Figure 3.1) is unexplained. It has been noted that reduced droplet size (from 2500 nm to 150 nm) by sonication increases the IgG2a response of pluronic emulsions (Bomford, et al. 1992) which include AF. These authors also find that when high levels of IgG1 antibodies were induced, the IgG2a isotype was absent. Figure 3.9 shows one mouse immunised with AF where there is a reasonable IgG2a level but minimal IgG1. It has also been noted (Allison, personal communication in Stieneker, et al. 1995) that the recommended method of preparation of these emulsions does not generate the optimal adjuvant effect and that this can be augmented by sonicating to reduce the droplet size. Together these findings suggest that the AF plus antigen emulsion given in the two different cohorts, although they were prepared by vortexing in the same manner both times, may have had different ranges of droplet sizes. It is also possible that the two AF cohorts are, by chance, samples from two tails of a normal distribution of population variability.
CHAPTER 4

CONSTRUCTION AND EXPRESSION OF MUTANT gp120

Introduction

A dramatic method of achieving the aim of reducing V3 immunogenicity is to construct a mutant with the whole loop deleted. There have been several studies on the functional effects on gp120 following major deletions. A particular truncated gp120 "ENV59" lacking V1, V2, V3, parts of the N- and C-termini, four disulphide bonds and eight N-linked glycosylation sites, was able to bind CD4 with high affinity (Pollard, et al. 1992) and could therefore be a suitable construct for presentation of the CD4 binding site as a subunit vaccine. Subsequently, others have demonstrated that deletion of V3 alone causes a decrease in the CD4 binding affinity (Wyatt, et al. 1993).

Therefore since the aim was to preserve, as much as possible, the normal conformation of the gp120 monomer, V3 was not deleted but substitutions were made designed to preserve as much as possible of the native structure of V3 and therefore that of gp120. For this purpose, serine was chosen as the substituting amino acid since it is (i) small - and therefore unlikely to sterically inhibit correct folding; (ii) uncharged - and therefore unlikely to inhibit correct folding by forming strong ionic interactions with other amino acids; and (iii) polar - therefore hydrophilic and not likely to be attracted towards the protein core and thus either again alter folding or prevent the loop from assuming its normal position. Antibody-antigen interactions have been estimated to rely substantially
(as much as 50%) on hydrophobic interactions (Novotny, et al. 1989)(Geysen, et al. 1988) although in individual examples electrostatic interactions may be more important (Lee, et al. 1992). Therefore based on its hydrophilicity and lack of charge, serine is potentially only weakly involved in antigen-antibody interactions and it was assumed for this work that serine was potentially only weakly immunogenic.

Glycine was another possible choice for substitution but glycine is often unacceptable as a substituting amino acid in an antibody/antigen reactive site causing reduction in the affinity of the interaction (Geysen, et al. 1988). Glycine is also unusually mobile due to increased rotational flexibility, since two of the groups on the alpha carbon, instead of one, are single hydrogen atoms and strings of glycines might therefore have made the loop hypermobile.

In order to characterise the effects of these V3 mutations on the global conformation of gp120, the secreted protein can be analysed immunologically with panels of monoclonal antibodies. For analysis of gp120 it was necessary to establish stable transfected cell lines for production of the recombinant protein since the transient expression of gp120 into COS cell\textsuperscript{1} supernatants was not detectable by ELISA. Among the many selectable markers available the glutamine synthetase (GS) selection system has been successfully used to produce high levels of protein expression in CHO cells (Bebbington, et al. 1992). GS is an enzyme catalysing the synthesis of glutamine from the substrates glutamate and ammonia and can be used as a selectable and amplifiable marker by the action of the specific GS inhibitor, methionine sulfoximine (MSX) even in cells, such as CHO cells, which have endogenous active GS genes (Sanders and Wilson 1984). CHO cells are widely used for production of recombinant proteins and, it was decided to used CHO cells with GS selection to establish stable gp120 expressing cell lines.

\textsuperscript{1}COS cells are monkey CV-1 cells stably transformed by a segment of SV40 early-region DNA. Plasmids carrying the origin of replication of SV40 are replicated to high copy-numbers in COS cells which can result in high levels of transient expression of foreign proteins.
In order to analyse the effects of V3 mutations on the conformation and immunogenicity of gp120, a series of mutant gp120 genes were constructed and used both for transfection of CHO cells and for immunisation of mice (Chapter 5).
Results

Construction of mutants

A V3-containing fragment of the gp120 gene was subcloned into pBluescript and a panel of nineteen mutants were made. Initially four mutants were selected for analysis of protein expression in CHO cells and for immunogenicity studies in mice. A flow diagram showing the sequence of experiments during construction of the mutants is shown in Figure 4.1.

Cloning of the Bgl II fragment. After each site-directed mutation it was necessary to sequence the DNA to check for random mutations, it was therefore desirable to work with a smaller fragment of DNA rather than the whole gp120 gene. V3 could be released by a Bgl II digest yielding a 580 base pair (bp) fragment which also included C3, V4, C4 and parts of C2 and V5. A vector was made for this Bgl II fragment by creating a unique Bgl II site within the multicloning site (MCS) of pBluescript SK+. The fragment and vector were ligated and the orientation of the Bgl II fragment with respect to the Sac I and Kpn I sites was ascertained by digestion with Pvu II. (Figure 4.2).

SmaI site mutagenesis. Since mutations were planned for both sides of GPGR, it was convenient to have a restriction endonuclease site within GPGR so that, by digestion and religating the loop sides in all combinations, a large number of mutants could be generated from a small number of mutagenesis steps. No such site existed in GPGR but it was possible to make a single base change which was silent and maintained GPGR in frame but generated a unique Sma I site. This is described below, but before this unique Sma I site could be made in V3 the Sma I site in the pBluescript MCS needed to be removed. This was achieved by digestion with Sma I and then with Xba I, filling in the
**Figure 4.1**
Flow diagram for construction and expression of wild type and mutant gp120s
Figure 4.2
Diagram to show cloning of the Bgl II fragment of gp120 into pBluescript SK⁺
overhanging Xba I end using Klenow polymerase followed by religation. These steps destroyed both the Sma I site and the Xba I site and removed the intervening Bam HI site (Figure 4.3). Before transformation of *E. coli* with this self ligated plasmid, a Bam HI digest was made to linearise any plasmid which had remained unaltered.

Oligonucleotide-directed site-specific mutagenesis was used to introduce the A to C silent substitution into GPGR to generate the unique V3 Sma I site. The original V3 sequence is shown in Figure 4.4

<table>
<thead>
<tr>
<th>Original sequence</th>
<th>G</th>
<th>P</th>
<th>G</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>With silent mutation</td>
<td>GGA</td>
<td>CCA</td>
<td>GGG</td>
<td>AGA</td>
</tr>
</tbody>
</table>

Single stranded (ss) DNA from pSK+BglIlgp120ΔSmaI and phosphorylated oligonucleotide 18 (OL-18) were used following the T7Gen mutagenesis kit protocol (Figure 4.5 and Table 4.1). Transformed colonies and, as negative controls, parental plasmid and pSK+Bgl II, were screened by hybridisation with ^32^P end-labelled OL-18 at 60°. Three of the positive clones were sequenced across the whole of the Bgl II insert to check the presence of the Sma I site and the absence of any undesired mutations. This plasmid with the unique Sma I site in V3 was named pV3SmaISK+ (Figure 4.5).

The pattern of variability within V3 described by LaRosa et al (LaRosa, et al. 1990) was used to select individual amino acids for mutation. It was decided to leave unaltered those amino acids which were relatively conserved in V3 since, due to their conservation, they may have important functions, one of which may be involvement in V3 loop secondary structure. GPGR was also unaltered as this motif is one of the most highly conserved elements of the V3 loop and the target of neutralising antibodies (Goudsmit 1988: Palker, et al. 1988: Rusche, et al. 1988), and so is probably a beneficial epitope to conserve.
Figure 4.3
Removal of the Sma I site from the multicloning site of pSK⁺ Bgl II gp120
Figure 4.4
DNA sequence of unmutated (wildtype) V3 loop from HIV-1 IIIB
Figure 4.5
T7 Gen mutagenesis. The creation of the Sma I site in GPGR is shown as an example.
Table 4.1

Oligonucleotides used in these experiments for mutagenesis, hybridisation and sequencing. Sequences are listed from 5' to 3' and restriction endonuclease sites are in bold and underlined (**CCCGGG** Sma I; **AGTACT** Sca I).

