Neutralizing Epitopes of HIV-1

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INTRODUCTION

Ideally, a vaccine against human immunodeficiency virus type 1 (HIV-1) would induce neutralizing antibody levels which would provide sterilizing immunity. The levels of antibody required however may not be achievable by vaccination (Gauduin *et al*., 1997; Parren *et al*., 1997; Moore and Burton, 1999; Shibata *et al*., 1999). Nevertheless, neutralizing antibody responses albeit of lesser, but still significant potency, will likely be essential in an HIV-1 vaccine in combination with broadly active cellular responses (Burton and Moore it *et al*., 1998). The necessity or benefit of stimulating B cells that produce neutralizing antibodies has been clearly established in murine infection models with retroviruses and other RNA viruses (Planz *et al*., 1997; Baldridge *et al*., 1997; Dittmer *et al*., 1998; Parren *et al*., 1999; Dittmer *et al*., 1999a; Dittmer *et al*., 1999b).

Effective vaccines have been developed against a number of viral diseases mostly by using empirical methods. Many of these vaccines, including those against smallpox, measles and polio, consist of live attenuated viruses. Live attenuated viruses have consistently provided protection against infection with simian immunodeficiency virus (SIV) and SIV/HIV-1 chimeras in non-human primates. Serious safety concerns exist however which preclude the use of such vaccines in humans (Ezzel, 1997; Baba *et al*., 1999). The immune correlates of protection against infection of macaque monkeys by live attenuated SIV have not been clearly defined and the role of antibodies and cytotoxic T-lymphocytes (CTL) have been questioned (Stott and Schild, 1996; Stebbings *et al*., 1998). Protection against SHIV (SIV/HIV chimeras expressing HIV-1 envelope glycoprotein) by vaccination with attenuated SIV argues against a role of antibodies (Shibata *et al*., 1997; Wyand *et al*., 1999). A series of elegant experiments in which protective immune responses against the Friend retroviral complex were dissected however have shown that T cells ($CD4^+$ and $CD8^+$) and B cells are required to act in concert to achieve protection against pathogenic challenge in mice vaccinated with an attenuated retrovirus (Dittmer *et al*., 1999a). Furthermore, whereas CTL were required to protect against lethal infection, neutralizing antibody responses appeared necessary to prevent persistent infection (Dittmer *et al*., 1999b).

Understanding neutralization of HIV-1 primary isolates is important for a knowledge-based approach for the development of a vaccine against HIV-1. Here we review HIV-1 neutralizing antibodies and their epitopes.

STRUCTURE AND FUNCTION OF THE ENVELOPE GLYCOPROTEIN COMPLEX

The HIV-1 mature envelope glycoprotein complex plays a pivotal role in the early events of virus attachment and entry into the target cell. Neutralizing antibodies found in the sera of infected individuals are primarily directed against this complex. The complex is arranged in a trimeric configuration of heterodimers, each consisting of a gp120 surface subunit non-covalently associated with a gp41 transmembrane subunit, *i.e.,* $(gp120\text{-}gp41)_3$. By comparing the amino acid sequence of gp120 subunits of different HIV-1 isolates, five variable regions (V1-V5) and five conserved regions (C1-C5) have been identified (Starcich *et al*., 1986; Modrow *et al*., 1987). A crystal structure of gp120 lacking the V1, V2 and V3 loops and the C and N termini suggests that the gp120 core is structurally organized into two major domains, the inner and outer domain, and a mini-domain termed the bridging sheet (Fig. 1A) (Kwong *et al*., 1998). The inner domain harbors both the N and C termini of gp120, which are involved in the interaction with gp41 (Wyatt *et al*., 1997), and is the probable site of trimer packing (Kwong *et al*., 1998). The outer domain displays an extensively glycosylated surface and as such is effectively concealed from the humoral response. The bridging sheet is composed of four antiparallel β-sheets extruding from the distal ends of the inner and outer domains. Together with additional contributions from the base of the V1/V2 stemloop structure this domain forms the conserved co-receptor binding-site. The CD4 binding-site (CD4bs) is located within a depression at the interface of the three domains and is relatively well conserved between HIV-1 isolates. Although coordinates for the V1, V2, V3 and V4 loops are missing from the structure, either because they were deleted from the gp120 core (V1, V2 and V3) or because of poor resolution (V4), their approximate positions can be placed in a model of gp120 based on experimental

data in combination with the position of the bases of the loops (Fig. 1B)(Parren *et al*., 1999).

The aminoterminal ectodomain of the gp41 glycoprotein consists of two α-helical regions that are connected by an extended disulfide-stabilized loop region. The aminoterminus of gp41 (residues 1–29) contains the hydrophobic, glycine rich "fusion peptide" which plays a critical role in the fusion of viral and target cell membrane. Three-dimensional structural analysis of peptides corresponding to portions of the ectodomain of gp41 reveal a symmetrical trimer in complex (Lu *et al*., 1995; Tan *et al*., 1997; Weissenhorn *et al*., 1997; Chan *et al*., 1997). In this oligomeric configuration, which probably represents the state after triggering of the fusion process (often referred to as the "hairpin" state), the aminoterminal α-helices form a central parallel coiled coil, around which the carboxyterminal α-helices are packed in an antiparallel arrangement.

