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HIV-1 Immune Escape and Neutralizing Antibodies:

Solid Phase Proteoliposomes Containing HIV-1
Envelope Glycoproteins as Antigens and Immunogens

and

HIV-1 Core Envelope Glycoproteins
Deficient in T-Helper Epitopes

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Abstract

The HIV-1 envelope glycoproteins gp120 and gp41 mediate binding and fusion of the virus to target cells. The envelope glycoproteins are exposed on the surface of the virus as trimeric spikes and are the major targets for neutralizing antibodies. The design of envelope glycoprotein-based subunit vaccines has been frustrated by many viral immune escape mechanisms. Trimeric envelope glycoprotein formulations hold promise to overcome limitations of monomeric envelope glycoproteins as immunogens. The generation of native, trimeric envelope glycoprotein complexes, however, remains a major challenge.

Here, solid-phase proteoliposomes containing native, trimeric HIV-1 envelope glycoprotein complexes that mimic the trimeric complex as it is found on the viral surface have been designed. In a comparative immunogenicity study, these proteoliposomes were shown to better elicit broadly neutralizing antibodies than gp120. A second trimeric envelope glycoprotein formulation, soluble YU2 gp140-GCN4 constructs, were also shown to better elicit broadly neutralizing antibodies in rabbits, extending a previous study in mice. These data support the hypothesis that trimeric envelope glycoprotein formulations are an advance over gp120-based immunogens.

To date, only four broadly neutralizing antibodies against the HIV-1 envelope glycoproteins have been identified. Here, three novel Fab antibody fragments binding to the CD4 binding site of gp120 have been identified from phage-displayed antibody libraries with proteoliposomes. These Fab antibodies display some breadth and potency in neutralizing HIV-1. Comparison of the neutralizing activity of Fab antibodies and whole antibodies directed to the CD4 binding site suggests that these Fab antibodies may significantly gain neutralizing potency as whole antibodies.

Many HIV-1 immune escape mechanisms complicate the elicitation of broadly neutralizing antibodies. Core gp120 envelope glycoproteins derived from primary isolate viruses were found to be deficient in T-helper epitopes. This finding is suggestive of yet another HIV-1 viral immune escape mechanism, the escape from recognition by $CD4^+$ T-helper cells.

1 Introduction

1.1 AIDS

Since the first reported cases of acquired immune deficiency syndrome (AIDS) in 1981, AIDS has become a pandemic of catastrophic proportions. Human immunodeficiency viruses (HIV) type 1 and type 2 are the etiologic agents of AIDS (10, 43). According to UNAIDS, there are an estimated 40 million people living with HIV today, predominantly of the HIV-1 type. In sub-Saharan Africa, AIDS is today the leading cause of death. The adult prevalence in many of these countries exceeds 10%. Eastern Europe and China are presently experiencing the steepest increase in new infections and may only be at the beginning of a disastrous epidemic.

With the availability of highly active anti-retroviral therapy (HAART), the progression of HIV-infection to AIDS can be slowed. However, while 500,000 people received HAART and 25,000 people died of AIDS or related diseases in the developed world last year, in sub-Saharan Africa, only 30,000 people received HAART and 2.3 million people died (www.unaids.org). The high cost of antiviral therapies and the complexities of their administration make it unlikely that these drugs will significantly alter the course of the HIV epidemic in developing areas of the world. Only a broadly effective vaccine will eventually be able to curb the global spread of HIV.

HIV infects and depletes primarily $CD4^+$ T-helper cells. HIV infection typically progresses through three stages: Acute, latent and AIDS (39). During the acute phase, a burst of viral replication coincides with rapid loss of $CD4^+$ T-helper cells. The end of the acute phase, which occurs within a few weeks of infection, is characterized by a drop in viral load and a rebounding $CD4^+$ T-helper cell count. During the subsequent latent phase, constant turnover of virus and $CD4^+$ T-helper cells occurs, resulting in a slow and gradual decline in $CD4^+$ T-helper cells. Eventually, the loss of $CD4^+$ T-helper cells becomes too severe and leads to the failure of the immune system to fend off pathogens that do not generally pose a threat to immunocompetent individuals. The emergence of opportunistic infections such as *Pneumocystis carinii*, cytomegalovirus, or neoplasms such as Karposi's sarcoma mark the onset of AIDS. Without treatment, AIDS patients rarely survive longer than a few years.

1.2 HIV

HIV belongs to the genus *lentivirinae* of the *Retroviridae* family. Based on sequence analysis and serologic properties, two types of HIV have been identified. HIV-1 is the prevalent strain in Africa, Asia, Europe, North- and South America. In some West African countries, HIV-2 is the dominant strain. Genetically related viruses that infect nonhuman primates, the simian immunodeficiency viruses (SIV), have been isolated and are believed to have given rise to HIV by cross-species transmission. In fact, HIV-1 exhibits more sequence homology to an SIV found in chimpanzees than it does to HIV-2. HIV-2, in turn, is most closely related to SIV isolated from sooty mangabeys (24, 42).

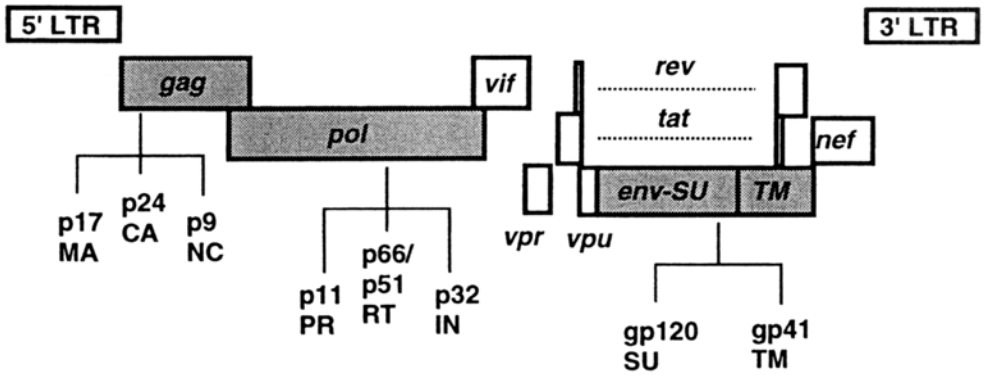

FIG. 1. Schematic diagram of the HIV-1 genome and its gene products. Virion structural genes are depicted as shaded, accessory genes as clear boxes (from Fields, Fundamental Virology). MA: Matrix protein, CA: Capsid protein, NC: Nucleocapsid protein, PR: Protease, RT: Reverse transcriptase, IN: Integrase, SU: Surface glycoprotein (gp120), TM: Transmembrane glycoprotein (gp41).

HIV is an enveloped virus with two identical copies of a single-stranded RNA genome of about 9.2 kb. The HIV genome encodes nine genes (*gag, pol, env, tat, rev, vif, vpr, vpu,* and *nef*) in three open reading frames. It is flanked on either side by identical long terminal repeats (LTRs) that contain non-coding sequences important for viral replication (Fig. 1). After entry of the virus into the host cell, the RNA genome is reverse-transcribed by viral reverse transcriptase and the provirus is integrated into the host genome by the viral integrase. The first viral gene products to be expressed by host-cell RNA polymerases are Tat and Rev, which upregulate the subsequent expression of

structural proteins. While Gag and Gag-Pol become localized to the inner surface of the plasma membrane, Env is extensively modified in the endoplasmic reticulum. The Gag and Gag-Pol polyproteins assemble the core particle containing genomic RNA and the accessory protein Vpr. The viral particle then buds from the cell surface, acquiring in the process a membrane containing envelope glycoprotein complexes. After budding, proteolytic processing of Gag and Gag-Pol by the viral protease generates the mature, fully infectious virus (38).

1.3 The HIV-1 envelope glycoproteins: Structure and function

The HIV-1 envelope glycoproteins gp120 and gp41 facilitate binding of the virus to its target cell and mediate fusion between viral and cellular membranes (121). Initially, the envelope glycoproteins are synthesized as highly glycosylated gp160 polyprotein precursors that undergo a sequence of additional posttranslational modifications (3, 36, 85). After oligomerization in the endoplasmic reticulum, the polyprotein is cleaved into the gp120 and gp41 subunits by cellular proteases. The envelope glycoproteins remain associated through noncovalent interactions. They are extensively glycosylated and transported to the plasma membrane. From there, the envelope glycoprotein complexes are incorporated into budding virions. Many lines of evidence suggest that the mature, functional envelope glycoprotein complex on the viral surface consists of a trimer of gp120-gp41 heterodimers. The ectodomain of HIV-1 gp41 crystallizes as a trimeric coiled coil with interdigitating alpha helices to form a six-helix bundle (25, 109, 116). The structure of the simian immunodeficiency virus (SIV) ectodomain has been solved by magnetic resonance and shown to be trimeric (22). Both the HIV-1 and SIV postfusogenic states of gp41 share close resemblance with the corresponding transmembrane proteins of viruses such as influenza (19) and Ebola virus (115). Each of these fusion determinants has been crystallized as helical bundles possessing trimeric coiled-coil motifs. The matrix proteins of HIV-1 and SIV that interact with gp41 also crystallize as trimers (48). The gp160 ectodomain of SIV (gp140) has been shown to be trimeric by biophysical analysis (26). Trimerization has also been reported for a number of gp120-gp41/gp140 ectodomain constructs (14, 122, 124).

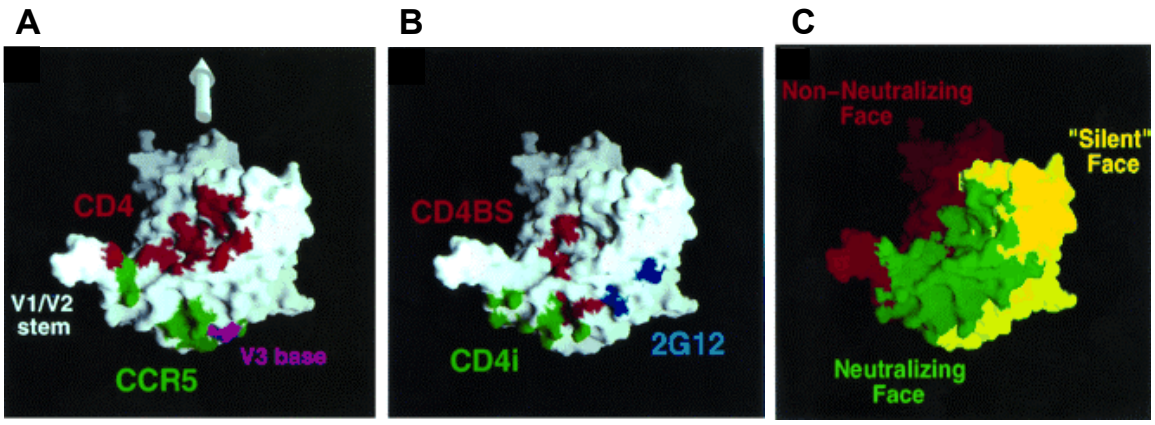

FIG. 2. Molecular surface presentation of HIV-1 core gp120 (from Wyatt and Sodroski, 1998). A. The arrow indicates the direction of the viral membrane. The inner domain is facing towards the left, and the outer domain, which is thought to be exposed on the assembled trimer, is on the right. The red surface indicates the footprint of CD4. Green indicates the surface thought to be involved in chemokine receptor binding. The base of the V3 loop is shown in magenta. B. Conserved gp120 neutralization epitopes. C. The antigenic faces of core gp120 are shown. The non-neutralizing face (red) encompasses most of the inner domain and N- and C-terminal regions interacting with gp41. This region is thought to be largely buried on the assembled trimer complex and interacts with non-neutralizing antibodies. The silent face (yellow) consists of heavily glycosylated regions on the outer domain and is poorly immunogenic. The neutralizing face (green) extends over both domains and the bridging sheet, as well as the V2 and V3 variable loops (not shown). This surface interacts with neutralizing antibodies.