<table>
<thead>
<tr>
<th></th>
<th>5'</th>
<th>3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript sense</td>
<td>GCGGATAACAAATTTACACACAGGAA</td>
<td></td>
</tr>
<tr>
<td>sequencing primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript antisense</td>
<td>CGCCAGGTTTTCCAGTCACGAC</td>
<td></td>
</tr>
<tr>
<td>sequencing primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OL-18</td>
<td>AGAGAGGACCCGGAGAGCAT</td>
<td></td>
</tr>
<tr>
<td>OL-2</td>
<td>AAAATAGGAAGTAGAGACACAG</td>
<td></td>
</tr>
<tr>
<td>OL-B</td>
<td>CAAGAAAAAGTAGTACATCCAGAG</td>
<td></td>
</tr>
<tr>
<td>OL-3</td>
<td>GGAACCCGGAGAGCATTACACACAG</td>
<td></td>
</tr>
<tr>
<td>OL-4</td>
<td>GGACCCGGAGATCATCTAGTACACAG</td>
<td></td>
</tr>
<tr>
<td>OL-C</td>
<td>TCTCCGGGTCCACTACGCTACTACTTTTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCTTGTATTGTGTGTGG</td>
<td></td>
</tr>
<tr>
<td>OL-D</td>
<td>TCTCCCGGTCCGCTACTATCCTTTTTTCTTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTATTGTGTGTGG</td>
<td></td>
</tr>
<tr>
<td>OL-E</td>
<td>TCTCCCGGTCCGCTACTACTTTTTTCTTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTATTGTGTGTGG</td>
<td></td>
</tr>
<tr>
<td><strong>Sca I</strong> sense PCR</td>
<td>TTGGGTAGCTAGTACTAATTGTACAGGACC</td>
<td></td>
</tr>
<tr>
<td>primer for the V3 loop</td>
<td>AAC</td>
<td></td>
</tr>
<tr>
<td><strong>Sca I</strong> antisense PCR</td>
<td>TTCATGCTAGTACTAATTGTACAGG</td>
<td></td>
</tr>
<tr>
<td>primer for the V3 loop</td>
<td>AAC</td>
<td></td>
</tr>
<tr>
<td>pKfdH sense sequencing</td>
<td>TGTGTGAATTGTGAGC</td>
<td></td>
</tr>
<tr>
<td>and PCR primer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
First two serine substitutions. Serine substitutions were initially planned to be made one at a time, using each newly created mutant as the parental sequence for the subsequent mutation in order to build up a string of serines on each side of GPGR. To make the first two substitutions, OL-2 and OL-B (Table 4.1) were phosphorylated and used in the T7Gen protocol with pV3SmaISK+ ssDNA. Mutant plasmids were identified by hybridisation with $^{32}$P end-labelled OL-2 or OL-B and three positive clones of each mutant were sequenced across the whole of the Bgl II insert. The desired mutation was present in all three clones of 'V3-2' and of 'V3-B'. However, in all six sequences, a frame shift mutation (a single base deletion) was detected in the C4 region of gp120 (not within V3, but making non-sense of C4, V5 and C5 including the C-terminus).

It was possible by digestion with Bsu 36I and Sac I to remove that portion of the Bgl II fragment which contained this frame shift deletion. This portion, being 3’ of the V3 sequences, did not affect V3 in any way. The excised DNA was replaced by corresponding sequences taken from pSK$^+$BglIigp120ASmaI (Figure 4.3) and after ligation, the normal reading frame of the Bgl II fragment was restored (Figure 4.6). The resulting constructs, V3-2 and V3-B, were sequenced again all across the Bgl II insert to confirm the correct sequences.

Problems with mutagenesis. In addition to the appearance of an undesired mutation at a site distant from the target sequence for the desired mutagenesis, frequent degradation of the ssDNA was a major stumbling block. This problem could be overcome by using alternative mutagenesis methods. A fragment of pBluescriptSK$^+$, from Sac I to Kpn I (which contained the Bgl II fragment with the site-directed mutations), was subcloned into the pBluescript KS$^-$ which has a different orientation of the phage fd origin of replication and opposite orientation of the MCS with respect to the T3 and T7 promoters present in the plasmid (Figure 4.7). These changes were necessary should a double stranded DNA mutagenesis kit be used. Since it would not be possible within a reasonable period of time to
**Figure 4.6**
Repair of V3-2 and V3-B by replacing the fragment containing the frameshift deletion.
Figure 4.7
Changing from SK+ to KS- vector
construct and then analyse the very large panel of desired mutants each with a
single extra serine substitution, it was decided to make a smaller number of
mutants. Each of these mutants would have a block of serines substituted for the
wild type sequence, and should any mutant cause a change in V3
immunogenicity individual substitutions could then be made to allow more
detailed analysis of their effects.

*Subsequent serine substitutions.* Mutation by PCR is a fast and efficient
method of introducing blocks of amino acid substitutions, while a potential
problem with this method is the creation of undesired mutations owing to the
infidelity of Taq polymerase. However, since DNA sequencing was required to
confirm the presence of the desired mutations between each mutagenesis step,
any Taq polymerase generated mutations would be detected. A diagram
showing the positions of the serine substitutions is given in Figure 4.8. (There is
another copy of Figure 4.8 inside the back cover of the thesis).

Oligonucleotides (OL-C; OL-D; OL-E; OL-3; OL-4 - Table 4.1) containing a
Sma I site (CCCGGG), the mutant sequence and 15 bases to anneal to the
template DNA were synthesised. Sense and antisense primers which annealed to
pBluescript were used to prime the synthesis of the complementary strands
(Figure 4.9). There are six codons for serine and the frequency of each in the
native gp120 BH 10 clone (from which the gp120 gene was originally taken) was
calculated in order to maintain the codon usage for serine. This was 43% AGT;
24% TCA; 18% AGC; 9% TCT; 6% TCC and 0% TCG. AGT was used wherever
possible but keeping the number of base substitutions to a minimum.

The PCR products (Figure 4.11) were digested with either Sac I or Kpn I
and cloned into the appropriate directional T vector (Figure 4.10). After selection
by Bgl II and Sma I digestion, three clones of each PCR fragment were sequenced
Serine substitutions in V3

Individual mutants

<table>
<thead>
<tr>
<th>Mutant Code</th>
<th>Mutant</th>
<th>Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No changes</td>
<td>E</td>
</tr>
<tr>
<td>2</td>
<td>S</td>
<td>D</td>
</tr>
<tr>
<td>3</td>
<td>S S K S S</td>
<td>C</td>
</tr>
<tr>
<td>4</td>
<td>S S S S K S S</td>
<td>B</td>
</tr>
</tbody>
</table>

Joined mutants

<table>
<thead>
<tr>
<th>Mutant Code</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>Wild Type</td>
</tr>
<tr>
<td>D</td>
<td>Mutant 4E</td>
</tr>
<tr>
<td>C</td>
<td>Mutant 4C</td>
</tr>
<tr>
<td>B</td>
<td>Mutant 4A</td>
</tr>
<tr>
<td>A</td>
<td>Mutant 1E</td>
</tr>
</tbody>
</table>

Figure 4.8

Diagram showing amino acid sequence of the V3 loop, the positions of the serine substitutions and the sequence of the four joined mutants studied so far during this work.

There is a copy of this diagram inside the back cover of the thesis.
Figure 4.9

on reverse
Figure 4.9
Mutagenesis by PCR. Diagram showing positions of primers and restriction sites
A: Mutations 5’ of Sma I in GPGR  B: Mutations 3’ of Sma I
Figure 4.10
Construction of directional T vectors to aid cloning of mutant V3 PCR products
Figure 4.11
Photograph of 2% agarose gel analysis of PCR products

Lane 1: Lambda (Hin dIII and Eco RI) size markers 500ng. Lane 8: Phi X 174 (Hae III) size markers 1000 ng. Lanes 2 and 9: No DNA controls. Lanes 3-7: Mutant C with MgCl₂ concentration titration. Lanes 10 - 14: Mutant D with MgCl₂ concentration titration.

The titration is with the addition of an extra 0.5 mM MgCl₂ to each subsequent reaction.
completely. Figure 4.12 shows an extreme example of a Taq polymerase generated mutation with an 11 base pair deletion in clone 8. Some other undesired mutations were found, but 1 to 3 fully correct sequences were confirmed from each set of three clones.

**Joining the mutant halves of V3 in all possible combinations.** The plasmids containing serine substitutions 3' of GPGR were made into vectors, while the plasmids containing serine substitutions 5' of GPGR were used to generate fragments for insertion into these vectors (Figure 4.13). To increase the efficiency of the ligation, Psp Al (which creates cohesive ends) was used instead of its isoschizomer Sma I (which creates blunt ends). Both fragments and vectors were purified on 1 % TAE gels and ligated in all possible combinations to generate 19 different mutant V3 constructs plus the wildtype.

<table>
<thead>
<tr>
<th>1A (Wildtype)</th>
<th>2A (V3-2)</th>
<th>3A</th>
<th>4A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B (V3-B)</td>
<td>2B</td>
<td>3B</td>
<td>4B</td>
</tr>
<tr>
<td>1C</td>
<td>2C</td>
<td>3C</td>
<td>4C</td>
</tr>
<tr>
<td>1D</td>
<td>2D</td>
<td>3D</td>
<td>4D</td>
</tr>
<tr>
<td>1E</td>
<td>2E</td>
<td>3E</td>
<td>4E</td>
</tr>
</tbody>
</table>

**Table 4.2**
Nomenclature of wildtype and V3 mutant constructs produced after ligation of the loop halves in all combinations. See Figure 4.8 for details of individual mutants.

4A, 4C, 4E and 1E, which are underlined in the Table 4.2, above are the mutants which were selected for the first protein expression and immunisation experiments (see below).
Figure 4.12
3 cloned PCR products of the same mutant. Clones 7 and 12 have the correct sequence but clone 8 has an 11 base pair deletion.
Figure 4.13
Diagram of vectors and fragments made for joining mutants in all combinations. All plasmids were digested with Sma I and Sac I
Transformed *E. coli* were probed (Figure 4.14) with the same oligonucleotides which had been used as primers in the PCR. Positively hybridising colonies of all constructs were screened with Bgl II to check the size of the fragment produced then sequenced - across V3 only - to recheck that the paring of 3' and 5' parts of V3 was correct for every one. Initially, no Sma I digest was made prior to sequencing, on the assumption that this site would be intact in successfully ligated plasmids. However, on sequencing the Sma I site was found to be defective in 5 of 13 (38%) clones; this must have been due to damage caused by Psp A1. An example of a V3 mutant (4C) with joined halves is shown in Figure 4.15.