The infection process is initiated by attachment of the virus to the target cell via the interaction between the gp120 subunit with the cellular receptor CD4. The subsequent interactions between gp120 and its co-receptors are complex and require conformational changes induced by binding to CD4 (Sattentau and Moore, 1991; Sattentau *et al*., 1993; Thali *et al*., 1993). Presumably, the V1 and V2 loops partially mask both CD4 and chemokine binding sites (Wyatt and Sodroski, 1998), and this masking is fully manifested only in mature oligomeric gp120 (Fig. 1B). Thus, conformational changes triggered by multivalent binding of oligomeric gp120 to a cluster of CD4 molecules displaces the V1, V2 and V3 loops and expose the co-receptor binding-site. The flexibility of the CD4 molecule allows the gp120, with its co-receptor binding-site exposed, to come near the co-receptor for interaction and thus bringing the viral and target cell membranes in close proximity. Upon binding to the co-receptor further conformational changes result in the destabilization of the gp120-gp41 interaction. This triggers gp41 to undergo its transition to form a "prehairpin" intermediate (Chan and Kim, 1998), which includes the insertion of the fusion peptide of gp41 into the target cell membrane and the possible dissociation of the gp120 subunits. The gp41 than undergoes additional conformational changes resulting in the formation of the "hairpin" structure and the fusion of the viral and target cell membranes, which finally results in the introduction of the nucleocapsid with the viral genome into the host-cell.

MATURE OLIGOMERIC ENVELOPE VERSUS VIRAL DEBRIS

The HIV-1 envelope exists in a number of antigenically distinct forms. It is synthesized as an envelope precursor molecule gp160, which oligomerizes and is cleaved into gp120 and gp41 (Allan *et al*., 1985; Robey *et al*., 1985). The mature functional $(gp120\text{-}gp41)_3$ oligomer on the virion surface tends to dissociate (shed) (Poignard *et al*., 1996a) resulting in the release of monomeric gp120 and exposure of gp41 spikes on the virion or infected cell surface. Antibodies against HIV-1 envelope in seropositive individuals may be elicited by any of these configurations. The following observations have led us to conclude that the majority of the response is elicited against the unprocessed gp160 or disassembled envelope (*i.e.,* viral debris) rather than the mature oligomer (Parren *et al*., 1997). 1. Antibodies against HIV envelope retrieved from HIV-1-infected individuals in general have a much higher affinity for unprocessed envelope than for mature oligomeric envelope (Parren *et al*., 1997). 2. Neutralization correlates with antibody binding to mature oligomeric envelope (Roben *et al*., 1994; Sattentau and Moore, 1995; Fouts *et al*., 1997; Parren *et al*., 1998), and primary isolate (see below) neutralizing antibody titers in sera from HIV-1 seropositive individuals are generally poor (Moore *et al*., 1996; Moog *et al*., 1997; Pilgrim *et al*., 1997). Viral debris rather than virions therefore appears to be the primary antigen specificity of antibodies against HIV-1 envelope in HIV-1 infected individuals.

T CELL LINE ADAPTED VIRUSES VERSUS PRIMARY ISOLATES

One of the most noticeable influences on HIV-1 neutralization is that of the origin of the virus producer cell (reviewed in Moore and Ho, 1995; Poignard *et al*., 1996b). The adaptation of HIV-1 to growth in immortalized CD4+ cell lines selects for HIV-1 variants that tend to have a strongly basic V3 loop (Fouchier *et al*., 1992), preferentially utilize CXCR4 as a co-receptor (reviewed in D'Souza and Harden, 1996; Moore *et al*., 1997; Berger, 1997), and have a high affinity for CD4 (Platt *et al*., 1997; Kozak *et al*., 1997). These T cell line adapted (TCLA) viruses are readily neutralized by sCD4 and a large spectrum of different monoclonal antibodies (mAbs) (reviewed in Moore and Ho, 1995). By contrast primary isolates, *i.e.,* viruses obtained by limited passage in primary cultures of activated peripheral blood mononuclear cells (PBMC), may use CXCR4 (termed X4 viruses), CCR5 (R5 viruses) or CXCR4 in combination with CCR5 (R5X4 viruses) (Berger *et al*., 1998). They generally have a reduced affinity for sCD4 and neutralizing mAbs, and generally display a high degree of resistance to neutralization by these ligands (Stamatatos *et al*., 1997; Fouts *et al*., 1997 and reviewed in Moore and Ho, 1995) (Fig. 2).