The amino acid sequence of gp120 reveals alternating variable (V1-V5) and conserved regions (C1-C5) (102). The variable regions V1-V4 are anchored at their bases with disulfide bonds and mask the CD4- and coreceptor binding sites. The atomic structure of a core gp120 deleted of variable loops V1, V2, and V3 as well as C-and N-terminal regions in complex with the receptor CD4 and the Fab fragment of the CD4-induced (CD4i) antibody 17b has been solved (54) (Fig. 2). The gp120 envelope glycoprotein is structurally organized into an inner and outer domain, linked by a bridging sheet minidomain. The inner domain is probably facing the axis of the trimeric complex and contributes most of the contacts with gp41 at the C-and N-terminal regions. The outer domain is heavily glycosylated and is thought to face away from the trimer axis. Four antiparallel beta sheets form the bridging sheet. The CD4 binding site is situated at the interface of the three domains and is relatively well conserved among viral strains. All three domains also contribute to coreceptor binding, with the V3 loop and the base of the V1/V2 stemloop structure providing additional contacts (81, 82).

The infection process is initiated by binding of the gp120 envelope glycoprotein

to the cellular receptor CD4. This binding event presumably displaces the variable loop regions V1 and V2 on the gp120 envelope glycoprotein and exposes the coreceptor binding site, allowing for subsequent binding to the cellular coreceptor, typically the chemokine receptor CXCR4 or CCR5. These interactions trigger further conformational changes that allow for the formation of a six-helix bundle formed by two potential alpha-helical regions, N36 and C34 in the gp41 ectodomain, with three C34 helices packing into the hydrophobic grooves of a trimeric N36 coiled coil. Formation of this six-helix bundle is proposed to bring the viral and cellular membranes into close proximity and allow the insertion of the gp41 fusion peptide into the host cell membrane (25, 53, 115).

Antigenically, the surface of the gp120 core can be organized into three different faces as defined by antibody binding (121) (Fig. 2). Conserved regions that are accessible to antibodies or neutralizing ligands comprise the receptor- and coreceptor binding sites and form the neutralizing face. The heavily glycosylated outer domain is virtually invisible to the host immune system and is termed the silent face. The non-neutralizing face is oriented towards the trimer axis, is not accessible to antibodies on the functional trimer, and elicits only nonneutralizing antibodies.

1.4 Antibodies in HIV-1 infection

Vaccine-induced protection from many viral infections is based solely on the elicitation of neutralizing antibodies. It is becoming increasingly clear that for the prevention of HIV-1 infection, antibodies are also likely to be a critical component of a vaccine-elicited immune response. The level of circulating neutralizing antibodies correlated with protection from viral challenge in several animal models (12, 17). Passive immunization with neutralizing antibodies has also been demonstrated to protect from the establishment of viral infection in primates when administered to the host prior to exposure with HIV-1 (5, 58, 61). Some HIV-1-infected individuals remain asymptomatic for long periods of time and largely contain viral replication. In some cases, these long-term nonprogressors exhibit high titers of broadly neutralizing antibodies that may contribute to the favourable outcome of infection. The envelope glycoproteins are the major targets for neutralizing antibodies *in vivo*. However, the elicitation of antibodies that broadly neutralize HIV-1 has proven to be exceptionally difficult. The identification

of only four broadly and potently neutralizing envelope glycoprotein-directed antibodies to date from extensive screens of human and rodent antibodies illustrates the elusiveness of this class of antibodies.

During HIV-1 infection, antibodies directed against the viral envelope glycoproteins are readily elicited. In most infected individuals, two classes of neutralizing antibodies emerge, strain-restricted and broadly neutralizing antibodies. The strain-restricted antibodies are generally directed towards the second variable (V2) or third variable loop (V3) of gp120 and appear early during infection (84, 92). These antibodies neutralize only a limited number of viral isolates. Only after the initial phase of high viremiae, when the infection has already been firmly established, do more broadly neutralizing antibodies appear (103). These are directed to conformational epitopes, some of which, the CD4 binding site antibodies (CD4BS), recognize the discontinuous CD4 binding site (78, 86, 111). Another group of antibodies that neutralize T-cell line adapted (TCLA) isolates and a subset of primary isolates recognize CD4 induced (CD4i) epitopes. These antibodies bind near the gp120 bridging sheet and interfere with gp120-chemokine receptor interaction (110, 119).

One phenomenon of antibody neutralization that has misled the focus of research for years is the greater neutralization sensitivity of viral isolates that have been extensively passaged *in vitro* in immortalized T-cell lines compared to primary, clinical isolates (31, 44, 60, 61, 66, 71, 117, 128). Passage in cell lines selects for viruses using the CXCR4 coreceptor, such as the molecular clones HXBc2, IIIB, and MN. These TCLA viruses can be neutralized by a wide range of antibodies. Viruses directly isolated from patients, such as the molecular clones SF162, BaL, ADA, 89.6, JR-CSF, JR-FL, and YU2, preferentially use the CCR5 coreceptor or both CXCR4 and CCR5 coreceptors. Neutralization of primary isolates generally requires much greater concentrations of neutralizing antibodies. This difference in neutralization resistance can most likely be attributed to the antibody-mediated immune selection pressure on primary isolate viruses. It appears that primary viruses, regardless of their choice of coreceptor, are more resistant to neutralization than TCLA viruses.

To date, only four monoclonal antibodies that broadly and potently neutralize HIV-1 primary isolates have been identified. All of these antibodies have unique

properties and despite intensive study, no general scheme of neutralization of HIV-1 has emerged. The CD4 binding site antibody IgG1b12 was identified from a phage-displayed IgG Fab library obtained from the bone marrow of a long-term nonprogressor with exceptionally high titers of serum neutralizing antibodies (21, 113). It binds the CD4 binding site and neutralizes a wide range of primary isolate viruses at nanomolar concentrations. The atomic structure of this antibody reveals that it binds to the CD4 binding site similarly to CD4, extending a long, finger-like complementarity-determining region 3 (CDR3) into the recessed cavity of the CD4 binding site (95). The second broadly neutralizing gp120-directed antibody, 2G12, binds a carbohydrate moiety on the outer face of gp120 and is a rare example of a carbohydrate-reactive gp120 antibody (112). The gp41-binding antibodies 4E10 (129) and 2F5 (69) bind in close proximity on the C-terminal ectodomain of gp41. The epitope of the 2F5 antibody is thought to be linear, comprising the amino acid sequence ELDKWA, however, to date this antibody could not be elicited by peptides containing the 2F5 epitope or any other immunogen.

1.5 T-helper cells, B-cells, and T-helper epitopes

Functional activation of the cellular and the humoral arm of the immune system requires interaction with activated $CD4^+$ T-helper cells (1, 51). At the same time, $CD4^+$ T-helper cells are the major target for HIV-1 infection. In the course of AIDS, $CD4^+$ T-helper cells specific for HIV-1 are preferentially infected and depleted. This loss of specific anti-HIV-1 $CD4^+$ T-helper cells has been suggested to be a major factor in the eventual failure of the host immune system to control the virus (34, 88).

The generation of mature antibody responses requires the interaction of B-cells with activated $CD4^+$ T-helper cells. Upon specific recognition of an antigen by the B-cell receptor, the antigen is internalized and proteolytically degraded into peptide fragments in the lysosomal compartment. Particular peptides, the T-helper epitopes, bind to the major histocompatibility complex class II (MHC class II) and are presented on the surface of the cell. The complex of MHC class II and T-helper epitope is then recognized by a specific T-cell receptor (TCR) on the surface of a $CD4^+$ T-helper cell. This interaction and a complex series of costimulatory signals lead to the activation of the B-cell (1, 51).

Most large proteins possess one or more contiguous stretches of amino acids that can function as T-helper epitopes (90). Sequence comparison of helper epitopes naturally presented by MHC class II and structural analysis of MHC and MHC-peptide complexes suggest key anchor residues necessary for MHC class II binding and TCR engagement (16, 50, 89, 90). Although each MHC allele is characterized by a number of unique polymorphic residues along the peptide-binding cleft that limit the number of potential peptide ligands, peptides that are able to bind promiscuously to a number of different MHC alleles have been described (23, 72, 99). This observation gave rise to the concept of "universal helper-epitopes", peptides that can serve as T-helper epitopes for a number of different MHC class II alleles. One such universal T-helper epitope consists of key residues necessary for the binding to MHC class II and TCR on a polyalanine backbone. This peptide (amino acid sequence AKFVAAWTLKAAA) was shown to bind ten out of ten tested HLA-DR molecules and was hence termed pan-DR-helper epitope (PADRE). The PADRE epitope was also shown to bind to certain mouse MHC class II alleles, in particular I-A^b expressed by C57BL/6 mice (2).

1.6 Immunogenicity of the envelope glycoproteins

Most envelope glycoprotein-based immunogens tested so far are based on monomeric gp120. The antibody responses obtained in these studies generally did not neutralize primary HIV-1 isolates. (9, 11, 12, 30, 61, 118) The poor ability of gp120 to elicit neutralizing antibodies is also reflected in the binding characteristics of neutralizing and nonneutralizing antibodies. Neutralization has been found to correlate with binding of antibodies to the mature envelope glycoprotein complex. No such correlation could be found with monomeric gp120 (41, 83, 97).

Attempts to generate mimics of the trimeric envelope glycoprotein complex to better elicit neutralizing antibodies have been frustrated by the lability of these complexes. A number of different strategies have been pursued to stabilize the trimeric complex as soluble gp140 constructs. The deletion of the proteolytic cleavage site between gp120 and gp41 prevents shedding of noncovalently bound gp120 subunits from the complex and is common to most gp140 ectodomain constructs. The insertion of cysteine residues at specific locations on the alpha helical gp41 regions that mediate

formation of the trimeric coiled coil have been used to stabilize the trimeric organization of the envelope glycoprotein complex (37). Heterologous trimerization domains such as the bacterial transcription factor GCN4 or the bacteriophage trimerization domain fibritin have also been employed to stabilize the trimeric complex (122-124). Intersubunit disulfide bonds between gp41 and gp120 have been inserted in order to stabilize the cleaved, fully mature trimeric complex (14). The immunogenic properties of these ectodomain constructs have been disappointing, perhaps because of their heterogeneity (35). Only one of these constructs, a gp140-GCN4 construct derived from the primary isolate YU2, has been shown to elicit neutralizing antibodies better than monomeric gp120 (125). Mice immunized with these constructs generated antibodies that showed significant neutralization of the homologous primary isolate YU2, heterologous neutralization of the primary isolate ADA and the TCLA isolate HXBc2, but little neutralization of other primary isolates. This may be seen as a proof of principle that suggests better immunogenicity of trimeric constructs compared to monomeric gp120. And yet, these molecules may not fully resemble the relevant trimeric envelope glycoprotein complex. It is conceivable that the extensive changes that need to be introduced in these molecules to stabilize the trimer may force the envelope glycoprotein in conformations they would not naturally assume. Many lines of indirect evidence suggest that the best envelope glycoprotein-based immunogen is going to be one that most closely mimics the native envelope glycoprotein complex as it is found on the viral surface. Broadly neutralizing antibodies are elicited in HIV-infected individuals, and a good correlation between neutralization and binding of these antibodies to the functional oligomeric envelope glycoprotein complex has been established (75, 83, 97). The best protection from homologous and heterologous SIV challenge that could be achieved to date was observed in a primate model after immunization with live attenuated virus (91).

Since inactivated virus in the case of HIV-1 may never be a safe immunogen (4, 6), the generation of virus-like particles (VLP) has been explored as a means of preserving the native envelope glycoprotein structure in its natural lipid environment (126, 127). These VLP, however, have been difficult to generate with envelope glycoprotein quantities large enough to elicit sufficient titers of antibodies.

1.7 Viral immune escape mechanisms

As a result of HIV-1 infection, a vigorous immune response occurs. Despite this immune response, the general slow progression to AIDS ensues. The immune system keeps the infection in check for many years until the virus eventually gains the upper hand. Both cellular and humoral effector mechanisms exert considerable immune pressure on the virus. The effect of the humoral response in particular is highlighted by the difference in neutralization sensitivity that can be observed between TCLA isolates and primary isolates (41, 65, 101). Viruses grown without antibody pressure in tissue culture become more neutralization sensitive. During chronic infection, however, HIV-1 and the host's immune system co-evolve for many years, during which the immune system selects for ever more neutralization-resistant viruses, particularly resistant to autologous antibodies.

Many escape mechanisms from antibody-mediated neutralization have been described. Maybe most importantly, the exceptionally high genetic variability of HIV-1 allows the virus to escape from antibody recognition by mutation of antibody epitopes and necessitates the constant adaptation of the antibody response to mutated generations of progeny virus. Only antibodies elicited by structures that are limited in their ability to mutate by functional constraints, such as the receptor binding sites, can potentially neutralize different viral strains. However, large regions of the envelope glycoprotein, the exposed variable loops V1 and V2, sterically block access to the receptor binding sites while themselves eliciting mostly strain-restricted antibodies (121).