*Subcloning into expression vector.* Initially only four mutants were selected for expression of mutant gp120 proteins. These four consisted of two of the potentially most radical mutants (4C and 4E) and two mutants complementary to these (4A and 1E). 4C has maximum serine substitutions on both sides of GPGR; 4E has, in addition to this, the two amino acid deletion immediately 5' of GPGR; 4A and 1E have maximum serine substitutions one side of GPGR and wild type sequences on the other. The Bgl II fragments were excised from pBluescript and subcloned back into the gp120 gene in pEE6HCMVgp120GS (Figure 4.16) which contains a hamster GS mini gene and the human cytomegalovirus promotor (HCMV) which has been shown to be a strong promotor in CHO cells and other cell types (Bebbington, et al. 1992).
Figure 4.14
Result of hybridisation with $^{32}$P labelled oligonucleotide showing the two stringencies of negative control. Faint hybridisation is occurring with the parental plasmid negative control. Not all of the apparently positive clones had the correct sequence. (In this experiment the number of colonies available did not fill the grid).
Figure 4.15
DNA Sequence of mutant 4C. The codons for substituted serines are in bold.

Sequence reads: 5' TGT ACA AGA CCC AAC AAC AAT ACA AGA AAA AGT AGT AGT AGC AGT AGT GGA CCC GGG AGA TCA TCT AGT TCA AGT AGT AAA AGC TCA AGT ATG AGA CAA GCA CAT TGT 3'
Figure 4.16
Diagram of the gp120 expression vector used (a) for establishing stable CHO cell lines secreting gp120 and (b) for injection into mouse skeletal muscle for expression of gp120 and an immune response.

**Glutamine Synthetase** - selectable marker for establishing CHO cell lines. **Col E origin** - origin of replication for E. coli. **β lactamase** - selectable marker for transformed E. coli. **HCMV promoter** - human cytomegalovirus promoter for gp120 gene. **SV40 poly A** polyadenylation signal from SV40.
Expression of wildtype and mutant gp120

Transfection of CHO cells and selection of foci. The four selected mutant gp120 plasmids, the wildtype gp120 plasmid and the gp120-negative control plasmid pEE14 were prepared by the 'Qiagen' purification method and used to transfect CHO cells by calcium phosphate coprecipitation. The transfected cells were subjected to 25 μM MSX in the initial selection step which resulted in approximately 10-20 foci per 10^6 cells. These foci were grown individually in 24-well plates and the supernatants were assayed in an ELISA for the presence of gp120. The gp120 was captured from the supernatant by sheep anti-gp120 (D7324) and detected with a rabbit polyclonal gp120 antiserum with baculovirus gp120 ADP607 Lot MY27R used as the standard. Approximately 90% were positive for gp120 and those with the highest level of gp120 secretion were selected for amplification.

GS gene amplification. The chosen foci were grown and split into 5 flasks and then grown in the presence of MSX at 25 μM, 100 μM, 150 μM, 200 μM and 250 μM. Foci appeared at all concentrations of MSX with the highest number of foci at the lowest MSX concentration. To choose which level of amplification to use, the highest concentration of MSX which had yielded 10 or more foci was chosen (P. Stephens, personal communication). These foci were grown and tested again in ELISA for gp120 secretion at 100-250 μM MSX resistance depending on the cell line. The gp120 concentration in these supernatants was apparently in the region of 1μg/ml, (Figure 4.17). The different lots of baculovirus recombinant gp120 (ADP 607) were variable in their gp120 concentration (H. Holmes, personal communication) and since this had been used as the standard in the ELISA a closer estimate of gp120 concentration in CHO supernatants was not possible.
Estimation of concentration of secreted gp120 in CHO cell line supernatants using baculovirus rgp120 as the standard

Figure 4.17
An example of an ELISA to estimate the concentration of the secreted gp120 in CHO cell supernatants. D7324 was used to capture the gp120; baculovirus recombinant gp120 was used as a standard. From this ELISA the concentration of secreted mutant 4E from this cell line was estimated to be in the range of 1-1.5 µg/ml.
Use of serum free medium and concentration by freeze drying. It was necessary to visualise the gp120 in order to detect any degradation which might have occurred, and, since the concentration of gp120 may have been lower than estimated, it was decided to concentrate it. In order to do this without also concentrating other proteins, an attempt was made to wean the CHO cells onto serum free medium. It was desirable to use a manufacturer’s serum-free medium rather than attempt to formulate our own and the best serum-free and glutamine-free (to permit selection for stable integration of the GS gene) medium available was “AIMV” (Gibco) although this is not specifically formulated for CHO cells. The cells were easily adapted from 10 % to 1 % FCS but they failed to thrive in AIMV supplemented with less than 1 % FCS. As an alternative, the cells were grown to 80 % confluence in their normal GMEM-S medium with MSX selection pressure and at this cell density the medium was aspirated, the cells washed twice with PBS and 20 ml of AIMV (without FCS and without MSX) was added. This AIMV was harvested 24 or 48 hours later at which point most of the cells were still alive. The harvested medium with gp120 was freeze dried and reconstituted in 1/20 volume. Western blots (Figure 4.18) indicated that there was no difference between the 24 hour and 48 hour samples in quantity or quality of gp120. Therefore 24 hours was used subsequently for harvesting serum free wildtype and mutant gp120 supernatants. These mutant gp120’s show no size change on SDS PAGE with Western blotting (Figure 4.19) and it was assumed from this that the mutation of the V3 loop had not adversely affected the overall processing of the protein.

Effect of freeze drying on gp120 structure. To establish whether freeze drying damaged the gp120, the available rat monoclonal antibodies to gp120 were titrated on the captured wildtype CHO supernatant gp120 both before and after freeze drying (Figure 4.20). The monoclonals were first titrated on the gp120 captured from the normal medium. Since their curves mainly did not extend to saturation, in the subsequent assay on the freeze dried gp120, the
Figure 4.18
Western blot of wildtype rgp120 from freeze dried serum free CHO cell supernatants 24 hours and 48 hours after the removal of the normal growth medium. Samples are Neat then 1:2 dilutions. Lanes 1 and 5: Neat. Lanes 2 and 6: 1:2 Lanes 3 and 7: 1:4 Lanes 4 and 8: 1:8 Lane 9: Baculovirus rgp120 500 ng The blot was probed with sheep anti gp120 C terminus (D7324).
Figure 4.19
Western blot of freeze dried (24 hours) wildtype and mutant rgp120 in serum free CHO supernatants.

Lane 1: Baculovirus rgp120 100 ng
Lane 2, 3 and 4: Supernatant from stable CHO cell line without the gp120 gene.
Lanes 5, 6 and 7: Wildtype gp120
Lanes 8, 9 and 10: Mutant 4E.
Lanes 11, 12 and 13: Mutant 4C.
Lanes 14, 15 and 16: Mutant 4A.
Lanes 17, 18 and 19: Mutant 1E
All dilutions are: Neat, 1:2 and 1:4 from left to right.
Figure 4.20
on reverse
Titration curve profiles for rat monoclonal antibodies on gp120 from normal medium and from freeze dried serum free medium

**Figure 4.20**
Titration curves (1:2 dilutions throughout) of rat monoclonal antibodies on captured wild type gp120 from different sources. **Column A** is gp120 secreted into the normal medium used for growth of the cell lines. **Column B** is gp120 from the same cell lines, secreted for 24 hours into serum free medium which was then freeze dried and reconstituted in 1:20 of the original volume. The starting concentration of the dilution series for the monoclonals is marked (all are in µg/ml). The curves in Column B begin at 1-2 factors higher (see text).
Figure 4.20 continued
starting concentration of the monoclonal was increased where possible. There is one monoclonal, A1 (directed to the C1 region of gp120), which appears to react less well with freeze-dried gp120. In general, allowing for (i) probable differences in the amount of gp120 captured between the two different experiments, (ii) the higher starting point of the 1:2 dilution series of the monoclonals and (iii) the fact that the assays were not performed simultaneously and therefore not read at the same time, there is apparently no obvious loss of binding of the other monoclonals to gp120. This set of monoclonals reacts with diverse epitopes on gp120 and as the overall profile of the titration curves is the same before and after freeze drying, it was concluded that no gross conformational shift affecting these epitopes had occurred during the freeze drying process. This freeze dried gp120, initially made for SDS PAGE and Western blot analysis was used in capture ELISAs so that all ELISAs could be performed on the same batch of gp120.

*Analysis of wildtype and mutant gp120 proteins by SDS PAGE and Western blots.* Figure 4.21 shows three reducing SDS gels with wildtype and mutant CHO secreted gp120 and baculovirus gp120 (ADP 607). One gel (A) was silver stained and the other two (B) were transferred to nitrocellulose (Hibond C extra) and probed with rabbit polyclonal antiserum to gp120 (ADP 421) or with sheep anti-gp120 C-terminus (D7324). The heavily overloaded band on the silver stained gel (A) is most likely to be the BSA in the AIMV medium. Lane 10 is an empty lane and the faint band in it is most probably fractional contamination from lane 9, similarly the faint band in lane 2 which contains baculovirus gp120 is probably contaminating BSA from lane 3. On the silver stained gel (A), no gp120 band is visible and this is at present unexplained, but on reducing SDS PAGE followed by western blot (B), the gp120 secreted from CHO cells runs at approximately 120 kDa, while the baculovirus recombinant protein runs at approximately 110 kDa. This size difference is likely to be due to the difference in glycosylation made in the two cell types. Both gp120s are typical broad bands.
Figure 4.21

A: Silver stained gel  B: Western blots probed with sheep anti gp120 C-terminus (D7324) and rabbit anti whole gp120 (ADP 421). A and B are from three different gels run on the same day with the same conditions. Samples were prepared as a batch for all three gels and loaded in the same lane order. **Lane 1:** High molecular weight size markers. **Lane 2:** Baculovirus rgp120 100ng. **Lane 3:** Control (i) Serum free medium only. **Lane 4:** Control (ii) Medium from stable CHO cell line without the gp120 gene. **Lane 5:** Wild type gp120. **Lane 6:** 4E. **Lane 7:** 4C. **Lane 8:** 4A. **Lane 9:** 1E **Lane 10:** empty lane
due to carbohydrate microheterogeneity. The blot probed with the sheep antibody shows a strong band at approximately 95 kDa but as this is present in all lanes, it must be a protein in the serum-free medium which cross reacts with the sheep antibody and not a CHO cell product and, more importantly, not a degradation product of gp120.