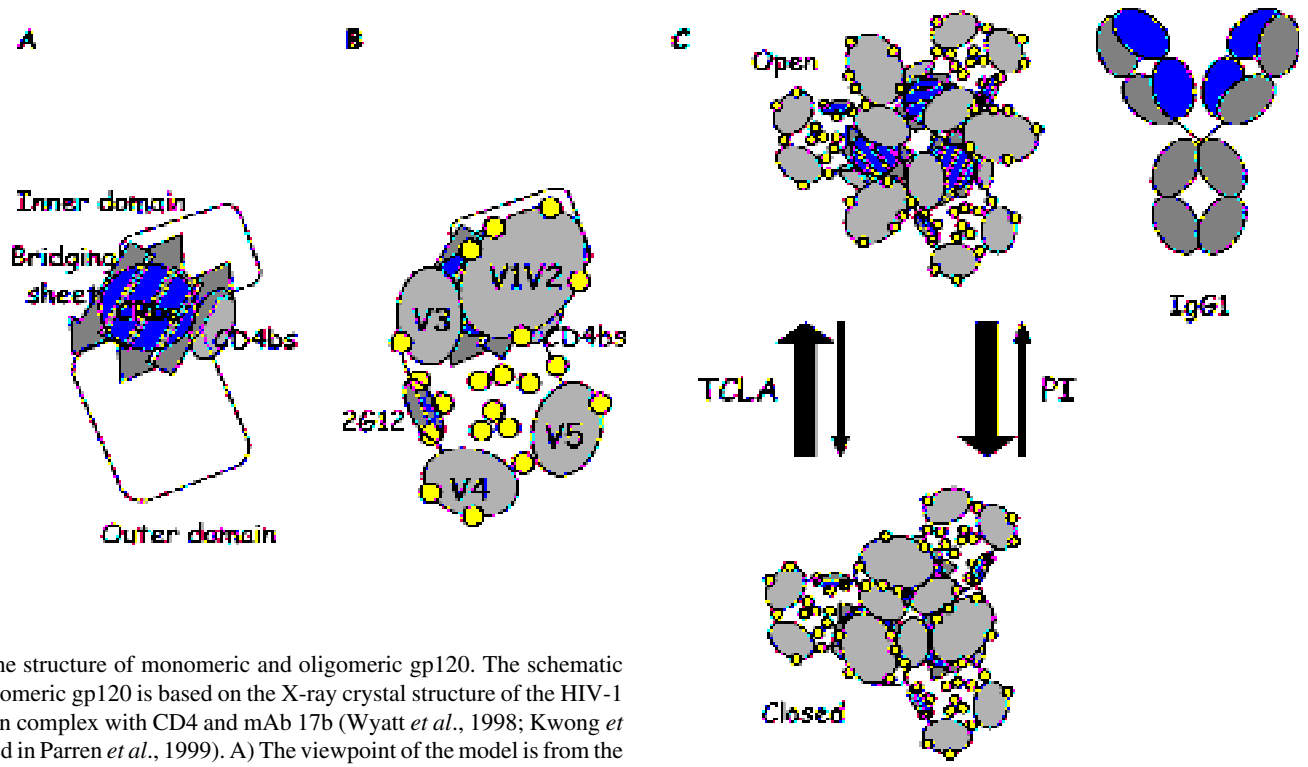

Fig. 1. A model for the structure of monomeric and oligomeric gp120. The schematic representation of monomeric gp120 is based on the X-ray crystal structure of the HIV-1 gp120 (HxBc2) core in complex with CD4 and mAb 17b (Wyatt *et al*., 1998; Kwong *et al*., 1998) and reviewed in Parren *et al*., 1999). A) The viewpoint of the model is from the target-cell membrane. Three structural elements are shown: the outer domain, the inner domain and the bridging sheet. The CD4bs (red oval) is located at the interface of the three domains. The conserved co-receptor binding-site (CRbs; yellow circle) is comprised of the bridging sheet with additional contributions from the base of the V2 loop. B) The location of the variable loops can be placed on the gp120 core, based on experimental data from mAb mapping and mutagenesis studies in combination with the position of the bases of the loops. The V1/V2 stemloop structure partially masks the CRbs and the CD4bs. The inner domain is involved in the interaction with gp41 and is the probable site of trimer packing. The outer domain is extensively glycosylated as indicated by the blue dots. The 2G12 epitope (purple oval) is located at the base of the V3 and V4 loop and probably involves carbohydrate structures. C) Model of mature oligomeric envelope from a target-cell viewpoint. The main characteristic of this model is that oligomeric gp120 can exist in different conformational states of which the two extremes (a closed state and an open state) are shown. In the closed state the masking of the CRbs and the CD4bs by the V1/V2 stemloop structure is fully manifested. The V3 loop also partially obscures the CRbs and the tip of the V3 loop is relatively inaccessible in this configuration. In the open state, the V3 loop is well exposed and the masking of the CD4bs and CRbs by the V1/V2 stemloop structure is less evident. Presumably, the oscilation of mature oligomeric gp120 of primary isolates has an equilibrium biased in favor of the closed state, whereas mature oligomeric gp120 of TCLA viruses is biased toward the open state. Neutralization correlates with antibody binding to oligomeric envelope (Roben *et al*., 1994; Sattentau and Moore, 1995; Parren *et al*., 1998) and can be understood in terms of epitope accessibility. Epitopes of the CD4bs, V3 loop and CRbs are accessible on TCLA envelope and antibodies against these sites neutralize TCLA viruses. These epitopes are relatively inaccessible on primary isolate envelope and primary isolates are therefore mostly resistant to neutralization by such antibodies. An immunoglobulin molecule (IgG1) is depicted next to the oligomeric envelope complex to demonstrate the relative size of the neutralizing agent and the accessible surface. With an accessible surface of approximately 3000 $Å^2$ on the neutralizing face of gp120, excluding the variable loops (Kwong *et al*., 1998), it can be hypothesized that there is space for three non-competing antibodies per gp120 molecule (with a binding area of approximately 800–900 $Å^2$).