Moreover, large surfaces of gp120 are covered by carbohydrate moities, with about half of the gp120 molecular weight being contributed by carbohydrates (Fig. 3). Since these carbohydrates are synthesized by the host glycosyltransferase system, they are considered "self" determinants by the host's immune system. Thus, the glycosylation renders the underlying foreign protein determinants of gp120 invisible to the immune system. The protective effect that even one single carbohydrate moiety can have is highlighted in the case of a gp120 mutant that deletes the glycosylation site at amino acid 301. Deletion of this single glycosylation site renders a number of primary viral isolates sensitive to neutralization by many antibodies that cannot neutralize the wild-type virus even at high concentrations (57).

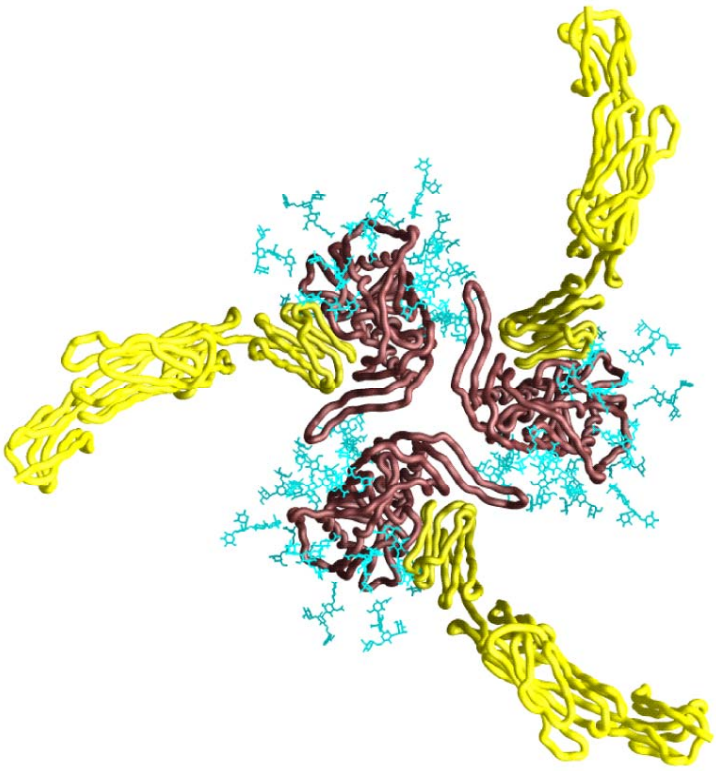

FIG. 3. Model of oligomeric core gp120 (modified from Kwong *et al*., 2000). Depicted is a Cα worm presentation of core gp120 (brown), the two membrane-distal domains of CD4 (yellow), and the gp120 carbohydrate cores (blue) as viewed from the target cell membrane. The carbohydrate cores shown represent about half of the high-mannose and complex N-linked glycan moieties. The long, finger-like structure extending counter-clockwise at the center of the trimer represents the V1,2 stem. The variable loops V1,2 and V3 are not shown.

The complex organization of gp41 and gp120 as trimers of heterodimers itself has important implications for immune diversion. It has been suggested that *in vivo*, disassembled envelope glycoprotein or viral debris may be the predominant immunogen (73). The interaction of gp41 and gp120 is noncovalent, thought to be labile, and allows gp120 to shed from the functional complex. Upon disassembly of envelope glycoprotein complexes, many surfaces that are occluded on the functional spike such as the gp41/gp120 interface and areas on gp120 buried along the trimer axis are presented to the immune system (Fig. 3). Both gp120 and gp41 may also change conformation upon dissociation. As a consequence, many antibodies elicited *in vivo* are raised against these decoy epitopes and do not bind the functional envelope glycoprotein complex.

Thermodynamic analysis of the interaction of gp120 with its receptor CD4 revealed highly unusual thermodynamics of this binding reaction (70). The changes in enthalpy and entropy of this reaction greatly exceeds that of other protein/protein interactions. The binding of CD4 to gp120 is characterized by an extraordinarily favourable binding enthalpy of –63kcal/mol. This change reflects a large number of bonding interactions during complex formation. The compensatory high entropy of this reaction of ~52kcal/mol is suggestive of large conformational rearrangements in gp120 upon CD4 binding. Since similar results have been obtained with deglycosylated core gp120 and fully glycosylated full-length gp120, these structural rearrangements are likely to occur at the interface of the inner and outer domain and the bridging sheet. These data have been interpreted to mean that monomeric gp120 in its free state is a very flexible molecule, possibly sampling many conformations. Only upon ligand binding does gp120 assume a fixed conformation. One implication of this flexibility of free gp120 for immunogenicity is that it may present a multitude of different conformations to the immune system, most of which may be irrelevant for virus neutralization.

Mutations in viral proteins that lead to loss of recognition by $CD8^+$ cytotoxic T lymphocytes have been documented and shown to allow for viral immune escape (15, 45, 76, 79). It is conceivable that viral proteins that lack potential T-helper lymphocyte epitopes for recognition by $CD4^+$ T-helper cells will also be selected for in an infected individual. Such escape mutants have been documented in the case of lymphocytic choriomeningitis virus. Adoptive transfer of $CD4^+$ T-helper cells from mice transgenic in the T-cell receptor for the immunodominant MHC class II-restricted LCMV epitope GP61-80 into C57BL/6 mice persistently infected with LCMV selected for virus mutants that failed to be recognized by $CD4^+$ T-helper cells (27). A similar mechanism has not yet been documented for HIV-1.

1.8 Scope of this thesis

The HIV-1 envelope glycoproteins are poor immunogens. HIV-1 has evolved a plethora of immune escape mechanisms to evade host immune surveillance. Most likely, we are only at the beginning of understanding the complexities of immune escape that need to be adressed and dismantled to generate better immunogens that elicit broadly

neutralizing antibodies. It appears that the envelope glycoproteins are protected from antibody neutralization by many layers of protection at virtually every level of immune effector mechanisms.

1. The generation of better mimics of the functional native envelope glycoprotein complex as it is found on the viral surface is a promising approach to limit the number of irrelevant epitopes that are presented to the immune system by, for example, immunogens based on monomeric envelope glycoprotein. A novel envelope glycoprotein complex formulation containing native, trimeric envelope glycoprotein complexes embedded in a lipid membrane, solid-phase proteoliposomes (PLs), have been generated, characterized, and tested for immunogenicity in mice and rabbits.

2. A great wealth of information has been drawn from the four broadly neutralizing antibodies that have been identified to date. The identification of additional neutralizing antibodies would greatly aid our understanding of neutralization mechanisms and viral immune escape, and may help in the identification of novel targets for vaccine design. To this end, proteoliposomes were used to screen phage-displayed Fab and scFv libraries for novel neutralizing antibodies.

3. Escape from T-helper cell recognition has been documented in a transgenic model for LCMV as a viral immune escape mechanism (27). For HIV-1, such a mechanism has not yet been described. Based on a comparison of the immunogenicity of envelope glycoprotein core proteins derived from TCLA- and primary isolates, escape from recognition by T-helper cells is suggested as yet another immune escape mechanism of HIV-1.

2 Materials and Methods

2.1 Envelope glycoprotein constructs

2.1.1 Envelope glycoprotein constructs used for the generation of proteoliposomes

The envelope glycoprotein constructs were derived from the primary R5 HIV-1 isolates YU2, JR-FL, and 89.6 and the X4, TCLA-adapted isolate HXBc2. The coding sequences for the YU2 and 89.6 envelope glycoproteins were obtained from the pSVIIIenv YU2 and pSVIIIenv 89.6 expression plasmid (106). The JR-FL envelope glycoprotein coding sequence, which contains a CD5 heterologous leader sequence in place of the normal JR-FL leader, was obtained from the AIDS repository and subcloned into the pcDNA 3.1(-) (Invitrogen) expression plasmid. The HXBc2 construct was codon-optimized for mammalian expression using overlapping primers and PCR and subcloned into pcDNA3.1(+) (Invitrogen). A sequence coding for the heterologous CD5 signal sequence was subcloned to replace the endogenous HXBc2 leader sequence. For all constructs, a cytoplasmic tail truncation was generated by introduction of a stop codon in place of the codon for amino acid 712 (704 for JR-FL) and the sequence coding for the C9-tag TETSQVAPA was added according to the QuikChange (Stratagene) protocol immediately before the stop codon. To create covalently linked gp120-gp41 glycoproteins, the proteolytic cleavage site between gp120 and gp41 was disrupted by replacing the arginines 508 and 511 with serines by QuikChange site-directed mutagensis. These modifications resulted in constructs encoding cleavage-deficient gp160ΔCT envelope glycoproteins that were subsequently used to generate the proteoliposomes. Amino acid residue numbers are designated according to the prototypic HXBc2 sequence. The introduction of the desired mutations was confirmed by sequencing.

2.1.2 Core gp120 envelope glycoproteins

The envelope glycoprotein constructs were derived from the TCLA HIV-1 isolate HXBc2 and the primary isolates YU2 and JR-FL. The coding sequences of the envelope glycoproteins were derived from the pSVIIIenv HXBc2, YU2, and JR-FL expression plasmids containing deletions of the V1 and V2 variable loops as previously described

(120). Briefly, the V1/V2 coding region was deleted from amino acids 128 to 194 (HXBc2 numbering) and replaced with a Gly-Ala-Gly sequence. Subcloning of sequences encoding these V3-containing core glycoproteins into the insect expression vector pMT (SmithKline Beecham Pharmaceuticals) containing the inducible metallothionein promoter was performed as follows. The gp120 core coding regions were amplified by PCR to remove the N- and C-terminal sequences (Δ82 and Δ492) and to append a 5' BamHI and a 3' XbaI site. The PCR products were digested with BamHI and XbaI. The pMT expression plasmid was digested with BglII and NheI to obtain compatible overhangs. The insert was then ligated into the pMT expression plasmid. For the generation of gp120 core constructs containing 5' and 3' PADRE sequences, the PADRE sequences (amino acid sequence: AKFVAAWTLKAAA) (2) as well as BamHI and XbaI sites were appended by PCR to the core-coding sequence in the pMT expression plasmid. The PCR products were digested with BamHI and XbaI and inserted into the pMT vector cut with BglII and NheI. The presence of the PADRE sequences was confirmed by DNA sequencing.

2.2 Expression of envelope glycoprotein constructs

2.2.1 Expression of gp160ΔCT

Plasmids expressing the gp160ΔCT glycoproteins (2μg DNA per 100mm dish of cells) were transfected into 293T cells using Effectene reagent (QIAGEN) following the manufacturer's protocol. For the expression of YU2 and 89.6 envelope glycoproteins, the HIV-1 Tat expressor plasmid pSVTat (0.5μg) was cotransfected with the plasmids expressing the gp160ΔCT glycoproteins. Thirty-six to fourty-eight hours after transfection, cells expressing the envelope glycoproteins were harvested with PBS containing 5 mM EDTA.

2.2.2 Core gp120 envelope glycoprotein

To establish Drosophila S2 cell lines that stably express core gp120, *Drosophila* S2 cells were transfected by the $CaPO_4$ method (93) using a 20:1 ratio of the gp120 construct-expressing pMT vector and the hygromycin B resistance-conferring plasmid pchygro. Following transfection, S2 cells were washed and maintained in MRD4 medium

(SmithKline Becham Pharmaceuticals) supplemented with 5% FCS and 0.1% pluronic F-68 (Sigma) at 25°C. After 48 hours, hygromycin B (Boehringer Mannheim) was added to a final concentration of 300μg/ml. When growth was observed in the cultures 3-4 weeks after transfection, the cultures were expanded and incubated in shaking flasks at 25°C. The cells were expanded and induced to produce recombinant envelope glycoprotein by pelleting the cells at 300xg and resuspending them in serum-free induction medium (MRD4 containing 750μM $CuSO_4$ and 0.1% pluronic F-68). The cells were then incubated with shaking at 25°C for five to seven days. The cell supernatant was collected and filtered through a 0.45μm filter system (Corning). The supernatant was passed over an F105 antibody affinity column prepared from F105 antibody covalently linked to Protein-A Sepharose (Amersham Bioscience). The column was washed with 10 column volumes of PBS/0.5M NaCl, followed by 10 column volumes of PBS. Bound protein was eluted with 5 ml of 100mM glycine-HCl pH2.3 and the eluate was neutralized by adding 2M Tris base. The eluate was then concentrated using a Centriprep YM-30 concentrator (Millipore). The protein concentration of the preparation was determined photometrically by determining the absorption at 280nm using extinction coefficients determined for each gp120 construct according to the primary amino acid sequence.

