Biological consequences of human immunodeficiency virus type 1 envelope polymorphism: does variation matter?

1995 Fleming Lecture

Delivered at the 134th Meeting of the Society for General Microbiology, 26 March 1996

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Introduction

Human immunodeficiency virus type 1 (HIV-1) establishes persistent infections in humans, in most cases leading to the development of AIDS. HIV-1 infects CD4+ lymphocytes, monocytes and dendritic cells in the peripheral blood and lymphoid organs, and microglia in the central nervous system (Gartner et al., 1986; Koenig et al., 1986; Pope et al., 1994). This virus tropism correlates with expression of the cell surface antigen CD4, which has been shown to be the principal receptor interacting with the virus surface glycoprotein, gp120 (Dalgleish et al., 1984; Klatzmann et al., 1984). However, cell surface expression of CD4 alone is not sufficient to confer susceptibility to infection by HIV-1. Recently, several members of the chemokine receptor family of G-protein coupled seven transmembrane spanning proteins were identified as additional coreceptors (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). Virus infection is accompanied by a progressive loss of CD4+ cells leading to immunodeficiency. Recent studies of virus and lymphocyte dynamics suggest that HIV infected lymphocytes have a reduced half-life of 2–3 days (Cao et al., 1995a; Ho et al., 1995; Wei et al., 1995). This rapid destruction of infected lymphocytes is only partially compensated for by lymphocyte production, leading over time to a progressive decline in CD4+ cells and a concomitant increase in virus burden. Understanding the molecular mechanisms of virus–host cell interaction is a prerequisite for understanding pathogenesis.

The envelope (Env) glycoprotein of HIV-1 is an integral membrane protein that projects from the surface of the virion and is responsible for interacting with cellular receptors and initiating fusion of the virus and cell membranes. Env is also the major target of the neutralizing antibody response and hence is considered the logical focus for vaccine research. I would like to take this opportunity to review both our own work and that of others in this fast moving field and attempt to highlight areas of future investigation.

HIV variation

Surprisingly large amounts of virus are present in the lymphoid tissues of HIV-infected individuals over the course of disease progression, even during periods of clinical latency (Embreton et al., 1993; Pantaleo et al., 1993). In the plasma, virus appears to turn over rapidly with a virion half-life of approximately 6 h and an estimated $10^9$ virus particles generated daily (Cao et al., 1995a; Ho et al., 1995; Wei et al., 1995). Since most single-stranded RNA viruses undergo $3 \times 10^{-5}$ mutations per nucleotide per replication cycle it is intriguing to understand why HIV appears more variable than other retroviruses. Earlier reports suggested that HIV reverse transcriptase was more error-prone (Mansky & Temin, 1995). However, it is now thought that the main driving force is likely to be the high levels of virus replication. With the production of $10^9$ virions daily and a genome size of $10^4$ nucleotides, virtually all possible mutations are generated daily (Wain-Hobson et al., 1995). This reservoir of genetic variants or ‘quasispecies’ enables the virus to adapt rapidly to its changing environment, for example by infecting ‘new’ target cells or replicating in the presence of drug/immune response.

HIV-1 has been genetically classified into the major (M) and outlier (O) groups. The M group is further divided into nine sub-types based on sequence diversity, where members of the same subtype differ by less than 10%, and those of different subtypes by 15% or more (Louwagie et al., 1993; Myers, 1994). At present, there is considerable effort in genotyping viruses recovered from recently infected individuals to gain insights as to the types of viruses being transmitted. To date, there is no clear association between biological phenotype, including tropism and sensitivity to neutralization, and genetic subtype leading us to question the relevance of genetic subtyping. However, this is unsurprising given that variation within an individual can result in a spectrum of viruses exhibiting differences in cell tropism, replication rate and cytopathicity (see below).

This extensive antigenic variation led Nowak et al. (1991) to mathematically model the time course of HIV infection, resulting in the ‘antigenic diversity threshold hypothesis’. The
authors suggested that increasing antigenic variation over time eventually overwhelmed the host’s immune system, resulting in AIDS. The authors predicted an association between disease progression and increasing levels of antigenic variation (Nowak et al., 1991; Nowak & May, 1993). However, recent observations show that genetic diversity is inversely related to rates of disease progression (Delwart et al., 1994; Lukashov et al., 1995; Wolinsky et al., 1996). These data argue that increased levels of antigenic variation merely reflect virus replication in the face of an active immune response.