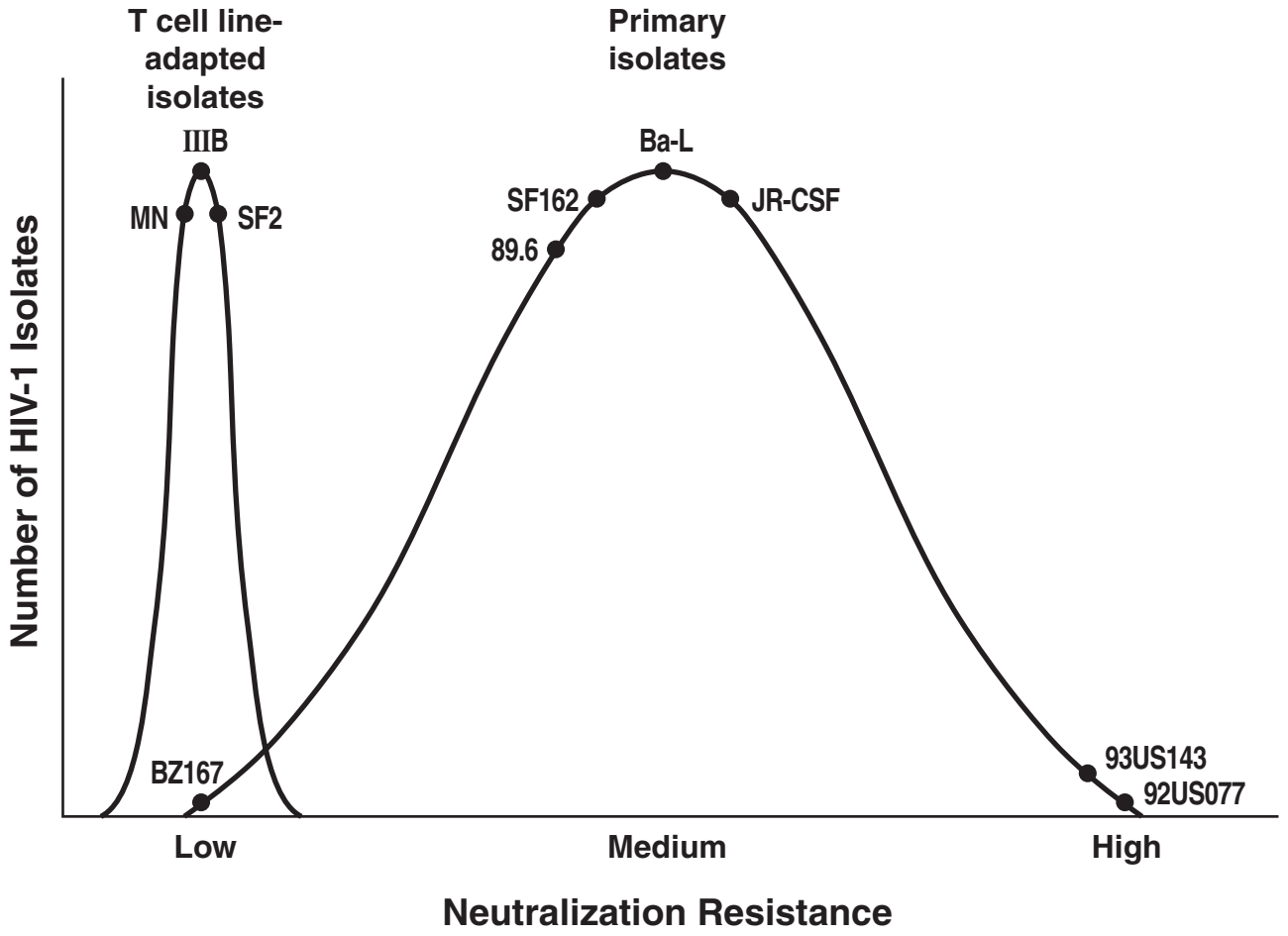

Fig. 2. An idealized depiction of neutralization resistance of HIV-1 isolates. TCLA isolates are highly sensitive to neutralization by antibodies and sCD4 in contrast to primary isolates which are relatively neutralization resistant. A bell-shaped curve was drawn to depict the fact that the degree of neutralization sensitivity can differ significantly between isolates. Some primary isolates (e.g., BZ167) (Moore and Montefiori, 1997) are just as sensitive to antibody mediated neutralization as TCLA isolates. Others may be extraordinarily resistant (e.g., 93US143 and 92US077) (Parren *et al*., 1998). In the evaluation of neutralization data, it is critical to note the neutralization phenotype of the isolate used. Neutralization of highly neutralization sensitive isolates by an antibody does not predict neutralization of relatively resistant isolates more representative of isolates encountered in human infection, and may therefore have little value for the evaluation of candidate vaccines.

The structural basis for the large difference in neutralization sensitivity between TCLA viruses and the majority of primary isolates can be understood as follows. It seems likely, based on the gp120 structure that the CD4bs on the primary isolate gp120 trimer is more completely masked by the V1 and V2 loops than that of TCLA viruses. The idea that gp120 oscillates between 'closed' and 'open' states is consistent with the dichotomy of primary and TCLA viruses: thus gp120 of primary isolates would have the equilibrium biased in favor of a 'closed' conformation, whereas TCLA gp120 would be biased towards 'open' (Fig. 1C). In this way, the virus *in vivo* would sacrifice some efficiency in receptor binding for increased resistance to antibody attack, whereas cell line-passaged virus would dispense with some now unnecessary antibody resistance mechanisms and adapt for more efficient receptor interactions instead. This notion appears to be generally applicable to lentiviruses, in that SIV, feline immunodeficiency virus (FIV) and equine infectious anaemia virus (EIAV) adapt to passage in cell lines in the same way as HIV-1 does (Baldinotti *et al*., 1994; Moore *et al*., 1995; Cook *et al*., 1995; Means *et al*., 1997; Montefiori *et al*., 1998).

Neutralization sensitivity is an important factor to take into account when evaluating HIV-1 neutralization studies. TCLA viruses are highly neutralization sensitive (Fig. 2) and it is well recognized that this has misled the HIV-1 vaccine field for many years (Cohen, 1993). These viruses nevertheless can be valuable as they can be used to eliminate a concept from further consideration, as a failure to inactivate TCLA HIV-1 *in vitro* or *in vivo* would be very discouraging (Burton and Moore, 1998). Success with TCLA HIV-1 however should not be overemphasized as inactivation of TCLA HIV-1 by an antibody in a neutralization assay or an *in vivo* challenge experiment would be encouraging, but would not predict activity against a primary isolate. It is important to note than neutralization sensitivities may also differ significantly between primary isolates, as indicated by the bell-shaped curve in Fig. 2. Some primary isolates are almost as sensitive as TCLA strains (e.g., BZ167)(Moore and Montefiori, 1997), whereas others may be exceptionally neutralization resistant (e.g., 92US077)(Parren *et al*., 1998). Arguably, the efficacy of HIV-1 neutralization particularly when evaluating candidate vaccines should be assessed with primary isolates of intermediate neutralization sensitivity and not with one of the outliers.