2.3 Deglycosylation of envelope glycoproteins

For the enzymatic deglycosylation of *Drosophila*-produced gp120, the proteins (0.5mg/ml) were treated with 0.1 unit/ml Endoglycosidase D and 0.25 units/ml Endoglycosidase H (Roche) in 0.5M NaCl, 100mM sodium acetate buffer, pH5.7 for 10 hours at 37°C. The deglycosylation of gp120 was confirmed by SDS-PAGE as reduction in molecular weight relative to WT gp120 protein.

2.4 Generation of proteoliposomes

Paramagnetic proteoliposomes containing the HIV-1 envelope glycoprotein were created according to methods established for solubilization and membrane reconstitution of the CCR5 chemokine receptor (64). Tosyl-activated Dynabeads (Dynal) were conjugated with the 1D4 antibody (National Cell Culture Center) according to the manufacturer's protocol. The 1D4 murine antibody recognizes the C9 epitope tag and

was used to capture the envelope glycoproteins on the Dynal beads. For the characterization of proteoliposomes, beads with a diameter of 4.5µm (M-450) were used, for immunizations and phage display, beads with a diameter of 2.8µm (M-280) were used.

Cells expressing gp160ΔCT envelope glycoproteins were harvested in PBS containing 5mM EDTA, washed in PBS and lysed in 5ml solubilization buffer (100mM $(NH_4)_2SO_4$, 20mM Tris-HCl (pH7.5), 1% (w/v) Cymal-5 and one tablet of Complete Protease Inhibitor Mixture [Boehringer Mannheim] per 50ml) at 4°C for 30 min on a rocking platform. For the preparation of $4x10^8$ M-450 or $2x10^9$ M-280 proteoliposomes, the amount of gp160ΔCT envelope glycoprotein produced by transient transfection of six 150 mm tissue culture dishes of 293T cells was found to be sufficient for saturation of the beads. Cell debris was pelleted by centrifugation for 30min at 14,000xg. The cleared lysate was incubated with 1D4-conjugated Dynal beads for 16h at 4°C on a rocking platform. After recovery of the beads, they were extensively washed in solubilization buffer.

Lipids were obtained as chloroform solutions from Avanti Polar Lipids. The following lipids were used: 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC), 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphoethanolamine (POPE) and Dimyristoylphosphatidic acid (DMPA) and Cholesterol at molar concentrations 45:25:20:10. The lipid mixture was dried in a 2-ml polyethylene tube under vacuum until all of the solvent was removed. PBS was added to the tube and a liposomal solution was obtained by ultrasonication for 5min in an ice bath using an Ultrasonic Processor (Heat Systems Inc.). Liposomal solutions of the head group-modified synthetic lipids 1,2-dioleyl-sn-glycero-3-phosphoethanolamine-n-(biotinyl) (Biotinyl-DOPE) and dioleoylphosphoethanolamine-lissamine rhodamine B (Rho-DOPE), at a final concentration of 1mg/ml, were prepared separately using the same protocol. For the formation of the lipid membrane, beads coated with gp160ΔCT glycoprotein were incubated for 15min at RT with 1ml solubilization buffer containing 2mg of lipid mixture, and, if fluorescence labeling or biotinylation was desired, with 1% of Rho-DOPE or biotin-PE. The detergent was slowly removed by dialysis for 24h at 4°C against PBS, using a 10kDa molecular weight cutoff dialysis membrane (Slide-A-Lyzer 10 K

[Pierce]). The excess of unbound lipid and residual detergent was removed on a magnetic separator in one washing step with PBS. Proteoliposomes were stored in PBS with 0.1% BSA/0.1% Na_2N_3 at 4°C for up to three months.

2.5 SDS-PAGE

2.5.1 gp160ΔCT eluted from proteoliposomes

Approximately $5x10^7$ Proteoliposomes and control beads devoid of gp160ΔCT were incubated at 100°C in reducing SDS sample buffer for 5min, separated on a 7.5%SDS-polyacrylamide gel and stained with Coomassie blue.

To determine that gp120 was present in the protein peaks eluted during molecular exlusion chromatography, samples from each peak were incubated for 5min at 100°C in sample buffer containing 2% BME and separated on a 7.5% SDS-polyacrylamide gel. To confirm that oligomeric forms of envelope glycoproteins could be detected in the high molecular weight peaks, the fractions were diluted in sample buffer lacking BME and separated on a 3-8% SDS-polyacrylamide gel. A sample from the peak consistent with a trimer was also analyzed in the presence of 2% BME.

2.5.2 Core gp120 envelope glycoproteins

For the detection of core gp120, 2µg of purified core gp120 was incubated at 100°C in reducing SDS sample buffer for 5min, subsequently separated on a 12% SDS-PAGE gel and stained with Coomassie blue.

2.6 Molecular exclusion chromatography

The gp160ΔCT glycoproteins captured onto Dynal beads were eluted from the beads for molecular exclusion chromatography under non-denaturing conditions as follows. The beads were incubated in 0.5M $MgCl_2$, 1% CHAPS, and 0.2mM C9 peptide (peptide sequence: TETSQVAPA) at 37°C for 30min. Approximately 5µg of eluted gp160ΔCT glycoproteins were loaded onto a Superdex 200 column (Amersham Pharmacia Biotech) in a 200µl volume. The column was then eluted with PBS containing 1% CHAPS at a rate of 0.5ml/min for 40min. The eluted protein was detected by measuring the optical density at 280nm (OD280) using a Varian ProStar System (Varian

Analytical Instruments). Fractions of the flow-through were collected at 2 minute intervals using a Varian Dynamax Fraction Collector. The fractions were further analyzed by reducing and non-reducing SDS-PAGE and Western blotting using a polyclonal anti-gp120 rabbit serum for detection of HIV-1 envelope glycoproteins. A mixture of high molecular weight protein markers (Amersham Pharmacia Biotech) was eluted under identical conditions to calibrate the column.

2.7 Western blot

For the analysis of fractions eluted by molecular exclusion, Western blotting was performed after SDS-PAGE under either non-reducing or reducing conditions. Proteins were electrophoretically transferred onto a 0.45 μm Hybond-C extra membrane (Amersham). The gp160ΔCT glycoproteins present in each column fraction were then detected by anti-gp120 rabbit serum at a dilution of 1:2000 and anti-rabbit IgG-horseradish peroxidase (HRP) (Sigma) at a dilution of 1:5000.

2.8 Flow cytometric analysis

For the comparison of antibody binding to either cleavage-defective or cleavage-competent gp160ΔCT envelope glycoproteins, 293T cells were transfected with plasmids expressing the two envelope glycoprotein variants. Approximately 10^6 cells per sample were harvested with PBS containing 5mM EDTA and washed once in FACS buffer (PBS, 2% FCS, 0.02% Na_2N_3). The cells were incubated for 1 hour at RT with the indicated amounts of antibodies in a volume of 100μl. After two washing steps in FACS buffer, the cells were incubated for 30min with a R-Phycoerythrin (PE)-conjugated F(ab')2 goat anti-human antibody (Jackson Immuno Research Laboratories, Inc., West Grove, PA) at a dilution of 1:200, washed twice and analyzed with a FACScan flow cytometer and CellQuest software (Beckton Dickinson). For FACS analysis of proteoliposomes, staining was performed as described above. Staining for membrane integrity of the proteoliposomes was performed using a PE-conjugated goat anti-mouse IgG (Boehringer Mannheim) or Avidin-FITC (Sigma) at a dilution of 1:200. The following ligands were used for staining of the envelope glycoproteins: soluble CD4, the potently neutralizing CD4BS antibody IgG1b12 (kindly provided by Dennis Burton), the

F105 CD4BS antibody (kindly provided by Marshal Posner), the strain-restricted neutralizing V3 loop antibody 39F, the non-neutralizing C1/C4 antibody A32, the non-neutralizing C1/C5 antibody C11, the CD4-induced 17b antibody (all kindly provided by James Robinson) and the broadly neutralizing gp41 antibody 2F5 (kindly provided by Hermann Katinger).

2.9 Animals, immunization, and serum preparation

2.9.1 Immunization with proteoliposomes

2.9.1.1 Mice

To determine the immunogenicity of M-450 Dynal beads and proteoliposomes, and to determine adjuvant requirements for immunization with proteoliposomes, groups of four BALB/c mice were inoculated as follows. For the immunization of group A, $0.5x10^7$ of M-450 conjugated with the 1D4 antibody was inoculated in 200µl MPL+TDM adjuvant (Sigma) emulsion subcutaneously. For group B, 2µg of affinity-purified YU2 gp120 was inoculated in 200µl MPL+TDM adjuvant emulsion subcutaneously. Proteoliposomes containing gp160ΔCT envelope glycoprotein ($0.5x10^7$ of M-450 proteoliposomes in 200µl volume) were either resuspended in 200µl of MPL+TDM adjuvant (group D) or the adjuvant solution was administered to the site of inoculation one day prior to inoculation with proteoliposomes (group C) to limit the exposure of proteoliposomes to the detergent-containing adjuvant. The inoculation was repeated three times (two times for group D) in four week intervals. Eyebleeding was performed seven days after inoculations. The blood was collected, incubated at 4^oC O/N to allow for clot formation, and serum was cleared for 10min at 14,000rpm in an Eppendorf microtube centrifuge. The cleared serum was stored at 4^oC.

2.9.1.2 Rabbits

Groups of five New Zealand White rabbits were inoculated intradermally with 1ml of MPL+TDM+CWS adjuvant emulsion (Sigma) containing the respective antigen. The amounts of YU2 gp120 and YU2gp140-GCN4 envelope glycoprotein were adjusted to the same molar quantity of the gp120 moiety. Thus, each rabbit received 18.9µg of

YU2 gp120 and 25µg of YU2gp140-GCN4. Animals inoculated with proteoliposomes received 1ml MPL+TDM+CWS adjuvant emulsion containing approximately $1.8x10^9$ M-280 proteoliposomes. Inoculations were administered 2, 6, 10, and 22 weeks after the initial inoculation. Ear bleeding was performed thirteen days after the second and seven days after the fourth inoculation and fifth inoculation (Table 1). The blood was incubated O/N at 4°C in a Vacutainer SST Gel Clot Activator (Becton Dickinson) and spun for 30min at 2000xg at 4°C. The cleared serum was collected and incubated for 30min at 55°C to inactivate complement. The sera were then stored at -20°C.

2.9.2 Immunization with core gp120 envelope glycoproteins

2.9.2.1 Mice

Female, four week old mice were purchased from Taconic. Groups of six C57BL/6 and BALB/c mice were inoculated subcutaneously with 20µg of gp120 resuspended in 200µl of MPL+TDM adjuvant (Sigma). The inoculation was repeated four times in four week intervals. Eyebleeding and serum preparation was performed as described above.

2.9.2.2 Rabbits

For the immunization of rabbits, 20µg of envelope glycoprotein was resuspended in 1 ml of MPL+TDM+CWS adjuvant (Sigma). Groups of five New Zealand White rabbits were inoculated intradermally with 1ml of the emulsified antigen. Inoculations were administered 4, 8, and 12 weeks after the initial inoculation. Earbleeding was performed thirteen days after each inoculation except the first. The blood samples were incubated overnight at 4°C in a Vacutainer SST Gel Clot Activator (Becton Dickinson) and cleared for 30min at 2000xg at 4°C. The cleared serum was stored at -20°C.