Biological consequences of HIV variation

**In vitro classification of virus isolates**

HIV is generally isolated from infected individuals by the infection or cocultivation of patient lymphocytes with donor phytohaemagglutinin-stimulated peripheral blood mononuclear cells (PBMC). Hence, PBMC are regarded as the ‘universal’ target cell(s) for in vitro propagation of HIV. HIV isolates may be categorized in vitro according to their ability to replicate in macrophages and established cell lines and to induce cytopathic effects (CPE). In summary, two groups of viruses may be defined: those which replicate in primary T cells, defined as macrophage or M-tropic; and those which are capable of infecting established T cell lines, defined as T-tropic. CPE consist of multinucleated giant cell (syncytium) formation and the lysis of single cells. These virus-induced CPE are likely to contribute to the destruction and loss of CD4 lymphocytes in vivo. Some viruses replicate slowly and do not induce multinucleated CPE (termed slow/low and nonsyncytial inducing; NSI); others replicate with faster kinetics and are able to induce syncytia in their target cells (termed fast/high and syncytial inducing; SI) (Asjo et al., 1986; Cheng-Mayer et al., 1988; Fenyo et al., 1988; Tersmette et al., 1989a). Generally, SI viruses are able to infect immortalized T cell lines (i.e. T-tropic) and NSI viruses replicate well in monocyte/macrophage cultures but fail to replicate in established T cell lines (M-tropic). Koot et al. (1992) reported that the human T-lymphotropic virus-I-transformed MT-2 cell line was the most sensitive ‘indicator’ cell for observing cytopathicity, whereby the majority of laboratories classify NSI/SI phenotypes based on virus replication in MT-2 cells. However, we have noted CPE in infected PBMC cultures when the same cultures fail to induce syncytia in the MT-2 cell line, suggesting that cytopathicity may not always correlate with T-tropism.

It is interesting to note that the earliest viruses to be isolated were derived from symptomatic individuals and were able to replicate in established T cell lines. Such viruses (e.g. LAI, RF, MN) were propagated extensively in cell lines and became the prototypic viruses studied by the majority of laboratories. However, it is now apparent that viruses propagated only in PBMC cultures, ‘primary’ viruses, differ from laboratory-adapted isolates in several important respects including cell tropism and sensitivity to neutralization by various ligands, which will be addressed in this lecture.

The most important question to be addressed is whether in vitro classifications correlate with pathogenesis in the host. Several authors report that the majority of viruses isolated from asymptomatic infected individuals are M-tropic and of the NSI phenotype (Asjo et al., 1986; Connor et al., 1993; Connor & Ho, 1994; Schuitemaker et al., 1992; Tersmette et al., 1989b). Several studies suggest that viruses isolated during the course of disease progression change their in vitro properties from NSI to SI phenotype, suggesting that the appearance of SI virus may be associated with a more rapid CD4 cell decline and onset of symptoms (Cheng-Mayer et al., 1988; Connor & Ho, 1994; Karlsson et al., 1994; Schuitemaker et al., 1992; Tersmette et al., 1989a). However, it is important to note that 40% of infected individuals die from AIDS-related symptoms without developing SI viruses, suggesting that SI virus is not essential for disease progression (Karlsson et al., 1994; Koot et al., 1992). Cell tropism is also relevant to understanding transmission: both Langerhans and dendritic cells have been suggested to be the primary cells targeted, suggesting that viruses exhibiting such tropisms may be preferentially transmitted (Soto-Ramirez et al., 1995; Spira et al., 1996). However, virus Env sequences amplified from a number of recently infected individuals failed to show consensus signature patterns for transmitting viruses (Zhu et al., 1993; Zhang et al., 1993). This may reflect our current lack of knowledge in interpreting biological phenotypes from primary sequence data.

**Virus–receptor interactions**

Recently, members of the chemokine receptor family of G-protein coupled seven transmembrane spanning proteins, LESTR and CKR-3 and 5, were identified as coreceptors for T-tropic and M-tropic isolates (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). This finding suggests that the NSI/SI phenotype ‘switch’ observed in some infected individuals may correlate with a change in coreceptor usage. Although the tissue and cell-type distribution of CKR-3 and CKR-5 are not completely characterized, current evidence suggests that they are likely to contribute to in vivo infection by M-tropic viruses. Doranz et al. (1996) reported that the SI M-tropic virus, 89.6, was able to use LESTR, CKR-3 and CKR-5, suggesting that this virus may be considered as an intermediate between the M- and T-tropic viruses. Given the differences between these coreceptors, if direct Env glycoprotein coreceptor interaction(s) occur they are likely to involve conserved conformational motifs not apparent from inspection of primary amino acid sequences. This is analogous to the interaction of chemokines with this family of different receptor proteins. It is likely that LESTR, CKR-3 and CKR-5 are not the only coreceptors utilized by HIV (Choe et al., 1996; Doranz et al., 1996). The virus population present within an individual has the potential to
adapt to use different members of this multi-gene receptor family, suggesting that selection for different coreceptor usage may increase virus diversity. The natural ligand(s) for LESTR is unknown; however, the chemokines RANTES, MIP-1α and MIP-1β, recently identified as the major CD8 soluble suppressive factors, bind the CKR-5 receptor (Cocchi et al., 1995). The ability of RANTES, MIP-1α and MIP-1β to selectively inhibit NSI virus infection conflicts with the earlier observations of Mackewicz et al. (1995) who reported that the CD8 soluble factors inhibited both NSI and SI virus infection. Clearly, the possibility exists that the natural LESTR ligand may also be secreted as a soluble protein and may selectively inhibit SI virus infection.