**Conformation of mutant gp120.** Figure 4.22 shows titration curves of rat monoclonal antibodies on captured wild type and mutant gp120. A mouse polyclonal gp120 antiserum was made up from sera from 5 protein immunised mice (k x d:QuilA) pooled in equal proportions. This was used as a reagent for estimating the amount of captured gp120 rather than the rabbit gp120-antiserum which had shown high background cross reactivity with the capture antibody. The control was supernatant from the gp120 gene-negative stable MXS resistant CHO cell line. Baculovirus gp120 was also included as another wild type gp120 for comparison. Rat Mabs D1, D2, D3 and D4 and mouse Mab ADP 3047 (grouped together on the first page of Figure 4.22) are directed to V3. These curves show an appropriate loss of binding to the mutant gp120s. D4 reacts well with the CHO wild type and baculovirus wild type gp120 but recognises none of the mutants. However, D2 and 3047 are both able to recognise mutant 4A in addition to wildtype gp120. For other regions of gp120 (grouped on the second page of the figure, and see also Figure 1.9 for the table of rat Mab epitopes) the curves show very little difference between wild type and mutant CHO gp120 (monoclonals A1 [C1 region], C1 [V1/V2 region], G2 [discontinuous V1/V2 and C4 regions], F2 [discontinuous, C3 and C4 regions], and B1 [C4/V5 region]). For other monoclonals (F1 [C4 region], and G1 [unmapped, conformation sensitive]) there is some difference between the curves for the different mutants.
Figure 4.22

on reverse
Analysis of the conformation of wild type and mutant gp120 secreted from stable CHO cell lines

Figure 4.22
Titration curves of rat monoclonal antibodies on captured CHO cell-line secreted wild type and mutant gp120 4A, 4C and 4E. Also included is captured wild type baculovirus gp120 and the supernatant from stable CHO cell line without the gp120 gene (control). Two mouse monoclonal antibodies are also titrated: 3047-directed to V3 and 326-directed to the CD4 binding site. A mouse polyclonal antiserum (composed of pooled tertiary response immune serum from 5 protein immunised [k x d: Quil A] mice) was used to estimate the amount of gp120 captured. These assays were made on two consecutive days - see also next page.
Figure 4.22 continued
Due to time pressure, the immunisation experiments with the wildtype and mutant gp120 DNA were made at the same time as the analysis of the expressed wildtype and mutant proteins. Mutant 1E failed to elicit a detectable IgG response (see Chapter 5). Since 1E has relatively minor changes compared with the other mutants (Figure 4.8) it was very surprising to find this stark difference in detectable IgG level. However, during analysis of 1E alongside the wildtype and other mutant proteins by SDS PAGE and Western blots, 1E protein was undetectable in the CHO supernatants (Figure 4.21). Subsequently DNA sequencing across the whole of the Bgl II fragment of 1E revealed the same frame shift mutation in the C4 region of gp120 as was originally identified in mutants B and 2 (which were later repaired). This means that this undesired mutation had occurred at an earlier stage than was detected in the construction of the mutants. The consequence of the frame shift in 1E is to create a stop codon 51 base-pairs downstream of the frame shift. This new stop codon causes (1) reduction in the total size of the 1E protein by 46 amino acids, (2) the loss of two N-linked glycosylation sites, (3) creation of non-sense sequences beyond the middle of C4 region, and (4) the loss of the original C-terminus which is necessary for capture of the protein in ELISA.

The absence of the 1E truncated protein (1EX) on the blot probed with the C-terminus specific sheep anti-gp120 (Figure 4.21) is therefore in agreement with the sequence data. However, it was hoped to detect the presence of 1EX with the rabbit antiserum since the large N-terminal part is still theoretically in frame. There is a faint band at approximately 130 kDa (Figure 4.21) but this is too large for the expected size of 1EX (approximately 112 kDa) and is also present in control lanes 3 and 4. The lack of detection of 1EX by the rabbit polyclonal antiserum suggests that 1EX is either not secreted or only in undetectable
amounts, that the rabbit antiserum is directed preferentially towards the C-terminal sequences of gp120, or that 1EX has a conformational change which profoundly affects many epitopes.

The conformation of wild type and mutant gp120. Surface plasmon resonance can be used to analyse the interactions between molecules in real time, and the rates of association and dissociation can be measured as well as the affinity constant (Van 1994). This technology might be superior to ELISAs for analysing the effects of the V3 mutations since conformational changes resulting from mutations are readily detected by this system (Dubs, et al. 1992; Gruen, et al. 1994; Mani, et al. 1994). The sensor chip could be coated with the capture antibody (D7324), but reasonably high concentrations (10 µg/ml) of the gp120 would be needed to achieve sufficient capture (G. Panayotou, personal communication) and these experiments were not possible at this time.

Considering the titration curves for the mouse polyclonal (Figure 4.22), the amount captured of each different gp120 is roughly equal. There may be more captured baculovirus gp120 and less of mutant 4C. Whether monoclons F1 and G1 are in fact demonstrating reduced binding to the mutant gp120’s is not clear from the ELISA of Figure 4.22 since the target antigen was not titrated and it is therefore possible that the differences observed are due to differences in the amount of target antigen.

Taken overall, the curves in Figure 4.22 indicate that the conformation of the mutants 4A, 4C and 4E is in general terms very similar to wildtype for the epitopes analysed. This does not exclude the possibility of local distortions in conformation which have been undetected by these antibodies. Analysis of the conformation of the secreted mutant and wildtype gp120 (not freeze dried) by J. McKeating using additional conformation-dependent rat monoclonal antibodies showed a definite reduction in reactivity with some mutants with some but not all antibodies. The epitopes for these mutation-sensitive antibodies have not yet been defined.
It is important to note that mutant 4A showed the least reduction in binding to the mutation-sensitive antibodies in J. McKeating’s experiments, demonstrating a parallel difference from the other two mutants as is seen in the experiments presented here. 4A is heavily substituted with serines only on the C-terminal side of GPGR. It is possible that the substitutions in 4A are less disruptive to the epitope(s) recognised by the mutation-sensitive antibodies and to monoclonals D2 and 3047 in Figure 4.22. The difference between the mutant 4A and the mutants 4C and 4E is the presence of wildtype V3 sequences on the N-terminal side of GPGR in 4A. The N-terminal side of GPGR is often part of B cell epitopes of V3 (Looney, et al. 1988; Meloen, et al. 1989; Simmonds, et al. 1990) and the epitope of Mab ADP 3047, which is able to recognise mutant 4A, has also been mapped to this region (IRIQRGPGPR)(Laman, et al. 1992). The results of these experiments indicate that substitutions with serine on the N-terminal side of GPGR are disruptive to gp120 and that mutations on the C-terminal side are more tolerable. There may be a local distortion in gp120 caused by mutation of certain regions of V3. More data relating to this hypothesis is presented in chapter 5.
Expression of V3 loop-phage gene VIII fusion proteins

Introduction

The following description is of an attempt to express the mutant V3 loops as a fusion protein with phage gene VIII. This fusion protein was intended as the target antigen for ELISAs with mutant immune serum (see Chapter 5) to test the innate antigenicity of the mutant loops which might reflect their immunogenicity in vivo and help to assess whether or not this immunogenicity had been reduced by various degrees of serine substitution. The wildtype V3 loop fusion protein might also serve as a more conformationally relevant antigen than even the long V3 peptide since similar constructs have been shown to structurally mimic the native V3 epitope (Perham, et al. 1995).

Veronese et al (Veronese, et al. 1994) created a fusion protein between the V3 loop of gp120 and the major coat protein of the filamentous bacteriophages (M13, fd and F1) and have used them successfully in ELISAs. The 50 amino acid major coat protein (geneVIIIp), is the product of phage gene VIII, and is expressed at about 2700 copies per phage, forming a ‘shingled tubular array’ around the phage genome (Greenwood, et al. 1991). Veronese et al showed that up to 6 amino acids could be inserted into the N-terminal extremity of geneVIIIp still allowing the resulting fusion protein to be 100% normally assembled. Insertion of over 6 amino acids sterically inhibited normal assembly but hybrid phages could be produced using superinfection with wild type (non-hybrid) phages. Many copies of the fusion protein were incorporated in the phage coat among the wild type copies of geneVIIIp. Veronese and colleagues used a 12 amino acid V3 insert into geneVIIIp and the resulting hybrid phages, used as target antigen in ELISA, gave stronger reactivity with immune serum than did
the V3 peptide. The expression vector, pKfdH, carries geneVIII with a Hpa 1 restriction site 9 base pairs (3 amino acids) from the mature N-terminus of geneVIIIp for cloning the desired sequence.

**Results**

To produce hybrid phages displaying copies of the wildtype and mutant V3 loops for use as antigen in ELISA, the V3 loops were PCR amplified using minipreps of wildtype and mutant gp120 in bluescript as templates. The primers (Table 4.1 page 125) each contained a Sea I restriction site and sequences which annealed to the unmutated extremities of V3. The PCR products (Figure 4.23) were cloned into a T vector, since digesting the PCR product directly with Sca I proved to be extremely inefficient.