2.10 ELISA

2.10.1 Detection of anti-gp120 reactivity in serum

To determine the anti-gp120 reactivity in the serum raised from immunizations with proteoliposomes or core-gp120, 100ng of affinity-purified mammalian-expressed YU2 gp120 (for the sera raised against proteoliposomes) or, for the sera raised against core-gp120, affinity-purified *Drosophila*-expressed core or full length gp120 derived from the HXBc2 or YU2 isolate was adsorbed onto each well of a high-protein-binding microwell plate (Corning) in PBS over night at 4^{o}C. After blocking the plates in 100µl blocking buffer (PBS/2% dry milk/5% FCS), serial serum dilutions in ELISA blocking buffer were incubated in each well for 1h at RT. After three washes with PBS/0.2% Tween-20, mouse serum binding to gp120 was detected as follows. A 1:2000 dilution of anti-mouse-IgG-biotin antibody (Sigma) in washing buffer was added for one hour, followed after three washes by an incubation with horseradish peroxidase (HRP)-conjugated Streptavidin (Sigma) at a 1:4000 dilution for 30min. For the analysis of rabbit sera, a secondary Anti-Rabbit-IgG-HRP antibody (Sigma) was added in washing buffer at a 1:2000 dilution for one hour at RT. For the detection of IgM titers in rabbit serum, an anti-rabbit-IgM-HRP antibody (Sigma) was used at a 1:2000 dilution. Following three washes, the ELISAs were developed with 100µl TMB Peroxidase substrate (KPL). The reaction was stopped by adding 100µl 1M HCl to each well. After 5min, the optical density at 450nm was read on a microplate reader (Molecular Devices). Endpoint titers were defined as last reciprocal serum dilution at which the absorption at 450nm was greater than two-fold over the signal detected with preimmune serum.

2.10.2 Detection of gp120- and gp41-binding of scFv and Fab antibodies

For the detection of gp120-binding of Fab and scFv antibodies by ELISA, Microplate wells (Corning) were coated overnight at 4°C with 50µl of PBS containing 100ng/well of the following antigens: Affinity-purified YU2 gp120, affinity-purified IIIB gp41, and, for the detection of unspecific binding, BSA (New England Biolabs). The wells were washed twice with PBS/0.02% Tween-20 and blocked with PBS/3% BSA for 1h at 37°C. After a single wash, 20µl of Fab- or scFv-containing supernatant and 20µl

PBS/1% BSA/0.02% Tween-20 was added to the wells and allowed to incubate at 37°C for 1h. For the detection of purified scFv and Fab antibodies, the desired antibody concentration was added in 100µl PBS/0.02% Tween-20. The wells were washed four times. For Fab detection, goat anti-human IgG F(ab')2 alkaline phosphatase (Pierce) diluted 1:500 in PBS containing 1% BSA was added, and the plate was incubated for 40min at RT. The wells were washed five times and developed by adding 50µl of alkaline phosphatase substrate (one tablet of disodium p-nitrophenyl phosphate [Sigma] to 5ml of alkaline phosphatase staining buffer [pH 9.8]). After 30min, the optical density at 405nm was read on a microplate reader (Molecular Devices). For the detection of scFv, mouse anti-HIS IgG (Santa Cruz Biotechnology) was added at a 1:500 dilution in PBS/0.02% Tween-20 and incubated for 40min at room temperature. After three washes, anti-mouse-IgG- HRP (Sigma) diluted 1:2000 in PBS/0.02% Tween-20 was added and incubated for 30min at RT. After three washes, the wells were developed and detected as described above.

2.10.3 Competition ELISA with sCD4 and D-mannose

Microplate wells (Corning) were coated overnight at 4°C with 50µl of PBS containing 100ng/well of *Drosophila*-expressed YU2 gp120. For sCD4 competition ELISA, the wells were incubated with sCD4 in 50µl PBS for 1h at RT. The antibodies were added to the wells at a concentration of 20µg/ml in 50µl PBS/0.02% Tween-20, thus obtaining a final antibody concentration of 10µg/ml. The wells were washed and developed as described above for Fabs.

For D-mannose competition ELISA, D-mannose (Sigma) was added to the wells in 50µl PBS/0.02% Tween-20. 50µl serum at a dilution of 1:250 in PBS/0.02% Tween-20 or 50µl antibodies at a concentration of 5µg/ml in PBS/0.02% Tween-20 were added to obtain a final serum dilution of 1:500 and a final antibody concentration of 2.5µg/ml. The wells were washed and developed as described above for rabbit serum.

2.11 Phage display

2.11.1 Panning of a naïve human phage-displayed scFv antibody library

The paramagnetic proteoliposomes were used to screen a naïve human single-chain antibody phage display library generated from a healthy uninfected donor. Before each round of selection, proteoliposomes were incubated with PBS/2% non-fat dry milk for 30min at RT. The proteoliposomes were adsorbed onto a magnet (Dynal) and incubated with the phage-displayed scFv library stock (approximately 10^{12} phage) for 30min at RT. After binding of the phage, the proteoliposomes were washed three times for 10min with PBS/2% non-fat dry milk after the first round of selection, four times for 10min with PBS after the second round of selection, and four times 15min after the fourth round of selection. For the first two rounds of selection, $4x10^7$ YU2 M-450 proteoliposomes were used. The following two rounds of selection were performed with $2x10^7$ proteoliposomes. The phage were eluted after each round by incubation with 100µl 0.1M HCL pH2.2 for 10min on a rocking platform. The eluted phage were neutralized with 1M Tris to pH7. After each round of panning, a counterselection step with $4x10^7$ beads conjugated with 1D4 antibody but devoid of gp160 was performed. The phage were infected into *E.coli* TG-1 bacteria and rescued by infection with M13 helper phage (Stratagene). After the fourth round of selection, phage produced by individual bacterial clones were tested for binding to gp120. Individual colonies were inoculated into a 96-well plate and induced to produce phage particles into the bacterial supernatant by infection with helper phage. The bacterial supernatants were tested for their ability to bind to YU2 gp120 by ELISA. Clones positive for gp120-binding were subjected to DNA sequencing to identify unique scFv antibodies.

2.11.2 Panning of biased human phage-displayed scFv and Fab antibody libraries

Six libraries generated from the bone marrow of HIV-1-infected asymptomatic donors (biased libraries) were panned with proteoliposomes as antigen. The library FDA-2 was panned as a Fab and a scFv library, the libraries DS, DL, DA, and MT were panned as Fab libraries only. For the panning of the FDA-2 Fab library, two separate libraries were prepared initially, one containing kappa light chains and one containing lambda

light chains. Libraries of either kappa or lambda light-chain variable-region fragments were panned separately for one round with each antigen and then combined. The DS, DL, DA and MT libraries were panned separately for one round of panning and then pooled for the three subsequent pannings. All libraries were subjected to four rounds of affinity selection against the respective antigen.

For affinity selections of the biased libraries, M-280 proteoliposomes were prepared as described. Per round of panning, 30-50µl proteoliposomes were blocked with 4% non-fat dry milk for 1h at 37°C. After adsorption onto a magnet, the proteoliposomes were incubated with the phage library (10^{11}-10^{12} phage diluted in 50µl of PBS/1% BSA). The proteoliposomes were incubated with the library for 1h at 37 °C on a rocking platform. After adsorption of the proteoliposomes onto a magnet, the proteoliposomes were washed twice with PBS alone, and once in PBS/1% BSA for different times according to the stringency desired in the particular experiment. The proteoliposomes were adsorped onto a magnet and the phage were eluted using 100µl 0.1M HCL pH2.2 with 1mg/ml BSA for 10min on a rocking platform. The eluted phage were then counterscreened for 30 min at RT on a rocking platform to absorb unspecific reactivities. The eluted phage were incubated with the following counterscreening reagents: 50µl of 1D4-coupled M-280 Dynabeads (pannings A, B, F, G, H, J, and K), 2.5µg YU2 gp120 coated onto the well of an ELISA plate (panning C), CCR5 Proteoliposomes (panning D), and YU2gp140-GCN4 complexed with sCD4 on proteoliposomes containing CCR5 (panning E). After counterscreening, the phage were infected into *E.coli* XL-blue and rescued with M13 helper phage.

From the enriched Fab phage pools after four rounds of selection, the pComb3 vector containing the Fab genes was obtained and engineered to secrete soluble Fab by polyclonal restriction digest of the library with NotI and NheI (New England Biolabs), thus deleting the bacteriophage coat protein geneIII. After religation of the vector, the library was transformed into *E.coli* XL1-blue (Stratagene). From the enriched scFv phage pools, the pComb3 vector was obtained and transformed into nonsuppressor *E.coli* strain TOP10 (Stratagene).

2.12 ScFv and Fab production

For small-scale production of Fab- and scFv-containing cell lysates for the screening of individual colonies for gp120- or gp41-binding, individual colonies of XL-1blue (Fab) and TOP10 (scFv) were inoculated into 10ml superbroth medium (SB [for 1 liter, 10g of 3-(N-morpholino)propanesulfonic acid (MOPS), 30g of tryptone, and 2g of yeast extract (pH 7)] containing 20mM $MgCl_2$, tetracycline (10µg/ml), and carbenicillin (50µg/ml) and incubated for 8h at 37°C. After adding 1mM IPTG to the cultures, they were incubated O/N at 37°C. The cells were pelleted, resuspended in 1ml PBS and lysed by three cycles of freezing in a dry-ice/ethanol bath and thawing. Cell debris was pelleted by centrifugation at 13,000xg for 10min and the cleared Fab- or scFv-containing supernatant was used for ELISA.

For large-scale preparations, a single colony of each Fab-expressing (scFv-expressing) clone of XL-1blue (TOP10) was used to inoculate an overnight culture of 10ml containing 20mM $MgCl_2$, tetracycline (10µg/ml), and carbenicillin (50µg/ml). The overnight culture was then used to inoculate 8 liters of SB medium containing 20mM $MgCl_2$, tetracycline (10µg/ml), and carbenicillin (50µg/ml). The cultures were incubated at 37°C with shaking at 250rpm until an optical density at 600nm of 0.8 was reached, after which time 1mM IPTG was added to each culture. The cultures were then incubated for a further 16h at 30°C. The cultures were pelleted by centrifugation at 8,000rpm for 15min in a Sorvall SLA-3000 rotor at 4°C, and the pellets were resuspended in 30ml of PBS containing 0.3 µM phenylmethylsulfonyl fluoride (PMSF). The bacteria were lysed by sonication (Misonix sonicator, 1/2-in. horn) four times at 50% cycle time (power 7 for 20s). The bacterial lysate was centrifuged at 17,000rpm in a Sorvall SS-34 rotor for 35min at 4°C. The supernatant was filtered (pore size, 0.8µm and then 0.2µm) and loaded onto a 5ml Protein G-Sepharose column for the purification of Fabs and a Ni-NTA column for the purification of scFv. The Fab was eluted with 0.2M glycine-HCl (pH2.2), neutralized with 2M Tris base (pH9). The scFv were eluted in 200mM Imidazole. Fab and scFv eluate was concentrated with a Centriprep YM-30 (Fab) and YM-10 (scFv) concentrator (Millipore) and dialyzed against PBS (pH 7).

2.13 HIV-1 single-round neutralization assay

The HIV-1 neutralizing activity of serum or antibodies was determined using a single-round virus entry assay, measuring infection by FACS detection of pseudotyped GFP reporter viruses or detection of infectious molecular clones by intracellular p24-Ag staining.

The intracellular p24-Ag neutralization assays were performed in 96-well culture plates. For each antibody or serum dilution, two wells were set up and combined to produce enough cells for precise quantitation by flow cytometry. 40µl of virus stock were incubated with the desired amount of purified antibodies in 10µl volume or 10µl of serum to obtain a serum dilution of 1:5. Approximately 20,000 $TCID_{50}$ (determined by a 14-day titration assay) of HIV-1 was added to each well. After incubation for 30min at 37°C, 20µl of PBMC ($1.5x10^5$ cells) was added to each well. PBMC were maintained in IL-2 culture medium containing 1µM indinavir, and the cells were fed on day 1 with 150µl of IL-2 culture medium containing indinavir. Since there were 150,000 PBMC per well, the resulting MOI was approximately 0.1.

PBMC were harvested for intracellular p24-Ag staining on day two. For intracellular p24-Ag staining, cells were transferred to V-bottomed plates and washed once in PBS containing 1% FCS. Cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD-PharMingen). Permeabilized cells were washed twice in V-bottomed plates using the wash buffer provided by the manufacturer and resuspended for 20min at 4°C with 50 µl of a 1:160 dilution of a phycoerythrin (PE)-conjugated mouse anti-p24 Mab (KC57-RD1; Beckman Coulter, Inc.), or a mouse IgG1 isotypic control antibody. After two additional washes, HIV-1- or mock-infected PBMC were analyzed with a FACSCalibur flow cytometer (Becton Dickinson), and data analysis was performed with FlowJo software (Tree Star). For the measurement of infection with pseudotyped GFP reporter viruses, infected cells were washed once in FACS buffer and directly detected. Live cells initially gated by forward and side scatter were analyzed for intracellular expression of p24-Ag or GFP. The number of p24-Ag-positive cells or GFP-positive cells was determined using a bivariate plot of fluorescence versus forward scatter; the gate was set on mock-infected cells. To enumerate infected PBMC, cells were washed, fixed and permeabilized, and stained with the KC57 anti-p24 antibody as

described above. After forward and side scatter gating, at least 50,000 events were counted. Final quantitation of p24-Ag-positive cells was done by subtraction of background events in mock-infected PBMC (usually less than 10 positives per 50,000 events). The percent neutralization was defined as reduction in the number of p24-Ag-positive cells and GFP-positive cells, respectively, compared with the number in control wells with individual prebleed serum or no antibody, respectively.