Another factor to be taken into account when attempting to correlate in vitro virus properties with virulence markers for disease progression in the host is whether an isolate is representative of viruses within the host. HIV is considered to exist as a population of distinct variants or ‘quasispecies’, and in vitro isolation has been reported to lead to a loss of genetic polymorphism (Sabino et al., 1994; Simmonds et al., 1990; Wain-Hobson, 1989). This is in contrast to the observations of Spira & Ho (1995) who reported that the most frequent env sequence obtained from cultured PBMC reflected the predominant sequence found within the plasma of the same individual 6 months later. We analysed virus populations by PCR length analysis pre- and post-culture and showed that the most predominant clones in the uncultured material failed to replicate in culture. However, proviral DNA may include defective genomes unable to replicate in culture. Shaw (1995) reported a low number of defective proviral genomes present in PBMC suggesting that this is not the explanation for the selective amplification of some clones in culture. In order to study tropism of the virus in vivo, we cloned gp120 ORFs directly from the brain and spleen of two individuals who died of AIDS with symptoms of dementia. This approach of cloning genes directly from infected tissue avoids the selection which occurs on in vitro isolation. Viruses expressing brain-derived glycoproteins were shown to be NSI and to infect glial cells via a CD4-dependent route using the coreceptors CKR3 and CKR5. Work is currently in progress to establish whether differences in coreceptor utilization are found between viruses expressing glycoproteins derived from different tissues.

Donaldson et al. (1994) reported a selective amplification of SI viruses in PBMC cultures. This hypothesis is supported by the observation that the chemokines RANTES, MIP-1α and MIP-1β, selectively inhibit NSI virus infection (Cocchi et al., 1995), suggesting a selective bias for SI virus replication in PBMC cultures. However, such selection is not routinely observed since the majority of virus isolates are of the NSI phenotype. One possible explanation is differential chemokine expression by different donor CD8 cells in vitro, such that some cells fail to express detectable levels of these soluble factors. Elevated levels of chemokine expression may be involved in controlling HIV load and replication in individuals who do not progress to AIDS and may explain why some individuals exposed to virus remain uninfected (Paxton et al., 1996).

### Envelope determinants of tropism and cytopathicity

The envelope glycoprotein is expressed as a gp160 precursor which is proteolytically cleaved to yield the extracellular and transmembrane domains, gp120 and gp41. The Env glycoprotein is extensively N-glycosylated, where carbohydrate constitutes approximately 50% of the molecular mass and probably obscures much of the protein's surface. The mature native envelope glycoprotein expressed at the infected cell surface is a noncovalently associated heterodimer, forming an oligomeric structure that probably comprises three gp120–gp41 heterodimers (Blacklow et al., 1995; Rao et al., 1995). Noncovalently associated oligomeric proteins typically interact over large areas, with hydrophobic interactions often playing important roles in subunit–subunit interactions (Hurtley & Heleneius, 1989). Interactions between adjoining gp120 molecules have been reported (Earl & Moss, 1993; Owens & Compans, 1990); however, extensive mutational analyses have shown the first 129 amino acids of the gp41 ectodomain to be principally responsible for oligomerization (Earl et al., 1990; Earl & Moss, 1993). The oligomeric nature of the Env glycoprotein has received much attention recently, since oligomerization may affect glycoprotein antigenicity (Earl et al., 1994; Broder et al., 1994; Richardson et al., 1996).

The gp120 molecule is responsible for the primary attachment of the virus to the cell surface receptor CD4, resulting in a number of conformational changes within the Env–CD4 complex which expose the gp41 fusion peptide (reviewed in Moore et al., 1993c). Thus, interaction of Env with CD4 triggers fusion of the virus and cell membranes via interactions with the coreceptors by a process termed ‘receptor-mediated activation of fusion’. To determine if extracellular gp120 is capable of conferring the fusion phenotype independent of gp41, we constructed a number of chimeric viruses, based on the T cell line-adapted molecular clone HXB2, expressing heterologous gp120 glycoproteins derived from infected PBMC cultures showing both NSI and SI phenotypes (Fig. 1a). Comparative biological characterization of the parental primary viruses and of the chimeras demonstrated identical patterns of cell tropism and cytopathicity for all clones derived from NSI cultures. However, chimeric viruses expressing gp120 ORFs amplified from PBMC cultures which gave rise to SI virus were shown to be of an NSI phenotype, suggesting either a bias in the cloning procedure or, more likely, that NSI clones constitute the predominant sequences present in cultures, agreeing with reports of a biased replication of SI clones in vitro. Furthermore, all the chimeric clones were able to replicate only in PBMC and failed to infect any of the T cell lines tested, suggesting that the viruses were unable to utilize the LESTR coreceptor present on the cell lines. To
address this possibility, we monitored the ability of the parental and chimeric viruses to infect cells transiently expressing CD4 and LESTR or CKR-5. The parental HXB2 virus was only able to infect cells expressing CD4 and LESTR, whereas all of the chimeric viruses failed to infect LESTR-positive cells and replicated in the CD4/CKR-5 cells. These data demonstrate that gp120 determines coreceptor usage and cytopathicity independently of gp41, confirming that gp120-mediated interactions with the cell surface define fusion and hence virus tropism(s) (J. Lewis, A. Murphy, P. Balfe & J. A. McKeating, unpublished).