THE NEUTRALIZING ANTIBODY RESPONSE TO THE HIV-1 ENVELOPE COMPLEX

With the recent elucidation of the X-ray crystal structure of gp120 (Kwong *et al*., 1998), together with earlier mutagenic and antibody competition studies (Moore and Sodroski, 1996; Ditzel *et al*., 1997), an antigenic surface map could be constructed, on which the spatial positioning of the neutralizing and non-neutralizing epitopes are revealed (Wyatt *et al*., 1998). Neutralizing epitopes on gp120 map to the surface of the envelope complex that is exposed in the oligomeric configuration and faces the target cell. For TCLA HIV-1 isolates strains the neutralizing epitopes include the CD4 and the co-receptor binding-sites, the V2 and V3 loops and the unique 2G12 epitope (Table 1). Far fewer epitopes are accessible on the mature envelope of primary isolates and neutralization of a range of isolates has only been observed with mAb b12 which recognizes the CD4bs and residues of the V2 loop (Burton *et al*., 1994; Roben *et al*., 1994; Mo *et al*., 1997) and mAb 2G12 which recognizes an epitope at the base of V3 and V4 loop.

Table 1. Neutralizing epitopes exposed on HIV-1 mature oligomeric envelope

Epitope recognized	Antibodies	Neutralization of TCLA strains	Neutralization of Primary isolates
gp120			
V3	loop2, 19b, 447/52D	Yes	No
CD4bs	F105, 21h, 15e	Yes	No
CD4bs/V2	b12	Yes	Yes
CD4i	17b, 48d	Yes	No
V2	C108G	Yes	No[1]
	L15, 697D	No	No[2]
base of V3 and V4 loop	2G12	Yes	Yes
gp41			
ELDKWA (residues 662–667)	2F5	Yes	Yes

[1] Strain specific neutralization
[2] Some weakly

An antigenic surface map has also been proposed for gp41 albeit less complete than for gp120 (Binley *et al*., 1996; Earl *et al*., 1997). These studies identified at least three conformational dependent epitope clusters present on native gp41, termed cluster I-III. Antibodies to epitope clusters I and II are readily detectable in sera from HIV-1 infected individuals, but most studies show that none of the three clusters neutralize TCLA strains or primary isolates at biologically relevant concentration (Sattentau *et al*., 1995; Binley *et al*., 1996). Presumably these epitopes are masked by the gp120 molecules in the mature oligomeric envelope complex and thus inaccessible for antibody binding. The only epitope that is exposed on mature oligomeric (gp120-associated) gp41 is defined by the neutralizing mAb 2F5 and is located in the membrane proximal part of the ectodomain of gp41 (Table 1 and Muster *et al*., 1993; Muster *et al*., 1994; Sattentau *et al*., 1995). Studies from one lab have suggested a neutralizing epitope within the cytoplasmic domain of gp41 (Chanh *et al*., 1986; Dalgleish *et al*., 1988; Evans *et al*., 1989), however this has not been confirmed by others (D'Souza *et al*., 1994; D'Souza *et al*., 1995).

NEUTRALIZING EPITOPES

V3

The third variable (V3) loop of gp120 was originally termed the principal neutralizing domain (PND), due to its dominant role in the neutralization of TCLA strains by sera from HIV-1 infected individuals and gp120 vaccine recipients. In contrast to the dominant role V3 loop specific mAbs play in TCLA strain neutralization, their role in primary isolate neutralization is insignificant (VanCott *et al*., 1995; Spenlehauer *et al*., 1998). This inability of V3 loop specific mAbs to neutralize primary isolates is thought to result from the relative inaccessibility of the V3 loop in the native oligomeric envelope complex of primary isolates as compared to that of TCLA strains (Bou-Habib *et al*., 1994). Furthermore, because of the hyper-variability of the V3 loop, the mAbs to this epitope display a highly strain-specific neutralizing activity.

Binding studies with monomeric gp120-CD4 complexes have demonstrated that mAbs to the V3 loop inhibit the interaction of this complex with the co-receptor (Wu *et al*., 1996; Trkola *et al*., 1996a; Hill *et al*., 1997). Although this would suggest neutralization at a post-attachment stage on intact virions, anti-V3 loop antibodies neutralize TCLA viruses by inhibiting HIV-1 attachment to the target cell (Ugolini *et al*., 1997; Valenzuela *et al*., 1997).

CD4bd

The majority of antibodies to gp120 recognizes discontinuous or conformationally sensitive epitopes, of which the CD4 binding domain (CD4bd) is the most prevalent (Moore and Ho, 1993). This epitope is defined by mAbs that competitively inhibit sCD4 binding to monomeric gp120. As with the anti-V3 loop antibodies, early neutralization experiments were biased by the use of neutralization sensitive TCLA strains. It has become evident that the majority of CD4bd antibodies that could neutralize TCLA strains were unable to neutralize primary isolates, with the exception of mAb b12. MAb b12 recognizes a conformation dependent epitope that overlaps the CD4bs with some involvement of the V2 loop (Roben *et al*., 1994; Mo *et al*., 1997). Uniquely among the CD4bd specific mAbs, b12 binds equivalently or better to the oligomeric form of the envelope glycoprotein (Roben *et al*., 1994; Fouts *et al*., 1997).

CD4i

A highly conserved but poorly immunogenic epitope is defined by mAbs that bind better to gp120 upon complexation with CD4. These antibodies, like 17b and 48d (Thali *et al*., 1993; Wyatt *et al*., 1995), were shown to inhibit the interaction of the gp120-CD4 complex with CCR5 (Wu *et al*., 1996; Trkola *et al*., 1996a), suggesting that the epitope was located in or near the co-receptor binding site. Mutational studies later confirmed that residues within the CD4i epitopes were crucial for co-receptor binding (Rizzuto *et al*., 1998). These residues are located primarily in the bridging sheet and may involve some residues in the V3 loop. Next to the obvious blocking of co-receptor binding, antibodies to CD4i epitopes have been shown to induce gp120 dissociation from gp41 (Poignard *et al*., 1996a). Like the V3 loop, the CD4i epitope is a neutralizing epitope only on TCLA strains of HIV-1. Neutralization of primary isolates by mAbs against the CD4i epitope has not been observed.