IC_{50}, IC_{80}, and IC_{90} values were determined as follows. Antibody dose-response curves were fit with a nonlinear function and the concentration of antibody required for 50, 80, or 90% reduction (IC_{50}, IC_{80}, IC_{90}, respectively) of infection was calculated by a least-squares regression analysis.

3 Results

3.1 Generation and characterization of proteoliposomes

3.1.1 Creation of gp160ΔCT proteoliposomes

293T cells transiently expressing the HIV-1 YU2, JR-FL, 89.6 or HXBc2 gp160ΔCT glycoproteins, which contain an alteration of the gp120-gp41 cleavage site and deletion of the gp41 cytoplasmic tail, were lysed in buffer containing Cymal-5 detergent. The gp160ΔCT glycoproteins were then captured onto Dynal beads conjugated to the 1D4 antibody, which recognizes a C9 peptide tag affixed to the gp160ΔCT C-terminus. Following addition of membrane lipids and dialysis of the Cymal-5 detergent, an artificial lipid bilayer is formed around the bead surface (Fig. 4). Thus, pure, properly oriented HIV-1 envelope glycoproteins, embedded in a natural membrane environment, were incorporated into an easily manipulable solid support.

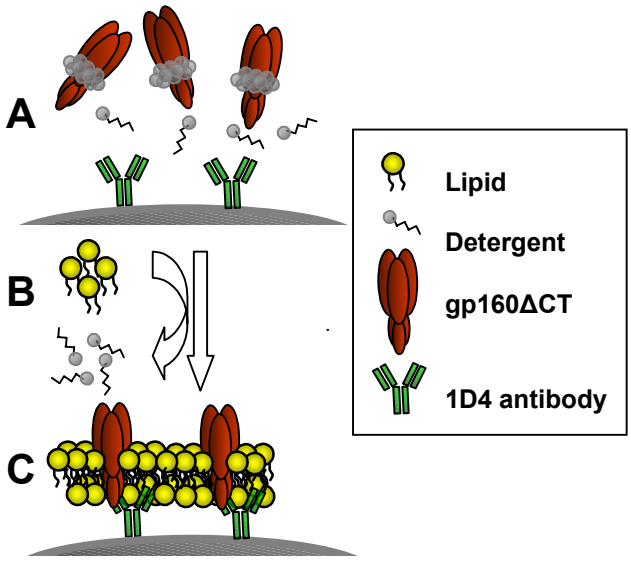

FIG. 4. Schematic diagram of the formation of proteoliposomes. (A) Beads conjugated with the 1D4 antibody recognizing the C9 tag were used to capture the C9 tagged gp160ΔCT glycoproteins from cell lysates. The beads were then washed extensively in detergent-containing buffer to remove non C9-tagged proteins. (B) The beads were incubated with detergent-solubilized lipid and dialyzed. (C) During dialysis in PBS, the detergent is replaced by a lipid membrane which assembles around the gp160ΔCT glycoproteins.

3.1.2 Analysis of proteoliposome protein composition

The gp160ΔCT proteoliposomes were boiled in sample buffer and analyzed by reducing SDS-PAGE to determine their protein composition. As a positive control, cell

lysates containing transiently expressed gp160ΔCT glycoproteins were immunoprecipitated with the F105 anti-gp120 antibody and protein A-Sepharose and analyzed in parallel. Proteoliposomes lacking the gp160ΔCT glycoproteins were treated similarly and served as a negative control. As seen in Fig. 5A, a band migrating at a position similar to the gp160ΔCT glycoprotein positive control band (lane 1) was observed among the proteins released from the gp160ΔCT proteoliposomes (lane 2). No such band was observed in this molecular weight range in the gp160ΔCT glycoprotein-deficient proteoliposome control sample (lane 3). Apart from a 50kD band corresponding to the 1D4 antibody heavy chain, a light chain band not retained on the gel, and the gp160ΔCT band, only minor impurities were detected in the gp160ΔCT proteoliposomes. The total amount of gp160ΔCT glycoproteins captured onto $5x10^7$ proteoliposomes was estimated to be 1-2 μg, as determined by the purified recombinant gp160ΔCT glycoprotein control of known concentration.

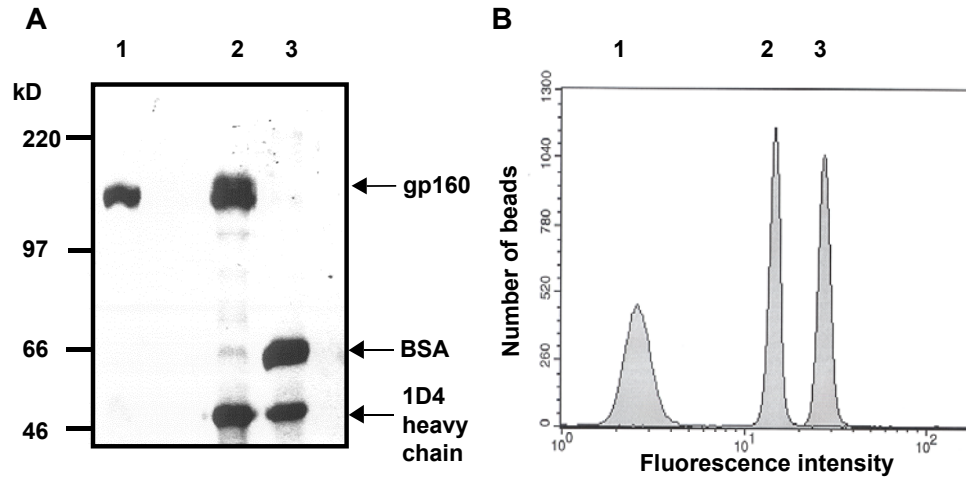

FIG. 5. Protein composition of proteoliposomes. (A) SDS-PAGE gel of gp160ΔCT glycoproteins eluted from $5x10^7$ proteoliposomes, stained with Coomassie blue. Lane 1 shows 1μg of affinity-purified gp160, lane 2 shows protein eluted from gp160ΔCT PLs and lane 3 shows protein eluted from beads conjugated with the 1D4 antibody. (B) FACS analysis of proteoliposomes stained with the IgG1 b12 antibody (peak 2) and HIV-1 patient serum (peak 3), compared to anti-human FITC secondary antibody alone (peak 1).

To examine the exposure of the HIV-1 envelope glycoproteins on the proteoliposome surface, the gp160ΔCT proteoliposomes were stained with the IgG1b12

anti-gp120 antibody and a mixture of sera from HIV-1-infected individuals and analyzed by fluorescence-activated cell sorting (FACS). The IgG1b12 antibody recognizes a conformation-dependent gp120 epitope near the CD4 binding site. The forward scatter versus sideward scatter plot showed mostly single proteoliposomes. Doubles and other multiples of proteoliposomes were generally observed to be less than 20% of the total events (data not shown). A gate was created to analyze only single proteoliposomes and was used for all further FACS analyses. The narrow distribution of the fluorescence intensity associated with each FACS peak following antibody staining suggests that the gp160ΔCT proteoliposomes have nearly uniform protein content (Fig. 5B).

3.1.3 Characterization of the size of the gp160ΔCT proteoliposome envelope glycoproteins

The gp160ΔCT glycoproteins were eluted from the reconstituted proteoliposomes under non-denaturing conditions by incubation with 0.2 mM C9 peptide in the presence of 1% CHAPS detergent and 0.5M NaCl. The eluted envelope glycoproteins were analyzed by size-exclusion chromatography on a Superdex 200 column equilibrated in PBS/1%CHAPS buffer. The chromatogram for the HIV-1 JR-FL gp160ΔCT envelope glycoproteins is shown in Fig. 6A. Parallel studies using the YU2 gp160ΔCT glycoproteins yielded a similar profile (data not shown). The fractions of the resolved JR-FL glycoproteins were collected and analyzed by SDS-PAGE and Western blotting (Fig. 6B).

The column was calibrated with molecular weight standards, allowing the apparent molecular size of the major peak to be approximated as 580kD. As the gp120 glycoprotein monomer was resolved by size-exclusion chromatography with an apparent molecular weight of 180kD (data not shown and (122)), a mass of 580kD is consistent with that of a trimeric gp160ΔCT envelope glycoprotein complex. The fastest migrating protein peak was detected just after the void volume and apparently consists of gp160ΔCT glycoprotein aggregates as determined by its migration pattern in the column and by the Western blot results (Fig. 6C). This aggregate peak has previously been

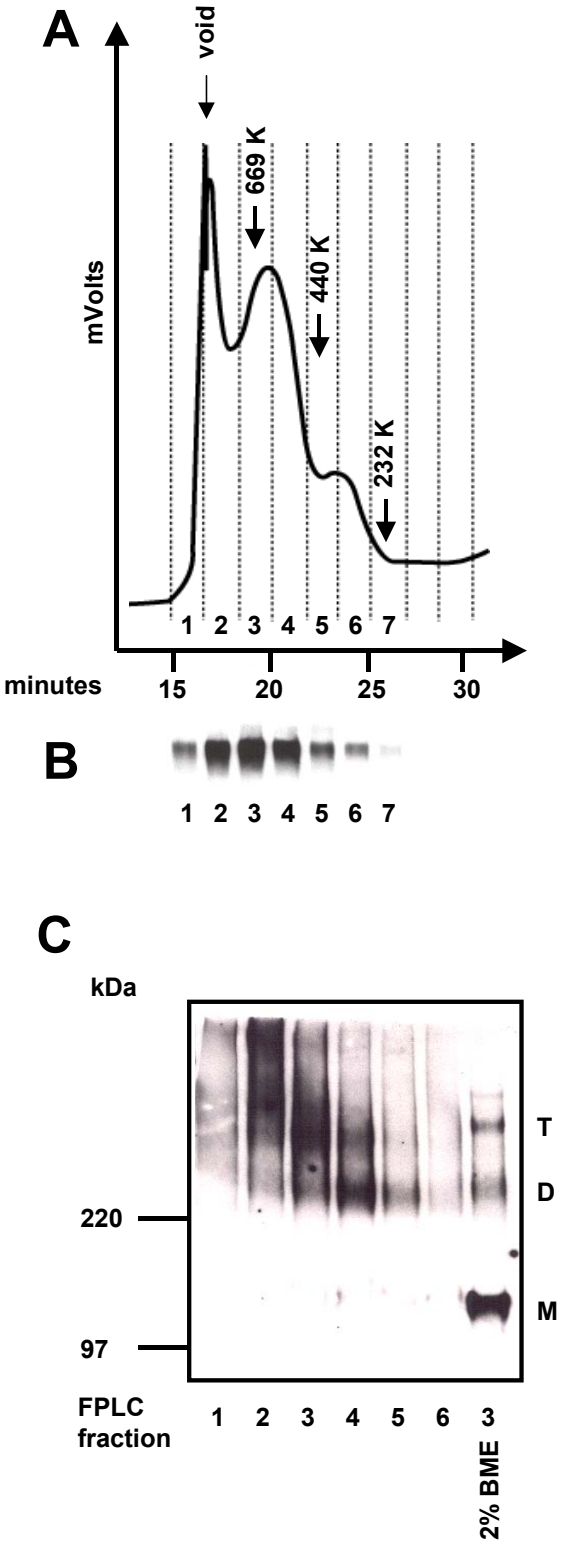

FIG. 6. Size exclusion chromatography and Western blot of JR-FL gp160ΔCT glycoproteins eluted from Dynal beads under native conditions. 293T cells transiently expressing the JR-FL gp160ΔCT C9-tagged glycoproteins were lysed in CHAPS containing buffer and incubated with Dynal beads conjugated with the 1D4 antibody. Beads were then washed and incubated in buffer containing 0.2mM C9 peptide and 0.5M $MgCl_2$ to elute the gp160ΔCT glycoprotein from the beads. (A) Approximately 5μg of JR-FL gp160ΔCT glycoproteins were analyzed on a Superdex-200 gel filtration column. (B) Eluted fractions were collected, analyzed by SDS-PAGE under reducing conditions (2% BME and 100^{o}C) and analyzed by Western blot with a polyclonal anti-gp120 rabbit serum. (C) Eluted fractions were analyzed on a 3-8% SDS-PAGE gradient gel under non-reducing conditions (minus BME and 37^{o}C) and under reducing conditions (2% BME and 100^{o}C), respectively, and detected by Western blotting using a polyclonal anti-gp120 rabbit serum. Protein bands of apparent molecular weights consistent with trimeric gp160ΔCT glycoproteins (T), dimeric glycoproteins (D) and monomeric glycoproteins (M) are marked as indicated.

observed in molecular exclusion chromatography of soluble GCN4-stablized gp140 trimers (122). Most of the gp160ΔCT glycoprotein eluted in fractions 3 and 4, with fraction 3 corresponding to the mass of 580kD. When all of the gel filtration fractions were subjected to non-reducing SDS-PAGE (with pretreatment in gel loading buffer at 37°C) followed by Western blotting, fractions 3 and 4 contained trimeric envelope glycoproteins, which were found to separate into trimers, dimers and monomers. The highest level of gel-stable trimeric envelope glycoprotein was detected in fraction 3 (Fig. 6C). Most of the glycoproteins migrating with molecular weight corresponding to trimers in this fraction could be almost completely reduced to a monomeric gp160ΔCT band by treatment with 2% β-mercaptoethanol (BME) and boiling, although bands migrating in a manner consistent with trimers and dimers could still be observed (Fig. 6C).