To model events taking place between virus and the cell surface we followed Env glycoprotein interaction(s) with soluble forms of the CD4 receptor (sCD4). The binding of sCD4 to the surface of cell line-adapted virions or infected cells resulted in a series of conformational changes: modulation of monoclonal antibody (MAb)-specific epitopes on gp120 (McKeating et al., 1993; Sattentau & Moore, 1991) (reviewed in Moore et al., 1993c), increased exposure of gp41 epitopes and dissociation of gp120 from gp41 (Allan et al., 1991; Moore et al., 1990). Similar CD4-induced conformational changes were reported for soluble gp120, suggesting similarities in the interaction of monomeric and oligomeric Env glycoproteins with CD4 (Clements et al., 1991; McKeating et al., 1993; Shotton et al., 1995; Wyatt et al., 1995). However, Moore et al. (1992) reported that several primary Env–CD4 interactions failed to induce gp120–gp41 dissociation suggesting either a ‘stronger’ gp120–gp41 interaction (McKeating et al., 1991) or a reduced Env–CD4 interaction. It seems unlikely that all of these in vitro measured effects are essential for fusion, although some may represent stages in the fusion process.

Variation in gp120 is found predominantly in five regions, V1–V5, which are interspersed between five conserved regions, C1–C5 (Modrow et al., 1987). These conserved regions are thought to constitute the functional core of the glycoprotein and include a number of hydrophobic residues. This core structure has been shown to include the minimal CD4 receptor binding site, such that deletion of the first, second and third variable regions of the Env glycoprotein results in a truncated protein capable of binding CD4 with an affinity comparable to that of the parental molecule (Jeffs et al., 1996; Pollard et al., 1992; Wyatt et al., 1993). Given the level of variation present in env, and the probable plasticity of the molecule, it is important to define interactions between domains and their possible role in glycoprotein function. Since no gp120 tertiary structural information is available, these interactions are being defined mainly from studies of the viability and antigenicity of defined envelope variants (Fig. 2).

The V1, V2, V3 and V4 regions form disulphide-linked
Table 1. Summary of defined neutralization epitopes

<table>
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<tr>
<th>Epitope</th>
<th>Immunogenicity of epitope and nature of elicited antibodies</th>
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<td>Immunized rodents</td>
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<td>CD4bs</td>
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<td>Conf</td>
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<td>gp41 aa 662–668</td>
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loops, which have been reported to interact both with one another and with conserved domains. To date, associations have been identified between the V3 and CD4 binding site (Moore et al., 1993; Willey & Martin, 1993; Wyatt et al., 1992); V2 and V3 (Andeweg et al., 1993; Koito et al., 1995); C1, C2 and C5 (Moore et al., 1993) and the V2 and C4 regions (Freed & Martin, 1994; Wang et al., 1996; McKeating et al., 1993). Mutagenesis of the V2 and V3 loops results in fusion-defective glycoproteins, suggesting a role for these regions in cell entry. The V3 loop has been reported to be the primary determinant of both macrophage tropism and cytopathicity, leading to the suggestion that 'signature sequence patterns' exist for the NSI and SI phenotypes (Chesebro et al., 1992; De Jong et al., 1992; Fouchier et al., 1992; Hwang et al., 1991; Korber et al., 1994a, b; Shiода et al., 1994). Since the variable loops have a role in determining tropism and in the induction of neutralizing antibodies, it is likely that amino acid changes in these regions may lead to both altered tropism and resistance to neutralization. McKnight et al. (1995) reported that a single amino acid change in the V3 region resulted in escape from neutralization and altered tropism from neural to macrophage cells.

We (Palmer et al., 1996) and others (Andeweg et al., 1993; Boyd et al., 1993; Groenink et al., 1993; Malykh et al., 1995; Westervelt et al., 1992) have suggested that the V2 region plays a role in determining cell tropism and cytopathicity. Env glycoproteins containing either site-directed mutations in V1V2 or 'foreign' V2 sequences display altered cytopathicity (Andeweg et al., 1993; Boyd et al., 1993; Freed & Martin, 1994; Koito et al., 1994, 1995; Sullivan et al., 1993). In addition, MAbs specific for the V2 region are capable of blocking both cell-free virus infection and cell–cell fusion (McKeating et al., 1993; Moore et al., 1993a; Gorny et al., 1994; Warrier et al., 1994; Shotton et al., 1995; Wu et al., 1995). Interactions of gp120 both with cell surface-expressed and soluble CD4 modulate the exposure of the V2 region (McKeating et al., 1993; Moore et al., 1993a; Shotton et al., 1995b; Wyatt et al., 1995). In summary, these data suggest that CD4–Env interactions induce a number of conformational changes within the V2 region which may be required for coreceptor interaction(s) and subsequent fusion of the virus–cell membranes.