V2

MAbs to the V1/V2 stemloop structure generally recognize conformational epitopes which are located in the central region of the V2 loop (Moore *et al*., 1993; McKeating *et al*., 1993; Gorny *et al*., 1994) and have been shown to neutralize TCLA strains relatively well (Warrier *et al*., 1994). So far mAbs to the V1 part of the stemloop structure have not been identified. Two mAbs directed against the V2 loop have been reported to be able to neutralize primary isolates (Gorny *et al*., 1994; Vijh-Warrier *et al*., 1996), although the range of isolates that can be neutralized is very limited (Pinter *et al*., 1998). The sequence variability, as with the V3 loop, and additionally a substantial length polymorphism make this epitope very strain specific (Wang *et al*., 1995).

2G12

Antibody competition studies identified a unique competition group that included a single antibody, 2G12 (Trkola *et al*., 1996b). Based on results from studies involving glycosidase treatment of gp120 and mutagenic alteration of N-linked carbohydrate sites, the epitope of this antibody is located at the base of the V3 and V4 loop and probably involves carbohydrate structures in the C2, C3, C4 and V4 domains (Trkola *et al*., 1996b). The inclusion of carbohydrate structures in the epitope might explain the rarity of this mAb. The 2G12 epitope is predicted to be oriented towards the target cell upon CD4 binding. This would allow the antibody to sterically impair further interactions of the membrane complex with the target cell.

2F5

The only gp41 specific mAb that displays neutralizing activity is 2F5 (Muster *et al*., 1993; Muster *et al*., 1994; Conley *et al*., 1994; Burton, 1997). This antibody recognizes an epitope that has been mapped to the linear sequence ELDKWA, which is located in the membrane proximal part of the ectodomain (residues 662–667) (Muster *et al*., 1993) and is the only epitope on gp41 that is exposed on the native oligomeric conformation of the HIV-1 envelope glycoprotein complex (Muster *et al*., 1993; Muster *et al*., 1994; Sattentau *et al*., 1995). MAb 2F5 does not interfere with virus attachment to the target cell, but neutralizes at a later stage (Ugolini *et al*., 1997).

NEUTRALIZATION MECHANISMS

The principal mechanism of antibody-mediated neutralization for HIV-1 is the inhibition of attachment of the virus to the target cell (Ugolini *et al*., 1997). This was found to be independent of the epitope cluster recognized by the neutralizing mAb (Parren *et al*., 1998).

Several mechanisms to inhibit attachment can be envisioned and have been proposed (Dimmock, 1995). Aggregation has been shown to be effective in neutralizing poliovirus or human rhinovirus (reviewed in Dimmock, 1995), however the observations that monovalent ligands neutralize as well as bivalent ones argues against a role of this mechanism in HIV-1 neutralization (Parren *et al*., 1998). Furthermore, the bell-shaped curve associated with this mechanism has not been described for HIV-1 (McLain and Dimmock, 1994) and aggregates could not be recovered in neutralizing antibody-treated HIV-1 preparations (McDougal *et al*., 1996). The absence of neutralization of amphotropic murine leukemia virus (AMLV) envelope-mediated infection of AMLV/HIV-1 envelope pseudotyped virions by a neutralizing antibody against HIV-1 finally suggests that virion aggregation is not a neutralization mechanism for HIV-1 (Schønning *et al*., 1999).

With one observed exception all effectively HIV-1-neutralizing mAbs block virus attachment to the target cell either by inhibiting the interaction with CD4 or the co-receptor (Wu *et al*., 1996; Trkola *et al*., 1996a; Ugolini *et al*., 1997). The absence of an epitope bias suggests that any antibody capable of binding to the limited surface of gp120 that is exposed in the mature oligomer (Fig. 1C) can effectively block interaction with the receptor binding sites (Parren *et al*., 1998). For attachment of virus to the target cell to occur, presumably multiple contacts in a localized area must be established. Coating of the viral surface with antibodies obstructs the close approach of the virus to the target cell, thereby preventing attachment and initiation of a fusion event (Parren *et al*., 1998). Such a mechanism is in good agreement with an elegant study on stoichiometry of mAb mediated neutralization. In this study, neutralization could be explained with an incremental model in which neutralization occurs incrementally as each envelope molecule binds mAb (Schønning *et al*., 1999). Although the studies above were performed with TCLA strains of HIV-1 rather than primary isolates for practical reasons, there is no indication that the general conclusions from these studies do not apply to primary isolates. To explain the relative neutralization resistance of primary isolates compared to TCLA strains, a theoretical model has been proposed (Klasse and Moore, 1996). It predicts that neutralization is the result of the reduction of the number of functional envelope molecules below a critical threshold. Neutralization resistance of primary isolates is explained by affinity of antibodies to primary isolate envelope and a higher number of envelope spikes per primary isolate virion (Klasse and Moore, 1996). Envelope density as a modifier of neutralization however is not consistent with an incremental model (Schønning *et al*., 1999). It has been demonstrated furthermore that neither increased spike density nor spike stability could account for the neutralization resistance of primary isolates (Karlsson *et al*., 1996). The mechanism of primary isolate neutralization and neutralization resistance therefore require further study. Convincing data however support an incremental mechanism in which coating of virions with antibody prevent attachment to the target cell and neutralization potency is determined by antibody affinity for the mature envelope oligomer.