3.1.4 Characterization of the proteoliposome membrane

The formation of the lipid membrane was examined by FACS analysis and fluorescent microscopy. According to our model, the murine 1D4 capture antibody would be expected to be partially occluded by a reconstituted lipid membrane. Thereby, binding

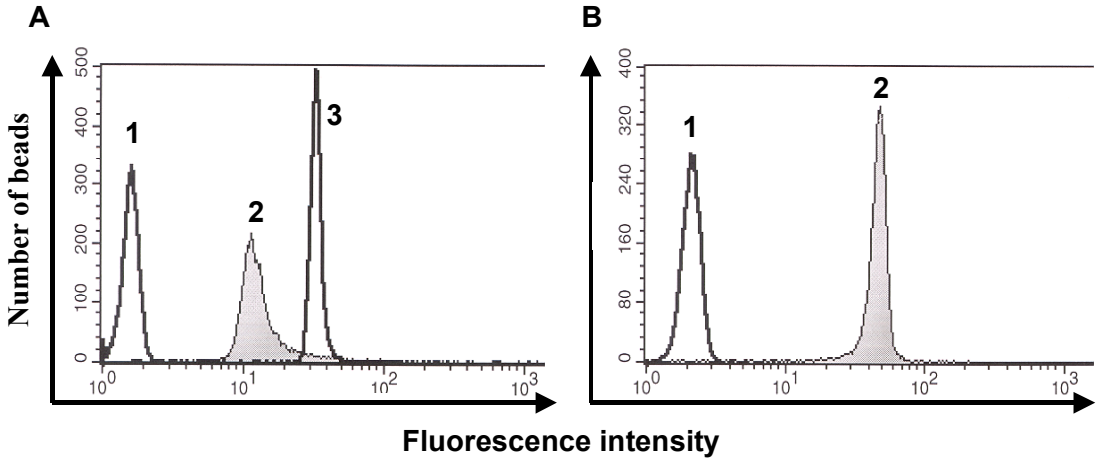

FIG. 7. FACS analysis of the reconstituted proteoliposome membrane. (A) Occlusion of the 1D4 antibody by lipid membrane reconstitution. gp160ΔCT proteoliposomes with (peak 2) and without (peak 3) a reconstituted membrane were probed with anti-mouse Ig-PE antibody. Peak 1 shows staining with the same antibody of non-conjugated beads. (B) proteoliposomes with a reconstituted membrane containing 1% biotinylated lipid (peak 2) and beads without a reconstituted membrane (peak 1) were probed with Avidin-FITC.

of anti-mouse IgG antibody should be impaired on proteoliposomes when compared to beads without a membrane. A more than 3-fold decrease of anti-mouse-PE signal could be observed on proteoliposomes containing a reconstituted lipid membrane versus beads without lipid reconstitution (Fig. 7, left panel, peak 2 compared to peak 3), indicating the presence of at least a partial lipid membrane.

The presence of the lipid membrane was also examined by FACS analysis after reconstitution of the membrane with biotinylated lipid at 1% (w/w) of total lipids. The proteoliposomes were stained with avidin-FITC and showed more than a 20-fold higher signal on fully reconstituted proteoliposomes compared to gp160ΔCT glycoprotein-containing beads without lipid reconstitution (Fig. 7, right panel). The presence of a lipid bilayer was then confirmed by visualizing the incorporation of rhodamine-conjugated lipid (rhodamine-DOPE) into the proteoliposome membrane by fluorescent microscopy (Fig. 8). Bright fluorescence could be observed in the rhodamine-labelled proteoliposomes (B) compared to negligible background fluorescent emission with untreated beads (A).

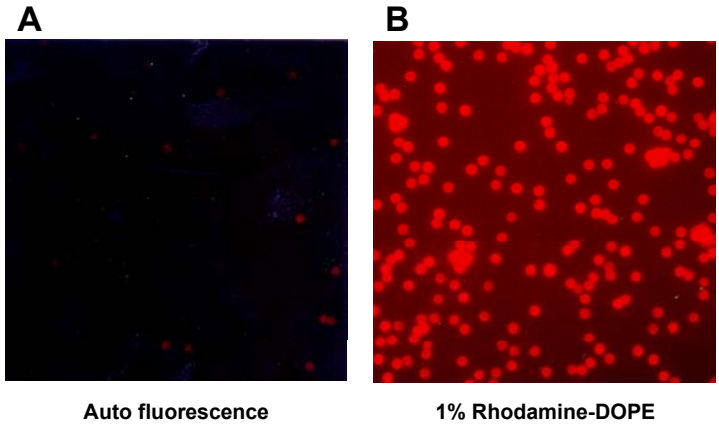

FIG. 8. Fluorescent microscopic image. (A) unconjugated beads and (B) proteoliposomes reconstituted with a membrane containing 1% DOPE-Rhodamine.

3.1.5 Antigenic characterization of the gp160ΔCT proteoliposomes

To confirm the conformational integrity of the gp160ΔCT glycoproteins on the surface of the proteoliposomes, binding of a panel of conformationally-sensitive ligands

to gp160ΔCT glycoproteins on proteoliposomes was compared with binding to gp160ΔCT glycoproteins expressed on the surface of transiently-expressing cells.

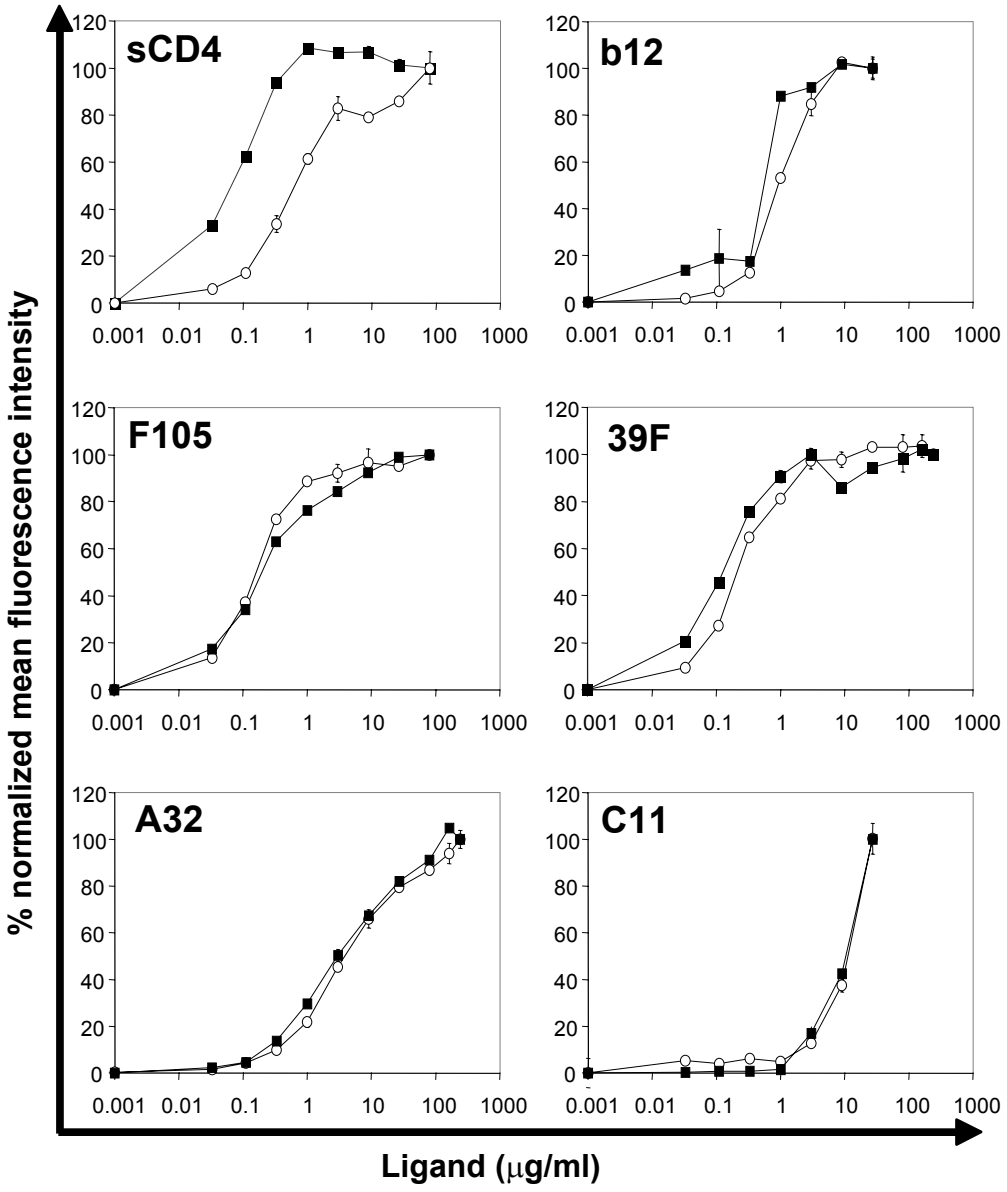

FIG. 9. Binding of a panel of anti-gp120 antibodies and soluble CD4 (sCD4) to YU2 gp160ΔCT glycoprotein expressed on 293T cells compared to YU2 gp160ΔCT glycoprotein on proteoliposomes. Cells (open circles) and proteoliposomes (closed squares) were incubated with increasing amounts of the indicated human antibodies, followed by detection with anti-human IgG-PE antibody. For detection of sCD4 binding to the glycoproteins, the rabbit anti-CD4 antibody T45 and anti-rabbit IgG FITC were used. Following staining, samples were analyzed by FACS. The binding of ligands to gp160ΔCT PLs was plotted as % normalized mean fluorescent intensity (MFI) at serially-diluted antibody concentrations. The % normalized MFI values were calculated according to the formula: [MFI-MFI(background)]x100/MFI(saturation)-MFI(background). Error bars indicate the range of values obtained for duplicate samples.

The antibodies were selected to probe several faces of the HIV-1 envelope glycoproteins, including the neutralization-relevant epitopes overlapping the CD4 binding site (CD4BS). The potently neutralizing CD4BS antibody IgG1b12, the less potent neutralizing CD4BS-antibody F105, and soluble CD4 (sCD4) were used to confirm the correct native conformation of the CD4BS. In addition, gp160ΔCT proteoliposomes were probed with the strain-restricted neutralizing V3-loop antibody 39F, and the non-neutralizing antibodies A32 and C11. The binding characteristics of all antibodies to the gp160ΔCT proteoliposomes compared to gp160ΔCT glycoproteins expressed on the cell surface were virtually indistinguishable (Fig. 9). The only difference in binding profiles was observed for soluble CD4 (sCD4). For the Proteoliposomes, sCD4 displayed an almost tenfold higher affinity than it did for gp160ΔCT glycoproteins expressed on cell surfaces. This affinity difference may be a consequence of better exposure of gp160ΔCT glycoproteins on the proteoliposomes due to the lack of the cellular glycocalix and cellular protein components. The observed affinities calculated for the antibodies were in the low nanomolar range, consistent with previously reported values (77, 83). For example, the affinity of the antibody IgGb12, as determined by the concentration necessary to achieve half-maximal binding, was calculated to be 6 nM. The affinity of the non-neutralizing C11 antibody was at least 10-fold lower than that of the neutralizing IgG1b12 antibody. This underestimates the affinity difference between the two antibodies because saturation binding was not achieved with the C11 antibody.