Following confirmation of the correct sequences, the Sca I fragment was isolated and subcloned into pKfdH linearised with Hpa I (Figure 4.24). To identify transfected colonies containing plasmids with a single insert in the correct orientation, the plasmid DNA was isolated and screened by PCR (Figure 4.25). From clones containing only single inserts, the orientation was ascertained using, as sense primer, an oligonucleotide which annealed to pKfdH in the region of gene VIII and a V3-loop antisense primer. Positive clones were sequenced to confirm the correct orientation of the inserted V3 sequence and to ascertain that no frame shift had occurred which would have made non-sense of part or all of the fusion protein (Figure 4.26). Plasmids containing a correct V3-loop fusion protein construct were identified for each of the 4 mutants 4A, 4C, 4E and 1E but not, unfortunately, for the wildtype V3 loop.

The rescued phages from cultures containing the 4A expression construct were analysed for the presence of 4A-V3-gene VIII fusion proteins in an ELISA using sheep anti M13 phage antibody and the mouse V3 specific monoclonal antibody (ADP 3047) which is able to recognise mutant 4A (Figure 4.22). Phages were easily detectable with anti-M13 antibody but the V3 monoclonal antibody failed to react with the putative ‘4A hybrid phages’. Veronese et al showed that
Figure 4.23
PCR products coding for Wildtype and mutant V3 loops 4E, 4C, 4A and 1E showing the 6 base pair deletion in 4E and 1E which removes the amino acids Q and R from the wildtype sequence.
Digest with Sca I
purify fragment and
ligate to pKfdH

Phage gene VIII
Hpa I

Screen by PCR for single inserts
Screen positive colonies again by PCR for orientation

pKfdH

Sequencing
Express fusion protein as part of hybrid-phage coat
Use hybrid-phages as antigen in ELISA for V3 reactivity

Figure 4.24
Cloning of V3 loop into vector pKfdH
Figure 4.25
90 colonies screened by PCR for the presence of a single insert. Most are positive, with a single band at 147 base pairs, about 10-15% are negative (no PCR product) and again about 10-15% show the presence of larger bands at 294, 441 base pairs and above indicating the presence of concatemers of 2, 3 or more inserts.
Figure 4.26
Cloned V3 loops 4A and 1E in pKfdH vector, for expression of the GeneVIIIIP-V3 fusion protein.
western blots are able to detect the presence of the fusion protein, if present, as a
fainter band of increased size. Due to the expected extremely low copy number
of the fusion protein in these experiments (described below), gels were
purposefully overloaded in order to have sufficient fusion protein present for
detection. Western blots using the sheep antibody detected no extra band in any
of the ‘hybrid phage’ lanes (results not shown).

**Discussion**

In the experiments presented here, the V3 loop section amplified by PCR
contained both the cysteines and the two asparagine glycosylation sites just
outside the loop to encourage V3 loop folding and possibly disulphide bond
formation. This attempt to present V3 in as native-like a configuration as
possible meant that the size of this insert was almost as large as geneVIIIp itself
and the fusion protein was likely to be incorporated at approximately 5-10 copies
per phage (T Terry, personal communication).

Based largely on discussion with Dr. T. Terry, it is probable that it is the
expression phase of the experiment which has failed. The size of the V3 loop
compared to the gene VIII protein means that the production of phages
containing the V3-geneVIII fusion protein requires optimisation of both inducer
IPTG concentration and multiplicity of infection of the helper phages. In her
laboratory, different inserts appear to require different conditions for expression
and this is at present unexplained. Fab antibody fragments fused to gene VIIIp
have been expressed successfully (Kang, et al. 1991) which means that there is
precedent for inserts substantially larger than the V3 loop to be inserted into the
phage coat, although large polypeptides (in this case single chain Fv) have been
found to be expressed more efficiently as fusions with the phage gene III protein
(Kretzschmar and Geiser 1995).
The manipulation of immunogenicity of individual epitopes is an important and expanding direction in vaccine design (Berzofsky 1991: Berzofsky 1993) not only for gp120 but also for many other important antigens. In the system above the phages coat the ELISA plates efficiently and have the potential to present the epitope either at a greater density (Markland, et al. 1991) or in a preferred conformation (Perham, et al. 1995) to the reacting antibodies. Based on the success of Veronese et al, if the copy number of the fusion protein is kept high by minimising the size of the inserted sequence, then it may be a robust and useful method for expression of individual well defined epitopes. Since these phage constructs can also be used as immunogens resulting in elicitation of neutralising antibodies with epitope specificity (Perham, et al. 1995) this expression system is potentially a powerful tool applicable to the analysis of individual epitope antibody responses.
CHAPTER 5

IMMUNOGENICITY OF MUTANT gp120 IN THE MOUSE

Introduction

As described in Chapter 4, certain V3 mutations had resulted in the loss of binding of some of the V3-specific monoclonal antibodies. A polyclonal response to the mutated gp120 may lack antibodies specific for these native epitopes and depending on the immunogenicity of the native V3 loop in the mouse, the loss of this subset of antibodies may or may not be detectable. To more fully understand the relationship between V3 immunogenicity and that of other regions of gp120 it would be necessary to measure the reactivity of sera from mutant gp120-immunised mice with the different forms of V3. The reactivity with the native V3 loop might reflect on the immunogenicity of V3 and possibly which regions of V3 are most important. The reactivity of the same sera with the mutant V3 loop would indicate whether the mutations had affected the immunogenicity of V3. One goal of this project is to record changes in the degree of response to constant regions of gp120 which may have resulted from the putative reduction in V3 immunogenicity. Not only constant regions, but perhaps other variable regions of gp120 may have altered immunogenicity and it was therefore necessary to measure the reactivity of the same sera with other epitopes of gp120 to see if the response to any of them had altered
Results

Mice were immunised with nucleic acids to avoid the time consuming preparation of recombinant mutant proteins in sufficient quantity, purity and with the native conformation. Although not as high titred as the response to gp120 following immunisation with protein plus certain adjuvants, nucleic acid immunisation (NAI) had proved successful in eliciting a strong immune response to gp120. As described in Chapter 3, the peptide reactivity of sera following NAI is increased and the mechanism for this is at present unclear, but since there was no apparent difference in epitope recognition between protein plus adjuvant immunisation and NAI, this was considered to be a quantitative rather than a qualitative difference.

Only a subset of the desirable assays were possible due to lack of availability of wildtype and mutant conformational V3 loops as target antigens for ELISAs, although an attempt had been made to produce recombinant wildtype and mutant V3 loop fusion proteins for this purpose as described in Chapter 4. The cost of buying bespoke V3 peptides to represent all different mutants was prohibitive especially if these were to be cyclised to present the loop in a more native-like conformation. In the absence of other alternatives, the mutant gp120 (CHO, freeze-dried) proteins and the wildtype gp120 (baculovirus) protein were used as the target antigens in ELISAs.

The kinetics of gp120-specific IgG development, IgG endpoint titre, subclasses of IgG and reactivity with denatured gp120 were measured using wildtype gp120 as the target antigen. The development of mutant gp120-specific IgG was measured using the relevant mutant (see below for definition) gp120 from CHO cells. To assay individual epitope responses to V3 and other regions of gp120 the same linear peptides were used as in Chapter 3.
Reactivity with whole wildtype gp120

Native gp120

6 groups of CBA/Ca (k haplotype) mice were injected with (1) Wildtype gp120 gene (2) Mutant 4E gene (3) Mutant 4C gene (4) Mutant 4A gene (5) Mutant 1E gene and (6) a control plasmid without the gp120 gene. Figure 5.1 shows the development of the serum IgG response measured on baculovirus (wildtype) gp120. Figure 5.2 shows the IgG endpoint titres at week 8. Immunisation with all mutant gp120 genes resulted in a gp120-specific IgG response except mutant 1E, the behaviour of which has been discussed above. There was no significant difference found between any of the other groups (F=2.76, p=0.61).

Denatured gp120 and subclasses of IgG

The same assays were made for the different subclasses of IgG as in Chapter 3 (Figure 5.3 cf. Figures 3.7 and 3.8). There was no difference between the mutants or between the wildtype and the mutants and the pattern of IgG1 and IgG2a was the same as that in the previous cohorts of mice. Similarly there was negligible reactivity with denatured gp120, again following the same pattern seen in the previous cohorts (Figure 5.4 cf. Figures 3.11 and 3.12).

Reactivity of sera with the relevant mutant gp120

Figure 5.5 shows the reactivity of serum IgG with the relevant mutant protein. "Relevant" is used in this context to indicate that the mutant protein used as the target antigen had the same V3 sequence as the mutant gp120 gene used to immunise that cohort of mice. The pattern of IgG reactivity remains similar to Figure 5.1 but 4E serum has shown an increased reactivity with its relevant mutant target antigen. There is still no statistically significant
IgG response kinetics in k haplotype mice immunised with wildtype and mutated Nucleic acids. Reactivity measured with baculovirus wildtype gp120.