The one exception on the principal neutralization mechanism is constituted by the gp41 specific mAb 2F5, which does not inhibit virus attachment to the target cell, but neutralizes at a later stage (Ugolini *et al*., 1997). The exact mechanism by which this epitope mediates neutralization of the virus is unclear, but the epitope is located near a conserved tryptophan-rich region that has been implemented in env-mediated fusion (Salzwedel *et al*., 1999). Mutations in the epitope had only a limited effect on cell-cell fusion, which makes it unlikely that this epitope is directly involved in the fusion process (Salzwedel *et al*., 1999). A hypothetical mechanism for neutralization could be that 2F5 interferes at some stage with the completion of the transition of the non-fusogenic state to the post-fusion state (Binley and Moore, 1997; Chan and Kim, 1998).

RELEVANCE OF *IN VITRO* NEUTRALIZATION DATA

The neutralizing activity of a mAb is measured *in vitro* in the absence of complement and antibody-dependent cell-mediated cytotoxicity. This would suggest an underestimation of the neutralizing efficacy of a mAb *in vivo*. However with the emergence of more data on the inactivation of the virus in animal models, it has become clear that there is generally a good correlation between the *in vitro* and *in vivo* results (Parren *et al*., 1995; Gauduin *et al*., 1995; Gauduin *et al*., 1997; Mascola *et al*., 1999; Shibata *et al*., 1999). The studies show that when a mAb is capable of neutralizing the challenge virus *in vitro* , sterilizing immunity can be obtained at concentrations in the order of 1–2 logs greater than those needed for 90 neutralization *in vitro* (Parren *et al*., 1997). To achieve protection, serum neutralizing antibody levels that are sufficient to inactivate virtually all the virus in an *in vitro* assay need to be achieved. Failure to obtain adequate antibody titers leads to establishment of infection and cell-to-cell virus transmission, which requires considerable higher antibody concentrations than those needed to block cell-free virus (Pantaleo *et al*., 1995). Once HIV-1 infection is established, even high levels of neutralizing antibody have no or only very limited effects on an ongoing infection (Poignard *et al*., 1999). This is not to say that pre-existing neutralizing antibody concentrations below the levels that would provide sterilizing immunity have no effect upon viral challenge. Studies on murine retroviruses and other RNA viruses have indicated that a reduction of the viral inoculum by neutralizing antibodies may provide a benefit by giving time to the cellular immune response to develop (Planz *et al*., 1997; Baldridge *et al*., 1997; Dittmer *et al*., 1998; Parren *et al*., 1999; Dittmer *et al*., 1999a; Dittmer *et al*., 1999b). A reduction of pathogenicity has furthermore been observed in passive neutralizing antibody transfer studies in macaques and chimpanzees. (Conley *et al*., 1996; Mascola *et al*., 1999).

ADDITIONAL EFFECTS OF POLYCLONALITY

The HIV-1 neutralizing activity found in the polyclonal antisera from infected individuals is the combined result of the neutralizing abilities of the distinct Abs. A handful of studies have looked at the combined effects of mAbs with different specificities on the neutralization of TCLA strains (Kennedy *et al*., 1991; McKeating *et al*., 1992; Tilley *et al*., 1992; Potts *et al*., 1993; Laal *et al*., 1994; Vijh-Warrier *et al*., 1996) and primary isolates (Mascola *et al*., 1997) *in vitro*. These studies demonstrate a neutralizing effect that is greater than the sum of separate neutralizing abilities of the tested Abs, or synergy. However, the observed synergy is generally weak and could only be shown with mathematical models. The biological relevance of this mechanism is unknown, as it is hard to interpret the situation *in vivo*. One passive antibody transfer study which assessed the combination of two neutralizing mAbs (2F5 and 2G12) together with HIVIG in a model with $SHIV_{89.6PD}$ in pigtail macaques, demonstrated a general correlation between the synergistic effects on neutralization observed *in vitro* and the protection *in vivo* (Mascola *et al*., 1997; Mascola *et al*., 1999). In severe combined immunodeficient mice reconstituted with human peripheral blood lymphocytes (hu-PBL-SCID mice), a cocktail of 2F5, 2G12 and b12 reduced the viral RNA titer during established infection, whereas b12 alone had no detectable effect (Poignard *et al*., 1999). This synergy however can be explained by the necessity of individual viruses to acquire multiple amino acid mutations for neutralization escape rather than by binding cooperativity effects.

NOVEL NEUTRALIZING EPITOPES

The primary isolate neutralizing antibodies identified so far are directed to epitopes which are present on the resting oligomeric envelope. It may be envisioned that epitopes which are exposed on the activated (after CD4 binding) or fusogenic state of the membrane complex (after complexation with the co-receptor) are interesting targets for antibody neutralization. The main epitope that becomes exposed after interaction with CD4 is the CD4i epitope overlapping the co-receptor binding-site. MAbs to this epitope isolated to date do not display primary isolate neutralizing activity at relevant concentrations. It can however not be excluded that more potent antibodies do exist.

The fusiogenic state of gp41 has previously been identified as a target for neutralization by studies using peptides that mimic the carboxyterminal helices of gp41 (Jiang *et al*., 1993; Wild *et al*., 1994). The peptides were able to inhibit the fusion process quite efficiently. It is possible that the dimensions of a full immunoglobulin molecule are a limiting factor in the accessibility of these neutralization sensitive sites. However, a recent study in which fusing cells were fixed using formalin, suggests the presence of yet unidentified neutralizing epitopes on the fusogenic state of the membrane complex (LaCasse *et al*., 1999). This observation suggests that the rarity of these types of antibodies may be due to a temporal rather than a physical constraint. However, to date no definite proof has been provided that the mouse sera recognize epitopes on the viral proteins and not cellular antigens (Montefiori and Moore, 1999).