Substitutional and length polymorphism within the V2 region suggest that its functional properties may be defined by interactions between discontinuous residues not readily apparent from inspection of linear sequences (Lamers et al., 1993; Palmer et al., 1996; Wang et al., 1995). Andeweg et al. (1993) suggested that a predicted α-helix in the N-terminal V2 region may be structurally important for syncytium formation. Furthermore, Groenink et al. (1993) concluded that both the mean length and location of potential glycosylation sites within the V2 domain, in addition to the number of positively charged amino acid residues in both V2 and V3, were predictive of an SI or NSI phenotype. We (Palmer et al., 1996) and others (Bosch et al., 1994; Cornelissen et al., 1995; Groenink et al., 1993; Lamers et al., 1993; Wang et al., 1995) reported that sequence variation within the V2 region is most frequently associated with length changes in the carboxyl region, often encoding repeated motifs containing potential glycosylation sites (Bosch et al., 1994; Palmer et al., 1996; Wang et al., 1995). However, since these naturally occurring sequences also exhibit substitutional polymorphism, it is impossible to directly assess the biological significance of length change alone by studying these proteins. We therefore designed a series of mutants to assess the effects of change in V2 length and number of N-linked glycosylation sites on virus phenotype and overall gp120 conformation. These data demonstrated that increases in V2 length did not affect replication rate, cytopathicity or cell tropism of the laboratory adapted virus HXB2 (Fox et al., 1996). In agreement, Wang et al. (1995) reported no association between cytopathicity and V2 sequence of several virus isolates. However, the variant viruses did show altered sensitivity to neutralization by sCD4 and MAbs specific for the V3 and CD4 binding site. One interpretation of these results is that longer V2 sequences of the mutant proteins show reduced interaction(s) with the conserved gp120 'core', thereby enhancing accessibility of the CD4 binding site. These data are consistent with a model where the V2 loop folds into the proximity of the C4 domain.
and thus partially masks this region from CD4. It is interesting
viruses replicated as efficiently as each other these data suggest
mation (Fig. 3).
that such conformational changes are not essential for virus
entry. However, these data show that changes in V2 length
modulate accessibility of the V3 and CD4 binding site,
suggesting that the V2 length polymorphism observed in vivo
may have significant effects on overall glycoprotein confor-
mation (Fig. 3).
To address the significance of such variation, we sequenced
the V1V2 region from infected PBMC obtained directly from
a number of infected individuals and analysed the effect(s) such
sequences would have on virus phenotype by transferring the
heterologous sequences into HXB2 (Fig. 1b). The majority of
chimeric viruses replicated, demonstrating that the sequences,
though genetically distinct, were capable of conferring a viable
phenotype. The viability of chimeras expressing distinct V1V2
sequences suggests that interactions between variable and
conserved regions may be flexible and depend upon conserved
conformational motifs not readily apparent from the primary
sequence. Several of the chimeric viruses were noncytopathic
and only able to replicate in PBMC cultures, demonstrating
that the V1V2 region alone is capable of determining both
cytropathicity and cell tropism.
Phylogenetic analysis of V1 and V2 sequences showed no
evidence for linkage between them, suggesting that these
variable loops evolve independently of each other (Lamers et
al., 1993; Palmer et al., 1996). To address whether these
variable regions could function independently of each other, a
number of sequences were selected, which conferred altered
tropism and/or cytopathicity to HXB2, for the construction of
chimeric viruses expressing only the heterologous V1 region.
Comparisons between these V1 and V1V2 chimeric viruses
demonstrate that viruses expressing heterologous V1 se-
quences exhibit different phenotypes both from the parental
HXB2 and from the chimeric viruses expressing the entire
V1V2 domain. A simple interpretation of these data is not
possible, however, as it is apparent that the V1 region is
capable of affecting virus replication and tropism. Antigenic
characterization of these V1 chimeric gp120 proteins demon-
strated that foreign V1 sequences disrupted V2 conformation,
whereby none of the V2 MAbs, specific for discontinuous
epitopes, recognized chimeric gp120 proteins. These data
imply possible interaction(s) between these two variable loops
such that amino acid changes in V1, resulting in phenotypic
changes, may act via a direct role of the V1 region or indirectly
by affecting V2 conformation. No mutagenesis studies have
been reported for the V1 region, largely due to a lack of
conserved features. However, Boyd et al. (1993) reported an
in vitro selected variant of the M-tropic JRCSF virus, capable
of replicating in T cell lines, with a single amino acid change
in V1. Furthermore, the block to JRCSF infection of T cell lines
was at the level of entry (Cann et al., 1992). Recent reports
confirm that JRCSF uses the CKR-5 coreceptor expressed on
macrophages, and hence a single change within V1 has allowed
the variant to utilize the LESTR coreceptor. These data suggest
that the V1V2 region may be involved in interactions with the
coreceptor post-CD4 binding.

Antigenicity of envelope glycoproteins and
identification of neutralization epitopes