Figure 5.1
Serum 1:100 IgG reactivity with baculovirus wildtype gp120. Mice were injected 4 times with plasmids carrying the Wildtype (n=8), Mutant 4A (n=8), Mutant 4C (n=8), Mutant 4E (n=7) and Mutant 1E (n=8) gp120 genes and with a control plasmid (n=8). Injections were 100μg DNA in 100μl volume at weeks 0, 2, 4 and 6 - one day after the serum sample had been taken.
Endpoint dilutions of gp120-specific IgG in k haplotype mice immunised with wildtype and mutated Nucleic acid

**Figure 5.2**
Log 10 endpoint dilution of gp120 specific IgG reactivity with baculovirus wildtype gp120. The endpoint was taken to be that serum dilution equal to the mean + 2 S.D. of a cohort (n=8) of mice immunised with the control plasmid.
IgG1 and IgG2a subclasses of gp120-specific serum antibody in k haplotype mice immunised with wildtype and mutated Nucleic Acids

Figure 5.3

gp120 specific IgG1 and IgG2a. Serum (1:300 and 1:900) reactivity with baculovirus recombinant (wild type) gp120.
Comparison of serum IgG reactivity with native and denatured baculovirus gp120 in k haplotype mice immunised with wildtype and mutated Nucleic Acids

Figure 5.4
Reactivity of serum 1:100 IgG from mice immunised with different mutant gp120 genes assayed on native and denatured baculovirus recombinant gp120. Native and denatured data are from separate ELISAs. Rat monoclonal antibodies were used as controls for denaturation and capture as described in Figure 3.13.
IgG response kinetics to relevant mutant CHO produced gp120

Figure 5.5
Serum 1:100 IgG reactivity of individual nucleic acid immunised mice with CHO cell line produced relevant mutant gp120. gp120 was secreted into serum free medium, freeze dried and concentrated x 20, then captured in ELISA with Sheep anti-gp120 D7324.
difference between Wildtype, 4A and 4E, \((F=2.42, p=0.115)\) however, 4C has significantly lower IgG at week 8 than the closest of these, 4E \((F=11.21, p=0.005)\). 4C was only just significantly raised above the controls \((F=4.68, p=0.048)\). The most noticeable feature is the apparent increase in reactivity of 4E serum with its relevant mutant target antigen compared with its reactivity with the wildtype antigen, however, it was not significantly increased.

**Peptide reactivity**

The endpoint titre of peptide reactive IgG (Figure 5.6) was in the same range as in the cohort of the same haplotype (k) of mice in Chapter 3 (Figure 3.20). The V3 peptide shows an appropriate lack of reactivity with sera from mutants 4C and 4E which were immunised with a gp120 gene containing the greatest extent of serine substitutions in V3. However, mutant 4A serum is not significantly different from Wild Type with respect to the endpoint IgG reactivity with the wildtype V3 peptide \((F=0.02, p=0.889)\). 4A was also significantly raised above 4C, the only other mutant showing any detectable V3 peptide reactivity \((F=8.3, p=0.012)\).
Figure 5.6
Log 10 endpoint dilution of peptide reactive IgG. The V3 long peptide is a 37-mer, the V3 short, C1 and C terminus peptides are 20-mers. The endpoint criteria were the same as those used in Figure 3.2.
Discussion

Reactivity with whole wildtype gp120

Although the mean IgG levels at week 8 are not significantly different, the figures showing the development of the IgG response (5.1 and 5.5) do indicate a trend in which 4A and wildtype may be similar and 4E and 4C may be reduced. Larger numbers would need to be used to increase the chances of detecting such a difference if it exists. The trend reflects the possibility that the N-terminal side of V3 may contain an immunodominant epitope.

It is very disappointing that the mutant 1E construct was flawed. 1E has maximum mutations on the N-terminal side and wild type sequences on the C-terminal side of GPGR and is therefore the complementary mutant to 4A. In future work using 1E and other mutants such as 2E, 3E, 2A and 3A (Table 4.2) it might be possible to delineate the precise regions of V3 which have detectable effects on it’s immunogenicity. It is possible that the N-terminal side of V3 is dominant or that GPGR itself is the main V3 epitope but that the recognition of GPGR can be disrupted by mutations in certain positions in V3 possibly by altering V3 conformation and the availability of GPGR.

Reactivity with the relevant mutant gp120

This data is difficult to interpret because the assay does not allow a clear comparison between the mutants. The results suggest that 4E serum shows a trend towards increased reactivity with its relevant target antigen (Figure 5.5) compared with its reactivity with baculovirus wildtype target (Figure 5.1), although this increase does not reach significance. However, the possible problem with the concentration of the captured antigen (discussed below) does not apply to 4E, and the implication of the apparent trend is that the V3 mutation in 4E has abolished some epitopes and created new ones. The loss
of some original epitopes is suggested by the reduced 4E serum reactivity with the wildtype target and the existence of new epitopes is suggested by the augmented reactivity of the sera with the 4E target over that seen with the wildtype target. The changes in total 4E-specific IgG levels may thus be reflecting the changes in 4E V3 reactivity and if so it may indicate that V3 contains a very powerful epitope which is possibly immunodominant. It is possible that these 'new epitopes' have been created by a local distortion in the conformation of gp120 in mutant 4E. Alternatively, however, the 4E mutation may have produced distortions at sites distant from V3 and that these are the locations of the putative new epitopes. Similarly, the substituted serines themselves may be involved.

The actual V3 mutation of 4E includes a deletion of amino acids Gln (Q) and Arg (R) adjacent to GPGR (Figure 4.8). In analysis of multiple isolates, V3 shows little length variation (Simmonds, et al. 1990) and it may be that V3 is intolerant of length changes which would be in contrast to the substantial length variation viably sustained by the V1/V2 regions (Bosch, et al. 1994) and in V4 and V5 (Simmonds, et al. 1990). From the database of HIV sequences (Meyers et al, Los Alamos National Laboratories, Los Alamos, New Mexico, U.S.A.) the consensus sequence for V3 lacks these two amino acids. IIIB is therefore untypical in the length of its V3 loop. However, since it is a viable strain of the virus, it must have been able to accommodate the Q-R 'insertion', possibly by cooperative changes in regions associated with V3 in the tertiary or quaternary structure. The removal of these two amino acids in 4E is thus a length change for IIIB and may have caused the suggested local or distant distortions. 4C has no such length change and this could be reflected in the suggested difference between 4E and 4C seen in Figure 4.22.

The amount of 4C captured in the ELISA shown in Figure 5.5 may be reduced compared with the other target proteins. Western blots with both the rabbit gp120 antiserum and the sheep anti C-terminus antibody confirmed
that 4C was considerably less concentrated in the freeze dried preparation, but at this stage of the experiments there was insufficient antigen available to make the necessary titrations. Several attempts were made to equalise the amounts of the different mutants captured by D7324 using rabbit polyclonal antiserum to detect them. Bearing this in mind it is not possible to say whether the apparently poorer reactivity with 4C protein compared with 4E is due to reduced concentration of target antigen or to lesser immunogenicity of 4C due to lack of putative new epitopes.

**Peptide reactivity**

The overall gp120 reactivity and the V3 peptide reactivity of sera following wildtype and 4A immunisations is comparable. In sera following 4C and 4E immunisation, the overall gp120 reactivity is reduced and V3 peptide reactivity is not measurable. Taken together, this suggests that V3 reactivity reflects total gp120 reactivity in all these cases. V3 may be therefore immunodominant in these mice when immunised with nucleic acids. Alternatively, the mutation of V3 may have caused conformational changes which adversely affected a different epitope and this epitope may be immunodominant. The N-terminal side of GPGR may contain or contribute to the immunologically important V3 epitope rather than the C-terminal side. This follows from the fact that 4A has the wild type sequence on the N-terminal side of GPGR and the observations that the reactivity of mutant 4A serum and of wildtype serum are not significantly different but those of 4C and 4E, which have N-terminal side mutations, are apparently reduced.

**Possible responses to serine substitutions or to carbohydrate**

It is possible that the increased reactivity of 4E antiserum with the 4E target is due to the existence of a response to the blocks of substituted serines which is detectable only on the mutant protein, however this is unlikely due to the physical characteristics of serine as described. The N-linked
glycosylation sites are not altered in any of the mutants, however, the immunogen in these experiments was gp120 generated and glycosylated in mouse cells and the target antigens in the two different assays (Figures 5.1 and 5.5) were gp120 glycosylated in insect cells and gp120 glycosylated in CHO cells respectively. Therefore the possibility that a difference in glycosylation has contributed to the observed differences in this assay cannot be entirely ruled out.
CHAPTER 6

GENERAL DISCUSSION

The aims of this project

The aim of this project was to test the hypothesis of epitope competition using gp120 as a model protein possibly resulting in the construction of a mutant(s) gp120 which might have application as part of a subunit vaccine for HIV-1, but the experiments presented here do not allow a firm conclusion to be drawn. These experiments suggest that V3 is immunodominant or is, at the very least, an extremely powerful epitope in the mouse which is an important consideration for future work since V3 is such a powerful and important B cell epitope in humans. We have successfully altered the immunogenicity of gp120 by substituting blocks of serines on either side of the GPGR motif in V3. The results strongly suggest that V3 immunogenicity has been reduced in some of these constructs.

Changes in the immunogenicity of constant regions of gp120 following V3 mutation have not been recorded. One of the most important constant regions is the CD4bs but two other constant regions epitopes have been identified (a region within C1 and the extreme C-terminus) whose reactivity is not lost in the mutants and the issue of possible epitope competition is still open.

Some of the mutant gp120 proteins show detectable conformational changes as a result of the serine substitutions which has important
implications for mutant gp120 constructs for use as subunit vaccines as discussed below. We have broadly located the region of V3 which appears to be most important in B cell epitopes, the N-terminal side of GPGR, and these results are consistent with those of others. However, NAI has not been used before for analysis of B cell epitopes of gp120 and it is important that this convenient method appears to be a relevant mode of immunisation for this type of analysis.

**Epitope specificity and design of gp120 subunit vaccines**

Analysis of the neutralising epitopes of gp120 has revealed ways in which intervention may be beneficial. Powerful neutralising antibodies to variable regions may have a role despite the usual strain specificity which limits their activity. With present and future knowledge of the conserved functional regions of gp120, it may be possible to identify epitopes which mediate neutralisation of divergent strains (Berzofsky 1991; Berzofsky 1993).

V3-specific antibodies correlate with the serum neutralising activity and with the clinical course (Fenouillet, et al. 1995). V3 variability itself may not be an insurmountable obstacle since small numbers of V3 sequences have been identified which may collectively represent the majority of isolates (Holley, et al. 1991). Some of the variability may be predictable (Holley and Karplus 1991; Korber, et al. 1993; Milich, et al. 1993) and it might therefore be possible in some way to utilise antibodies with mutual escape-excluding specificities analogously to using drug combinations.