NON-ENVELOPE MEDIATED NEUTRALIZATION

Several examples of neutralization *in vitro* have been described, which are mediated by antibodies that are not directed to the envelope complex. The majority of non-envelope neutralizing antibodies is directed at cellular membrane proteins. During the budding process HIV-1 acquires a variety of cellular proteins on its membrane (Arthur *et al*., 1992; Tremblay *et al*., 1998), although the diversity of the proteins present on the membrane is probably an overestimation due to contamination of the virion preparations with microparticles (Raposo *et al*., 1996; Gluschankof *et al*., 1997; Bess *et al*., 1997; Dettenhofer and Yu, 1999). Antibodies to several of these host cell derived proteins have been shown to neutralize *in vitro*. Most notable are the antibodies to the receptor-ligand pair ICAM-1 (intercellular adhesion molecule-1; CD54) and LFA-1 (leukocyte function-associated molecule-1; CD11a/CD18) (Gomez and Hildreth, 1995; Rizzuto and Sodroski, 1997). Also antibodies to HLA-DR, β2-microglobuline and HLA class I have been shown to neutralize *in vitro* (Arthur *et al*., 1992). As inhibition of attachment is a major mechanism of neutralization of HIV-1 (Ugolini *et al*., 1997) it may be that antibodies to these proteins present on the virion membrane interfere with binding of the envelope complex to the receptors on the target cell. Alternatively, the incorporation of MHC class II enhances virus entry into the target cell (Cantin *et al*., 1997). By blocking this interaction the infection process is less effective, which may contribute to the neutralizing activity observed.

Protection against infection with SIV grown in human cells has been consistently observed in monkeys after active or passive immunization against host cell components (Arthur *et al*., 1995; Stott and Schild, 1996). The importance of antibody mediated neutralization via these self-antigens in humans is unclear. It is suggestive that the extent of HLA mismatch between mother and child and relative rarity of certain HLA haplotypes in commercial sex workers have been shown to correlate with the risk for seroconversion. However, antibodies against HLA class I allotypes do not appear to contribute to resistance against HIV-1 infection in exposed uninfected sex workers (reviewed in Plummer *et al*., 1999).

Host-derived cyclophilin A is specifically incorporated into HIV-1 virions through interactions with the gag protein and is required for infection. It has been suggested that cyclophilin A may play a role in virus-cell fusion and that antibodies against cyclophilin A may inhibit HIV-1 infection (Sherry *et al*., 1998). Additional studies however are necessary to explain how cyclophilin A, which is localized inside the virion, may become accessible to neutralizing antibodies. It has been suggested that anti-cyclophilin A antibodies could play a role in a vaccine against HIV-1 (Sherry *et al*., 1998). As cyclophilin A is a self-protein, however, it is very unlikely that effective and safe responses could be elicited.

During natural infection the viral regulatory protein Tat is released from productively infected cells (Ensoli *et al*., 1993; Westendorp *et al*., 1995; Chang *et al*., 1997) where in turn it may transactivate virus replication in the neighboring cells (Frankel and Pabo, 1988; Ensoli *et al*., 1993). Extracellular Tat also induces co-receptor expression and thereby facilitating HIV-1 transmission (Li *et al*., 1997; Huang *et al*., 1998). Antibodies against Tat were shown to inhibit $HIV\text{-}1_{IIIb}$ replication *in vitro* and correlate with non-progression *in vivo* (Re *et al*., 1995; Zagury *et al*., 1998). These results indicate a possible role for anti-Tat antibodies in controlling HIV-1 infection. In a study in cynomolgus monkeys vaccinated with a Tat vaccine however control of pathogenic $SHIV_{89.6P}$ infection did not correlate with Tat-neutralizing antibody levels (Cafaro *et al*., 1999).

CONCLUSIONS

HIV-1 sensitivity to neutralization is determined to some extent by the cell in which the virus was grown, less so by the target cell and co-receptor used. Increased accessibility of epitopes on the mature oligomeric envelope of TCLA viruses probably determines their relative sensitivity to neutralization as compared to primary isolates (summarized in Fig. 1). The principal mechanism of neutralization of HIV-1 most likely is an incremental mechanism in which coating of virions with antibody prevents attachment to the target cell and neutralization potency is determined by antibody affinity for the mature enve-

lope oligomer.

A number of common neutralizing epitopes have been identified on TCLA HIV-1 gp120. Most of these epitopes, however, including relatively immunogenic epitopes overlapping the CD4bs and V3 loop have shown to be mostly irrelevant for the neutralization of HIV-1 primary isolates. Only two epitopes on gp120 appear to be accessible on primary isolate envelope and conserved on a broad spectrum of isolates: an epitope which involves residues of the CD4bs and the V2 loop recognized by mAb b12 and an epitope at the base of V3 and V4 loop recognized by mAb 2G12. Both these epitopes are poorly immunogenic and antibodies with b12 and 2G12 specificity are rarely elicited in the humoral response after HIV-1 infection. A single neutralizing epitope has been defined on HIV-1 gp41. This epitope recognized by mAb 2F5 is relatively conserved, poorly immunogenic and is accessible on TCLA as well as primary isolate oligomeric envelope.

Neutralization of HIV-1 *in vitro* is a good measure of the antiviral activity of a given antibody preparation. There is a good correlation between neutralization *in vitro* and protection: antibody concentrations that neutralize all the challenge virus in an *in vitro* assay can provide sterilizing immunity *in vivo*. Pre-existing neutralizing antibody concentrations at insufficient levels to provide sterilizing immunity may decrease pathogenicity by reducing the viral inoculum and clearing infected cells, thereby allowing more time for the cellular immune response to mature. Once HIV-1 infection is established, however, even high levels of neutralizing antibody have no or only very limited effects on an ongoing infection.

There are many indications that a vaccine that would effectively elicit high affinity antibodies against conserved epitopes accessible on mature oligomeric envelope of HIV-1 primary isolates would have a major impact on HIV-1 transmission. Poorly immunogenic epitopes defined by mAb b12, 2G12 and 2F5, represent the relevant vaccine targets on the HIV-1 envelope structure identified to date.

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