Antigenicity of laboratory adapted virus antigens
Gp120 is the major target for the neutralizing antibody
response, where epitopes within V2, V3 and the discontinuous
CD4 binding site have been shown to induce antibodies
capable of neutralizing laboratory isolates (McKeating et al.,
1992; reviewed in Fenyo et al., 1996). Immunization of rodents
with recombinant gp120 derived from laboratory adapted
prototypic strains demonstrated that both the V2 and V3
regions are immunodominant inducing isolate- or type-specific
antibodies, whereas antibodies specific for the discontinuous
CD4 binding site were generated at low frequencies (Mc-
Keating et al., 1996c). In contrast, the majority of human MAbs
distinguish discontinuous epitopes overlapping the CD4 bind-
ing site, suggesting a significant difference between infected
individuals and immunized rodents in the immune responses
generated (Table 1). Vaccine efficacy trials in humans based
on recombinant prototypic soluble glycoproteins have been
disappointing, in that vaccinee sera neutralized only laboratory
adapted isolates closely related to the immunizing strain and
failed to neutralize primary isolates (reviewed in Fast et al.,
1995). The ineffectiveness of these immune responses may be
due to the use of monomeric antigens leading to the induction
of antibodies unable to recognize native oligomeric glyco-
protein, the immunodominance of the variable loops resulting
in type-specific neutralizing antibodies or the use of laboratory
adapted virus antigens. To address the latter possibility, we
immunized rats with recombinant antigen (W61D) derived
from a primary SI isolate, including gp120 and gp160 from
which the gp120 cleavage signal had been removed. Prelim-
inary data suggests that the gp160-immunized animals have
generated an immune response which preferably reacts with
oligomeric Env, whereas the gp120 immune sera reacted
poorly with all antigens tested. Furthermore, all of the MAbs
generated from the gp160-immunized rats recognize conser-
vated conformation-dependent epitopes present on the majority
of primary Env glycoproteins tested (unpublished data). These
data are in agreement with that reported by Earl et al. (1994).
One interpretation of these data is that the variable
loops are not as immunogenic in primary gp-160 immunized
rats; alternatively, the variable loop sequences of the im-
munogen are more related to those of other primary viruses
tested. The majority of studies reporting the antigenicity of
'oligomeric' glycoproteins have utilized antigens in which the
gp120—gp41 cleavage signal was removed to prevent dissocia-
tion (Richardson et al., 1996). Since the N and C termini of

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gp120 are thought to interact with gp41 in the oligomeric form, the effect such modifications may have on conformation and antigenicity are unclear (Helseth et al., 1991; Schulz et al., 1992). However, the gp120–gp41 complex may be more stable for primary viruses than for laboratory adapted viruses (Groenink et al., 1995; Sattentau et al., 1993). Thus, synthetic gp120–gp41 antigens based on primary Env sequences may retain oligomeric structure when expressed in vitro.

Some primary viruses are sensitive to neutralization by a limited number of human MAbs and polyclonal sera (Burton et al., 1994; Conley et al., 1994; Mascola et al., 1994, 1996; Moore et al., 1995; Sattentau et al., 1993; Trkola et al., 1995). However, the induction of such antibodies in response to recombinant antigen is rare (McKeating et al., 1996c). We (Jeffs et al., 1996) and others (Pollard et al., 1992; Wyatt et al., 1993) have reported that removal of the variable loops resulted in a molecule, PRI2, capable of binding sCD4 with an affinity comparable to that of the full-length protein. Furthermore, removal of these loops increases accessibility of the C1 and C4 regions to MAbs (Jeffs et al., 1996; McKeating et al., 1996a). We therefore investigated the immunogenicity of the PR12 protein with the intention of re-directing the immune response from the variable loops to the more conserved regions critical for gp120 function. Immunization of rodents with PRI2 induced a cross-reactive neutralizing response, whereas immunization with the full-length gp120 protein induced a type-specific neutralizing response, as reported by others. These results are consistent with the immunodominant nature of the variable regions, which generally lead to the induction of antibodies which only neutralize homologous virus.

In summary, we have shown that removal of the V1–V3 loops from LAI gp120 results in a protein with altered immunogenicity compared to the full-length protein. Differences were found both in the ability of the immune sera to recognize glycoproteins of diverse origins and in their ability to neutralize viruses expressing these glycoproteins. These data lead us to conclude that the immune response to PR12 is directed toward conserved epitopes present on the majority of virus glycoproteins. None of the MAbs derived from the PR12-immunized rats were able to neutralize virus infectivity and hence the epitopes responsible for the induction of this broad cross-neutralizing activity remain to be elucidated. In an attempt to increase the neutralizing response elicited by PR12, we are presently immunizing rats with deleted forms of oligomeric gp160 and soluble gp140 proteins. Future experiments will compare immune responses generated to both monomeric and oligomeric forms of these Env glycoproteins. It is hoped that these modified immunogens will have enhanced immunogenicity and be models for future vaccine trials in humans.

Variation in patterns of glycosylation

The Env glycoprotein is highly glycosylated and potential sites are located predominantly in the variable loops (Leonard et al., 1990). Several reports suggest that these glycan modifications are essential for correct processing, folding and oligomerization of the glycoprotein (Fenouillet & Jones, 1995; Li et al., 1993; Wu et al., 1995). It is interesting to note that deletions and/or insertions in the variable regions frequently encode repeated motifs containing potential glycosylation sites (Bosch et al., 1994; Palmer et al., 1996; Wang et al., 1995). We (McKeating et al., 1993; Shotton et al., 1995) and others (Back et al., 1994) have reported that addition or removal of such glycans can affect the ability of MAbs both to bind and to neutralize. It is possible that antigenic regions on primary Env glycoproteins may be masked by glycans (Gabriel & Mitchell, 1993). However, no clear discrimination can be made between primary and T cell line-adapted viral Env sequences both with respect to the number and location of potential glycosylation sites. It is possible that cell type-dependent glycosylation patterns may result in proteins with different antigenicities. In support of this hypothesis, we have observed marked differences in the ability of MAbs specific for the C1, V3 and C4 regions to recognize the same gp120 protein expressed in either human embryonal kidney or Chinese hamster ovary (CHO) cells. Further comparisons between solubilized viral Env and recombinant gp120 expressed in several cell lines and phytohaemagglutinin-activated PBMC suggest that processing and glycosylation patterns differ. Our data would suggest that CHO-derived antigen is a poor mimic of viral PBMC-derived protein. These data suggest that care should be taken in the choice of cell type used for the production of recombinant antigens for vaccine trials.