The apparent paradox of the existence of a powerful neutralising epitope on gp120 is eloquently outlined by Nara et al (Nara, et al. 1991). It is possible that the easily shed gp120 monomer is functioning as a decoy molecule to draw the immunological attention away from similar epitopes on the oligomeric complex (Sattentau and Moore 1995). It is thought that
dissociation of gp120 from gp41 may be mediated by conformational changes which depend on CD4 binding (Pinter, et al. 1993). Nabs to V3 and Nabs to CD4bs synergise in their activity (Cavacini, et al. 1993; Montefiori, et al. 1993; Thali, et al. 1992; Tilley, et al. 1992) which implies a reciprocal allosteric interaction (Pinter, et al. 1993) between V3 and the CD4bs. Therefore, V3 may be more exposed on the soluble monomer subsequent to conformational changes which allow it to dissociate from the virion anchored gp41 and this may allow V3 to mediate the proposed decoy mechanism.

Point mutations which subsequently disrupt the binding of neutralising antibodies (Nabs) is a frequent finding (Klasse, et al. 1993; McKeating, et al. 1993; Veronese, et al. 1993; Yoshiyama, et al. 1994). The observation that limited substitutions can turn previously neutralising antibodies into enhancing antibodies (Kliks, et al. 1993) also makes the elicitation of a neutralising response potentially hazardous. These point mutations may be within the binding site of the Nab affected or may act at a distance by mediating conformational changes which affect the Nab epitope. As previously described the binding of Nabs to the CD4bs enhance the binding of Nabs to V3 (and vice versa) and these antibodies synergise in their neutralising activity. This indicates that there may be a conformational change (Pinter-1993, Robertguroff-1994) which leads to an increase in accessibility of V3 which may allow it to mediate the subsequent step(s) required for fusion. gp120 may utilise its conformational flexibility to mediate both viral escape and its other functions.

Evidence for carbohydrate participation in neutralisation epitopes is increasing, including O-linked structures (Boyd and James 1992; Gorny, et al. 1994; Hansen, et al. 1991a) although the extent of carbohydrate involvement is unknown. Significant attention may need to be directed to the carbohydrate structures of gp120 since apart from the amino acid variability, it is also possible that there may be patterns in the variability of carbohydrates.
A precedent for this is the observation that the set of glycoforms generated is not a random mixture as originally supposed but has a definite profile dependent on the cellular environment in which the glycosylation occurred (Lis and Sharon 1993). Antibodies to carbohydrates which are neutralising for lymphocyte infection can act as infection enhancing antibodies for monocytes (Hansen, et al. 1991b). More optimistically, Lee (Lee, et al. 1994) et al have shown that epitopes mediating enhancement can be removed by site directed mutation and leave neutralising epitopes unaffected, however, the problem of viral mutation in vivo still remains. Since the pattern of glycosylation can influence the formation of neutralisation epitopes (Benjouad, et al. 1992: Gram, et al. 1994), it is possible that gp120 may also be able to escape neutralisation through effects on the glycosylation mechanisms of the ‘host’ cell. This has implications for the methods of synthesis of subunit vaccines.

The CD4bs is conserved and generates broadly crossreactive neutralising antibodies (Thali, et al. 1991) and these are prevalent in sera from infected individuals (Hariharan, et al. 1993: Moore and Ho 1993). While other constant regions can also be the target of Nabs (Nakamura, et al. 1993) the CD4bs may be the most important of these in vaccine design. The mutant gp120 constructs described here were designed to avoid the problem of V3 variability by disabling its immunogenicity. Subsequently other variable regions could be similarly disabled with the eventual aim of engineering a molecule to which the neutralising response is dominated by constant epitopes. Following immunisation with this construct, even though incoming viruses would have native V3 loops, it is possible that the circulating neutralising antibodies to constant region(s) may prevent viral entry to cells and constitute sterilising immunity. A possible disadvantage of these particular ‘disabled’ V3 loops is that the synergy in neutralisation between V3 and the CD4bs may be lost unless the V3 response was to GPGR.
The same problem may apply to the ENV 59 construct of Pollard et al (Pollard, et al. 1992) which lacks the V3 loop but retains GPGR.

Changes in epitope exposure and the creation of new epitopes is a widely observed phenomenon and an important consideration in the design of subunit vaccines. Sattentau and Moore (Sattentau and Moore 1995) describe five broad groups of epitopes which can generate Nabs. Important among them are those epitopes which are present only on the native oligomeric form (Broder, et al. 1994: Sattentau and Moore 1995). There have been many observations of conformational changes which create new neutralisation epitopes following the interaction of gp120 with antibodies or with CD4. These include V3 specific antibody complexed gp120 (Denisova 1995) and CD4 complexed with gp120 (Kang, et al. 1994: Kang, et al. 1993: Thali, et al. 1993).

Unprocessed gp160 does not display the same epitopes as monomeric gp120 (Abacioglu, et al. 1994: Pinter, et al. 1993) and virus infectivity requires that gp160 is cleaved to produce gp120 and gp41 (McCune, et al. 1988). It is therefore unlikely that gp160 presents epitopes which are identical to those presented by gp120/41 oligomers. This may be reflected in the relative failure of gp160 immunisation compared with gp120 immunisation (Berman, et al. 1990: Earl, et al. 1994: Pincus, et al. 1994: VanCott 1995) indicating that gp160 is far from ideal as an immunogen, although the case for using it is argued strongly (Montefiori, et al. 1993: Rovinski 1995: VanCott, et al. 1995).

Collectively these findings indicate the importance of using an immunogen which presents epitopes which are encountered by the immune system when the oligomeric gp120/41 complex is present and functioning. A sugar-based crosslinking strategy (Chen, et al. 1991) has been used for efficiently producing homomultimers of CD4. This method or others might be adaptable to the gp120/41 complex and its higher order structure to produce an immunogen from which gp120 is unable to dissociate and subsequently
elicit responses to the monomer. Along these lines, crosslinked gp120-CD4 has successfully induced antibodies to epitopes which are cryptic on monomeric gp120 but exposed following CD4 binding (DeVico, et al. 1995). The mutant gp120 constructs described here, or others with similar antigenically disabled V3 loops, may need to be presented in the context of the native oligomer. Preferably, the oligomer would be crosslinked to prevent subsequent presentation of oligomer-cryptic epitopes to foil the putative role of gp120 monomer as a decoy. If the crosslinking were able to prevent conformational change in the immunogen, the induced response might include antibodies which, by binding to the oligomer on the virus, prevented the conformational changes required for infection and were therefore neutralising.

It has been suggested that the infection-enhancing/neutralising activity of some antibodies to gp120 may depend on whether or not they cause dissociation of gp120 from gp41. High affinity binding to the CD4bs may cause dissociation (Moore, et al. 1990) whereas lower affinity may not cause dissociation but mediate viral attachment to the cell surface where subsequent detachment of the antibody from the virus allows infection of the cell (Levy 1993). Since there is a distinct danger of inducing infection enhancing antibodies following vaccination and if it is correct that enhancement is mediated by lower affinity interactions, it may be essential to induce only high affinity antibodies. To this end, either the constructs described here or those of Pollard et al (Pollard, et al. 1992) in which the CD4bs has been proved to be functional, might be used in a dissociable form. It might be possible to present the gp120 in such a way that only those clones of B cells which had high affinity interactions with gp120 could respond. Those with lower affinity, putatively unable to cause the conformational changes required for dissociation of gp120 from gp41, would be deprived of antigen and be less likely to receive T cell stimulation. This scenario would require
the covalent attachment of gp41 to a solid phase support which was too large
to be endocytosed by B cells, with the gp120 non covalently associated with
gp41 as normal. This idea obviously requires a great deal of refinement before
its feasibility can be judged. Apart from the induction of enhancing
antibodies there may be further dangers associated with vaccination. In some
cases, the existence of maternal antibodies hampers the development of the

**Animal Models**

The most valuable animal model for AIDS would be one in which the
virus HIV-1 caused an AIDS-like disease. The rhesus macaque
(*Macaca mulatta*) and the cynomolgus monkey (*M. fascicularis*) develop an
AIDS-like illness but only after infection with SIV, not HIV-1 and the
chimpanzee (*Pan troglodytes*) although susceptible to HIV-1 infection, does
not develop disease. Studies in chimpanzees could also be misleading since it
appears that the potency of their neutralisation response to HIV-1 is ten fold
greater than that of humans and yet the duration of the human response may
be significantly longer (Berman, et al. 1994). Recently the pigtailed macaque
(*M. nemestrina*) has been shown to be both susceptible to HIV-1 and to
develop an ‘AIDS-like’ disease, characterised by an acute and then persistent
infection together with lymphadenopathy, skin rash and febrile episodes

The severe combined immune deficiency (SCID) mouse reconstituted
with human primary lymphoid tissue (SCID-Hu mouse) is, however, an
alternative to the simian immune system models. These mice are infectable
with HIV-1 and primary responses to V3 can be elicited (Ifversen, et al. 1995).
They can be rendered resistant if the human donor was immunised with a
vaccinia-gp160 construct followed by rgp160 (Mosier, et al. 1993) and passive
protection in SCID-Hu mice can also be achieved (Gauduin, et al. 1995), as it can for chimpanzees (Emini, et al. 1992).

In vitro findings must ultimately be tested in vivo, in man, and the use of animal models as in intermediate step is a very complex issue. Fortuitously, the intense research activity into the human lentivirus HIV-1 has led to an increased understanding of other animal lentiviruses including FIV and BIV (Gonda 1992). It is my personal feeling that the scientific community should not be remote from responsibility towards other species, even in the pursuit of scientific understanding. In my own opinion we should direct our attention towards the development of models for the human immune system such as the SCID-Hu mouse, and away from endangered species (Prince, et al. 1988) such as the chimpanzee. It may transpire that the SCID-Hu mouse response to HIV-1 is no more artificial than that of the chimpanzee, and it is potentially more immunologically relevant since it involves interactions between this virus and cells of the human immune system.

**T cell epitopes**

There is still controversy on the subject of the presentation of T cell epitopes. Some authors find that the flanking sequences in the native protein can affect the processing and therefore the presentation (Eisenlohr, et al. 1992: Leclerc, et al. 1993: Shivakumar, et al. 1989) while others find no influence of flanking regions (Ahlers, et al. 1993: Thomson, et al. 1995). The existence of multideterminant regions and promiscuous T cell epitopes in HIV-1 proteins (Hale, et al. 1989) offers the chance of inducing broad cross reactivity by vaccines containing minimal numbers of epitopes. However, there is the possibility that the existence of such promiscuous epitopes is an adaptive response of the virus. These promiscuous peptides may have less than
optimal affinity for each MHC allele to which they bind. A medium affinity peptide present in potentially large quantities may successfully compete with higher affinity peptides for the MHC binding groove. The virus may therefore be protected from high affinity T cell recognition.

**Nucleic Acid Immunisation**

The major issue in the field of NAI is the potential hazard of the persistent DNA. Although Wolff et al (Wolff, et al. 1992) searched diligently for evidence of plasmid DNA integration into the host cell chromosome, this has not been found but the risk of insertional activation of oncogenes or of other transformation events remains. Potentially RNA could be used to avoid this danger (Zhou, et al. 1995). In order to optimise the uptake of DNA after injection and possibly augment the resulting immune response investigations have been made into the effects of dose, volume, age and gender (Davis, et al. 1993: Ulmer, et al. 1994). Although higher doses of DNA (400 μg) have been used in the experiments presented in this thesis, it is possible to achieve protective immunity with the intramuscular injection of as little as 1μg of DNA (Ulmer, et al. 1994) or 2 x 0.4 μg DNA on gold particles delivered to the epidermis by the 'gene gun' (Fynan, et al. 1993). DNA can also be effectively delivered intravenously and mucosally (Fynan, et al. 1993). Systemic delivery of DNA in cationic liposomes results in the transformation of cells in multiple tissues especially the lungs and spleen (Zhu, et al. 1993). However, the expression of injected naked DNA appears to be limited to skeletal and cardiac muscle (Acsadi, et al. 1991: Davis and Jasmin 1993).

Since tolerance to specific antigens can develop it is possible that some of the mice in the nucleic acid cohorts, which apparently did not respond with gp120-specific IgG had responded by becoming tolerant to gp120. Genetically modified myoblasts carrying genes for foreign proteins can be injected into
skeletal muscle where they subsequently fuse with mature myofibres (Barr and Leiden 1991: Dhawan, et al. 1991: Yao and Kurachi 1992). Subsequently, steady state levels of greater than 1ng/ml (close to human physiological levels) of human growth hormone (Dhawan, et al. 1991) have been found possibly indicating tolerance induction in the immunised animal. However other studies, which have measured the serum levels of recombinant proteins, record peaks as high as 1µg/ml which then decline simultaneously with the rise in specific antibody (Yao and Kurachi 1992). Myoblast injection might possibly be a tool for investigating the putative induction of tolerance to endogenously synthesised foreign antigens following NAI.

**Summary and conclusion**

To summarise the findings of these experiments:- V3 appears to be immunodominant in these mice. Mutation, by serine substitutions in V3 can reduce the overall immunogenicity of gp120 which may be due to reduced V3 immunogenicity but this is not conclusive. The mutant gp120 constructs can fold but in some cases an altered overall conformation is detectable by monoclonal antibodies to all regions which still bind to the mutated molecules but with reduced affinity. The antibody responses to constant regions of gp120 mutants and the wildtype are detectable Alterations in the immunogenicity of these regions has not been recorded but this issue remains open.

In conclusion, the experiments described in this thesis indicating subtle but detectable conformational changes in gp120 following mutations of V3 highlight an essential theme. It may be vital, for the induction of an antibody response to gp120 which will be beneficial, that the immunogen is presented in that conformation which will induce only a subset of antibodies, without precluding stimulation of T cell responses. Whether it is presented as protein
plus adjuvant, nucleic acid or other future mode of immunisation, it is possible that this will remain the most important consideration in the design of a subunit vaccine for HIV-1 gp120.
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The Immune Response to HIV gp120 Induced by Nucleic Acid Immunization (NAI)\textsuperscript{a}

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The HIV-1 envelope glycoprotein, gp120, is involved in virus infection through binding to CD4 on T cells.\textsuperscript{1} This has made it a candidate protein for AIDS vaccines.\textsuperscript{2} Structurally, gp120 consists of constant regions, with very little variation between different virus isolates, and hypervariable regions which exhibit strain-specific differences. Although the hypervariable regions contain epitopes capable of eliciting neutralizing antibodies, the high mutation rates represent a mechanism of virus escape from immune surveillance. We are attempting to construct mutant gp120 molecules in which the highly conserved principal neutralizing determinant (PND) of the V3 loop\textsuperscript{3} is maintained, but the immunogenicity of the hypervariable residues of the V3 loop is reduced.

The antibody response to baculovirus-derived recombinant HIV-1 type IIIB gp120 was examined in different strains of mice. The levels of gp120-specific serum immunoglobulin G (IgG) elicited depended both on the choice of mouse strain and on the adjuvant used. Furthermore, the antibodies preferentially recognized discontinuous epitopes on HIV-1 gp120 (Peet \textit{et al.}, manuscript in preparation). In nucleic acid immunization (NAI) the antigen is synthesized \textit{in vivo} and may be processed differently to proteins used in conventional immunization. To examine if this could influence the nature of the immune response to HIV-1 gp120, recombinant plasmids expressing gp120 were injected directly into mouse muscle.

Mice of the CBA/Ca strain (6–12 weeks old) were preinjected with either 25 μl of 0.5% bupivacaine (1-butyl-N-(2,6-dimethylphenyl)-2-piperidinecarboxamide hydrochloride) one day before, or with 100 μl 10 mM cardiotoxin (from \textit{Naja niger collis} snake venom) seven days before, the first injection of plasmid DNA. Two different recombinant gp120 plasmids were used: pEE6.gp120GS containing the entire gp160 with a stop codon introduced at the extreme C-terminus of

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FIGURE 1. Mice given (a) bupivacaine and pEE14 control plasmid, (b) bupivacaine and pEE14:PA,gp120 (dotted line indicates a control mouse given pCDM8), (c) bupivacaine and pEE6:gp130GS, and (d) cardiotoxin and pEE6:gp120GS.
gp120 in order to express HIV-1 gp120 with the viral signal sequence, and pEE14tPA.gp120.3 where the secreted gp120 sequence has been linked to the signal sequence of human tissue plasminogen activator. Both plasmids contain the human cytomegalovirus (hCMV) promoter. pCDM8 or pEE14 without gp120 were used as control plasmids. pCDM8 was obtained from Dr. B. Seed (Massachusetts General Hospital, Boston) and the remaining plasmids were obtained from Dr. P. Stephens (Celltech Ltd. UK). Each mouse received injections of 100 µl (1 mg/ml) Qiagen-purified plasmid into the hind leg at 0, 2, 4 and 6 weeks. The antibody response to HIV-1 gp120 at a serum dilution of 1/100 was determined using a capture enzyme-linked immunosorbent assay (ELISA) with baculovirus-derived HIV-1 gp120 as the antigen.

Control plasmids did not provoke a gp120 response (Fig. 1a). However, intramuscular injections with HIV-1 gp120 expression plasmids resulted in a varied but long-lasting response in the injected mice. When followed for up to 26 weeks significant levels of gp120-specific IgG could be detected (Fig. 1b). The antibody response to gp120 showed substantial variation between different individual mice. In most animals appreciable levels of gp120-specific IgG were detectable after three injections with gp120 plasmid DNA. It should be noted that the IgG response was determined using baculovirus-produced gp120 which may differ immunologically from gp120 synthesized in mammalian cells due to glycosylation differences. However, although it is possible that the low responders preferentially have antibodies to epitopes on gp120 not readily available on insect cell-derived gp120, a more likely explanation would be that either the plasmid DNA failed to be expressed or, possibly, that tolerance to the antigen was induced in these mice.

The poor or nonresponsiveness seen in a few of the mice seemed not to be due to the choice of facilitator or the particular signal sequence used (which could affect the efficiency of HIV-1 gp120 secretion). However, whilst no striking difference in either the IgG levels or the number of responding mice was observed between the mice preinjected with the facilitator bupivacaine or cardiotoxin (Figs. 1c and 1d), injection with the dose of bupivacaine used was not well tolerated, with some animals dying of cardiac arrest immediately following administration. In contrast, no adverse effects were seen in the mice preinjected with cardiotoxin. These observations suggest that, because NAI is usually carried out on young mice in which the window between suboptimal and lethal dose of bupivacaine is fairly narrow, cardiotoxin may be the preferred facilitator.

The tissue plasminogen activator signal sequence results in a much improved production rate of recombinant gp120 in Chinese hamster ovary (CHO) cells compared with constructs using the viral signal sequence (P. Stephens, personal communication). However, no striking differences in the HIV-1 gp120-specific IgG levels were observed in the mice injected with pEE14tPA.gp120 versus pEE6.gp120GS.

In conclusion, an appreciable specific immune response was mounted against HIV-1 gp120 following intramuscular injection of plasmid DNA which was affected neither by the signal sequence used nor by the choice of facilitator.

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