Primary virus glycoproteins

Primary viruses are generally insensitive to neutralization by sCD4 and antibodies which are capable of neutralizing laboratory adapted virus isolates (Ashkenazi et al., 1991; Daar & Ho, 1991; Moore et al., 1995; Turner et al., 1992; Wrin et al., 1995). Understanding the mechanism(s) of this resistance is of great importance, both in terms of developing effective immunotherapies and in vaccine design. The recent reports that laboratory adapted viruses differ from primary NSI viruses in their utilization of the LESTR coreceptor are unlikely to account for the differences in sensitivity to neutralization, since primary SI viruses, defined by their ability to infect LESTR-expressing MT-2 cells, are similarly resistant to neutralization (Fenyo et al., 1996). It is now apparent that the study of T cell line-adapted viruses may lead to the development of therapeutics which may be ineffective in vivo. One example of this is the failure of sCD4-based therapies in clinical trials despite their effective neutralizing activity in vitro for laboratory adapted viruses (Daar & Ho, 1991).

Several reports have shown that short-term PBMC cultures retain some of the genetic variability present in vivo, in contrast to the relatively invariant virus population present after long-term propagation in T cell lines. The majority of reports...
relating to primary virus glycoprotein function have studied
single proteins, with the implicit assumption that a single clone
would be representative of the virus population (Ashkenazi et
al., 1991; Daar & Ho, 1991; Moore & Ho, 1995; Turner et al.,
1992). In order to address the characteristics of primary virus
populations and their interactions with CD4 and neutralizing
antibodies, we have studied multiple gp120 glycoproteins
derived from several primary isolates. Characterization of
these proteins demonstrated that an ‘isolate’ consists of a
polymorphic population of antigenically distinct viruses.
Considerable variation was observed both in neutralization
epitopes and in the CD4 binding site, suggesting that these
regions are not well conserved and therefore may not
constitute suitable targets for vaccine design (Murphy et al.,
1996). The variation observed within the CD4 binding site was
surprising given the numerous studies reporting the highly
conserved nature of this site (Olshovsky et al., 1990; Sattentau
et al., 1986). In contrast, the V3 region was conserved amongst
all the clones derived from primary virus isolates (LaRosa et al.,
1990). However, despite antigenic conservation of V3 epitopes
none of the V3 MAbs tested were able to neutralize the
primary virus stocks or the chimeric viruses expressing the
same gp120 ORFs. These data are consistent with previous
reports of the inaccessibility of the V3 region on the primary
oligomeric protein (Bou-Habib et al., 1994; Earl et al., 1994;
Stamatatos & Cheng Mayer, 1995). Furthermore, we have
shown that thrombin, a protease recognizing the GRAF motif
present within the V3 loop, is less active with primary gp120
proteins compared to the laboratory adapted MN strain (Fig.
4). This differential activity cannot be ascribed to differences in
the protease recognition site (J. Lewis, A. Murphy, P. Balfe &
J. A. McKeating, unpublished). In contrast to previous reports,
these data imply that the V3 region on primary Env
glycoprotein is partially occluded in both the monomer and
oligomer. Thus, exposure of the variable loops may differ
between primary and laboratory adapted isolates and may
contribute to the observed differences in neutralization (Figs 2
and 3).

Several reports considering the resistance of primary
viruses to neutralization by sCD4 have proposed that this is
due to a reduced affinity of the envelope for CD4, apparent in
the oligomeric form of the glycoprotein, but not in the
monomer (Ashkenazi et al., 1991; Turner et al., 1992). However, this conclusion was based on the failure to demonstrate differences in the affinities of monomeric gp120 proteins from primary and laboratory cultured viruses for sCD4 (Turner et al., 1992). Our results suggest that such conclusions may be misleading, since monomeric gp120 proteins derived from single primary virus isolates display a wide range of relative binding affinities for CD4, such that the overall population may demonstrate a reduced net affinity. It is interesting to note that over 50% of the gp120 proteins cloned from two viral RNA preparations demonstrated minimal binding of CD4, suggesting that CD4-independent routes of infection may exist. To address this possibility, and to determine if these proteins were derived from replication competent viruses, we transferred a number of gp120 ORFs into the infectious molecular clone HXB2. Preliminary data demonstrate that the majority of chimeric viruses expressing proteins with low or negligible CD4 binding are nonviable; however, one virus expressing gp120 with a minimal affinity for CD4 replicates as efficiently as another clone expressing gp120, cloned from the same PBMC, with an affinity for CD4 equivalent to HXB2. These data suggest that some of these ORFs were derived from infectious virus.

Sullivan et al. (1995) proposed that the conformation of the primary virus oligomer, which confers neutralization resistance, also results in a reduced affinity for CD4 and decreased efficiency of virus entry into cells. This model is supported by the observations of Kabat et al. (1994) who compared the rate-limiting steps in infection of primary and T cell line-adapted viruses. These authors demonstrated that primary virus infection was limited by the density of CD4 on the target cell, whereas infection by T cell line-adapted viruses was independent of the level of CD4 expression. These data suggest that T cell line-adapted viruses have acquired a more efficient way of interacting with the cell surface receptors. However, it is not known whether LESTR/CKR-5 coreceptor expression levels will also limit primary virus entry. The mechanism(s) of envelope glycoprotein–coreceptor interaction(s) are unknown, but it will be interesting to assess whether primary glycoproteins with reduced affinities for CD4 will show altered interactions with the relevant coreceptors.

Generally, primary virus isolates are resistant to neutralization by the majority of MAbs, although a small number of human MAbs have been identified which are capable of neutralizing such isolates (Burton et al., 1994; Conley et al., 1994; Muster et al., 1993). Several of these antibodies are currently being evaluated for immunotherapeutic use in humans (Katinger, 1994), including the human MAbs IgGB12, 2G12 and 2F5. However, our data show antigenic variation within all of these epitopes which may limit the success of such MAbs as immunotherapeutic agents. However, some primary viruses are sensitive to neutralization by a limited number of polyclonal human sera. It is interesting to note that there is no correlation between genotype and neutralization type, in that some viruses are sensitive to neutralization by serum from individuals infected with viruses of diverse clades. Likewise, some individuals, including those classified in ‘non-progressor’ cohorts (Cao et al., 1995b), elicit an antibody response that can neutralize viruses from diverse clades (Fenyo et al., 1996; Kostrikis et al., 1996; Nyambi et al., 1995). These results clearly indicate that antibodies capable of neutralizing divergent primary viruses exist in response to a natural infection. The question remains as to whether such antibodies can be generated by a recombinant antigen. At present, the epitopes recognized by such cross-neutralizing antibodies are unknown; it is imperative that future studies be targeted at understanding the nature of such antibodies.

In order to study the contribution and recognition of gp41 epitopes by the neutralizing human immune response we studied the sensitivity of a number of HXB2 viruses, chimeric for gp120 glycoproteins derived from a primary isolate, to neutralization (McKeating et al., 1996b). Both the parental primary and chimeric viruses were insensitive to neutralization by sCD4 and a panel of MAbs, demonstrating that transfer of the gp120 protein alone is sufficient to confer a ‘neutralization-resistant’ phenotype to the clone HXB2. However, the chimeric viruses were more sensitive to neutralization by polyclonal human sera, suggesting that association of primary virus gp120 with the HXB2 gp41 transmembrane protein resulted in an altered conformation affecting the presentation of neutralization epitopes recognized by polyclonal human sera. Alternatively, neutralizing human antibodies may be targeted toward epitope(s) located on gp41 (Muster et al., 1993; Wenisch et al., 1989).

Concluding remarks

The question of what immune responses are essential for protection against HIV infection is fundamental. Although this lecture has concentrated entirely on the humoral immune response, cytotoxic T cells are undoubtedly critical for controlling virus replication. The development of vaccines capable of eliciting cross-reactive immune responses effective against clinically relevant viruses is the ultimate objective of much of the work discussed here. Clearly, the recent discovery of the much sought after secondary coreceptors will lead to the development of small molecule chemokine mimics and will refocus studies analysing the molecular mechanism(s) of virus entry and cell tropism. Some of the pertinent questions will be: do the surface-exposed variable loops mediate interactions with the coreceptor(s)? Do soluble chemokines inhibit infection with virus by direct competition, internalization, phosphorylation or changes in the affinity state through G-protein uncoupling? Is there significant genetic polymorphism in the CKR receptors which may account for the relative resistance of some individuals to HIV infection? If so, do polymorphic CKR receptors differentially stimulate chemokine secretion? Does HIV induce aberrant signalling events via these coreceptors.
which may have pathogenic consequences? Further understanding at the molecular level of differences between T cell-adapted and clinically relevant primary viruses may yield new insights into the biology of virus–cell interactions and hence suggest new avenues for therapeutic intervention. Recent progress in the characterization of oligomeric glycoproteins has suggested new approaches for the study of HIV antigenicity, which may lead to novel vaccine candidates in the future.

I would like to thank Robin Weiss in whose laboratory much of this work was initiated for his encouragement, encyclopaedic knowledge of retroviruses and his intuitive ability to ‘know’ the right experiments to perform. The progress would not have been possible without the support, hard work and advice of Peter Balfe. A special mention to Harvey Holmes for his wonderful parcels of goodies. In addition, I would like to acknowledge my debt to all my colleagues in the lab. who do the ‘real’ work: Daniel Fox, Catherine Jessop, Adam Jones, Julie Lewis, Anthea Murphy, Katie Oliver, Christopher Palmer and Christine Shotton at ICR. Much of this work has been performed in collaboration with: Jeff Almond, Cath Arnold, Jonathan Ball, Jon Clewley, Eva-Maria Fenyo, Simon Jeffs, Jackie May, Francine McCutchen, Susan Zolla-Pazner and finally all the people I should have acknowledged and have forgotten. This work has been supported by the Medical Research Council, Programme EVA and The Lister Institute for Preventive Medicine.

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