3.1.6 2F5 antibody binding to gp160ΔCT proteoliposomes

The epitope of the broadly neutralizing antibody 2F5, ELDKWAS, is situated proximal to the viral membrane in the gp41 ectodomain. To determine the influence of the reconstituted membrane on this important neutralizing determinant, we used the 2F5 antibody to probe proteoliposomes reconstituted with a membrane and proteoliposomes coated with gp160ΔCT glycoprotein but devoid of a membrane. Since sequence variation in the HIV-1 YU2 strain alters 2F5 antibody recognition, proteoliposomes with gp160ΔCT glycoproteins derived from the primary isolate JR-FL and the T-cell line

adapted isolate HXBc2 were examined. The 2F5 antibody bound to HXBc2 gp160ΔCT glycoprotein proteoliposomes with a reconstituted membrane with approximately a ten-fold higher affinity than it did to HXBc2 gp160ΔCT glycoprotein on beads without a membrane (Fig. 10, upper right panel). The same could be observed with JR-FL proteoliposomes, although the effect was less pronounced (Fig. 10, upper left panel). To show the specificity of the enhanced 2F5 binding observed in the presence of the reconstituted membrane, binding of the gp120-specific antibodies F105 and 2G12 to gp160ΔCT proteoliposomes with and without a membrane was examined. No affinity difference could be detected using these antibodies (Fig. 10 lower panel).

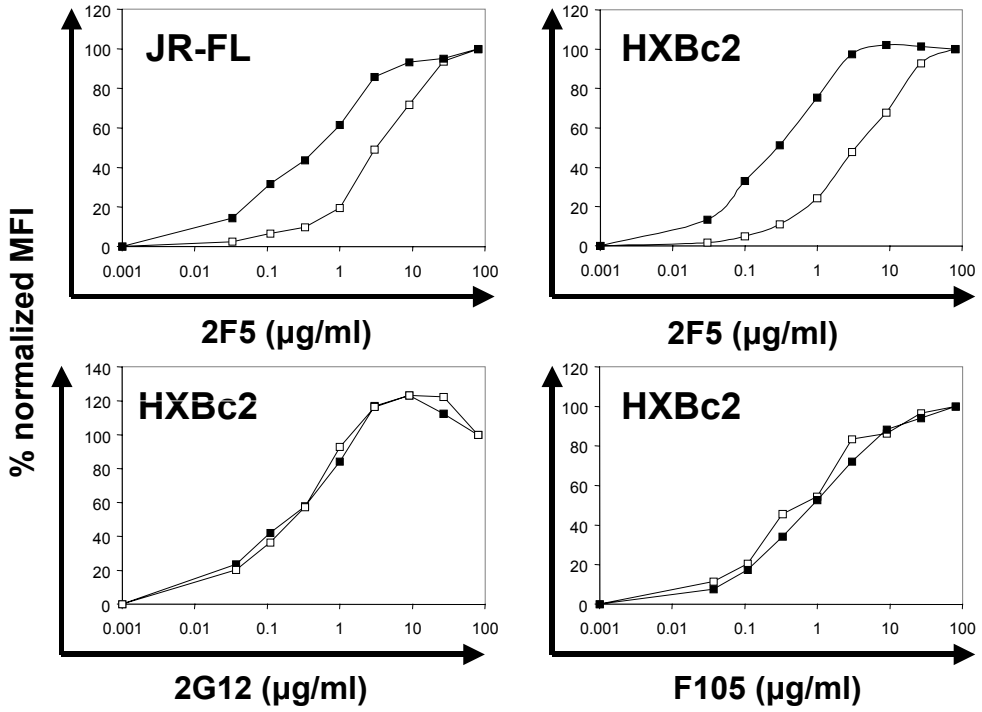

FIG. 10. Upper panel: Binding of the gp41 antibody 2F5 to gp160ΔCT from JR-FL (left) and HXBc2 (right) on beads without a membrane (open squares) and fully reconstituted proteoliposomes (closed squares). Proteoliposomes and beads were probed with increasing concentrations of 2F5 antibody and anti-human IgG-PE antibody and analyzed by FACS. The MFI was plotted as % maximal MFI at the given antibody concentration. Lower panel: Binding of the antibodies 2G12 (left) and F105 (right) to the PLs (closed squares) and beads (open squares) was performed as described for 2F5 binding above.

3.1.7 CD4 induction of the 17b epitope

One functional conformational change characteristic of native gp120 is the induction of the 17b antibody epitope by CD4 (106). To test whether the gp160ΔCT glycoproteins on proteoliposomes exhibit this property of gp120 expressed on the cell surface, gp160ΔCT glycoprotein proteoliposomes and 293T cells expressing cleavage-competent envelope glycoproteins were preincubated with sCD4 prior to binding of the 17b antibody. 17b binding was detected by staining with a PE-conjugated anti-human IgG antibody followed by FACS analysis. The binding of the 17b antibody was increased by 63% after preincubation with sCD4 on cleavage-competent gp160ΔCT glycoprotein expressed on cells compared to a 46% increase on gp160ΔCT Proteoliposomes (Fig. 11).

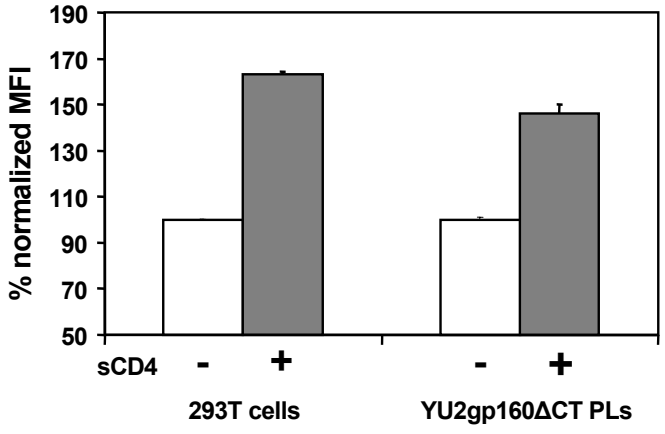

FIG. 11. Induction of the 17b epitope by sCD4. Binding of the 17b antibody to gp160ΔCT glycoprotein on 293T cells and PLs was compared. Cells and PLs were incubated with and without sCD4 prior to binding of the 17b antibody and analyzed by FACS. Staining without preincubation with sCD4 was set as 100% and increase of 17b binding is shown for 293T cells (open bars) and proteoliposomes (solid bars).

3.1.8 Characterization of cleavage-deficient gp160ΔCT glycoproteins

Deletion of the cleavage site for the host protease furin results in the expression of uncleaved gp160ΔCT glycoprotein precursor proteins on the surface of transfected cells. This modification abrogates the dissociation of gp120 from the cell surface and therefore increases the amount of envelope glycoproteins retained on the cell surface or captured on the surface of the proteoliposomes. In addition, a cytoplasmic tail deletion was introduced into the gp160 glycoprotein in order to increase cell surface expression (ΔCT). Cell surface expression levels of cytoplasmic tail-deleted envelope glycoproteins

increased 8-fold over full-length constructs containing the intact gp160 cytoplasmic tail (data not shown).

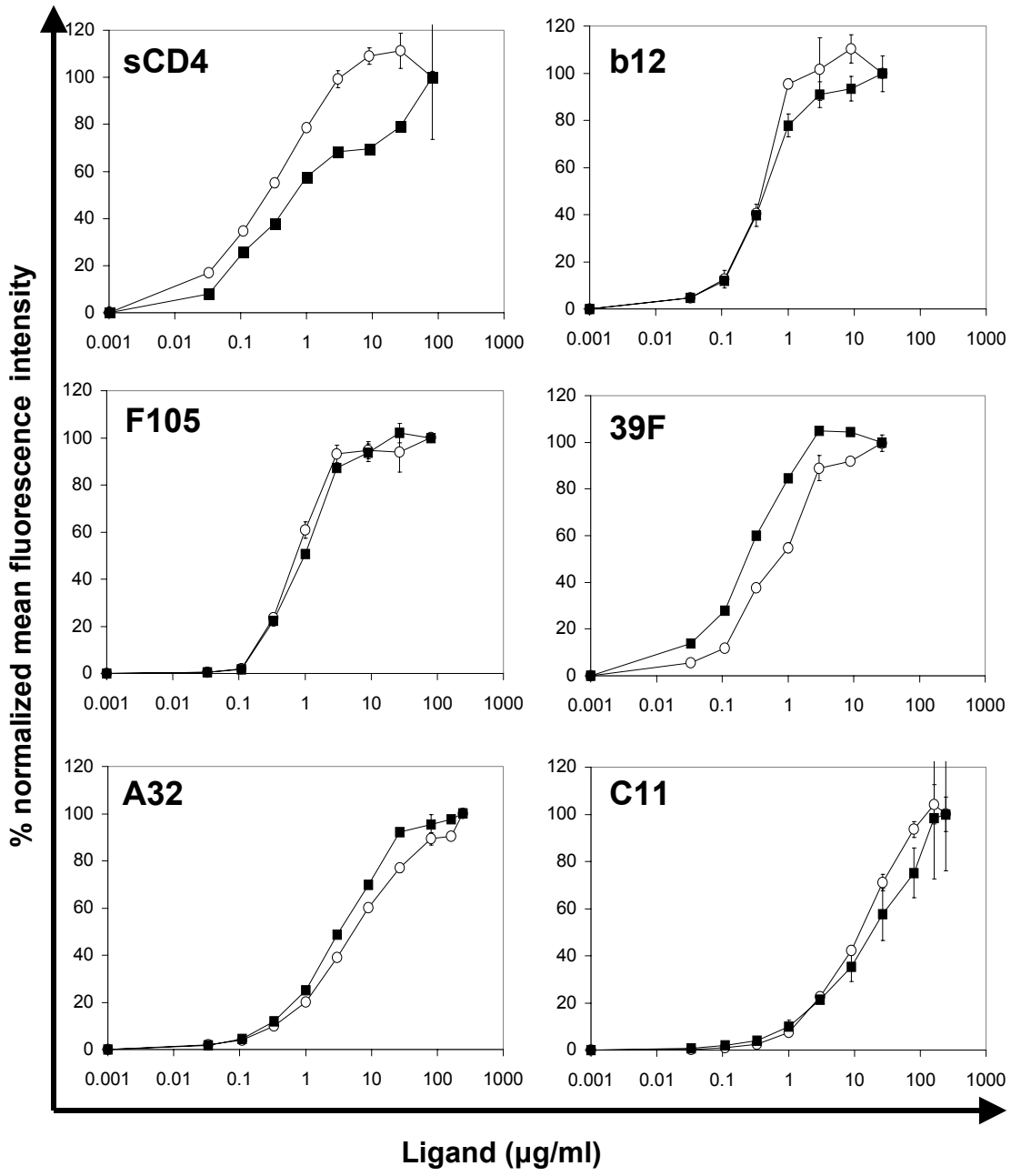

FIG. 12. Effect of proteolytic cleavage of HIV-1 envelope glycoprotein on ligand binding. Cleavage-competent YU2 gp160ΔCT glycoprotein (closed squares) and cleavage-defective YU2 gp160ΔCT glycoprotein (open circles) was expressed on 293T cells. Cells were incubated with increasing amounts of the indicated ligands followed by detection with anti-human IgG-PE. The anti-CD4 polyclonal rabbit antibody T45 and anti-rabbit IgG-FITC was used for the detection of sCD4 binding. Normalized MFI was calculated as described for Fig. 9.

It is possible that the deletion of the cleavage site might distort envelope glycoprotein conformation and result in a conformation not representative of functional, cleaved envelope glycoprotein. To assess the effects of the cleavage site deletion, the binding properties of a panel of conformationally-sensitive ligands to cleavage-defective and cleavage-competent gp160ΔCT glycoproteins expressed on the cell surface were analyzed. FACS analysis was performed by staining the gp160ΔCT expressed on 293T cells with increasing concentrations of ligands. No significant affinity difference was observed between cleavage-competent gp160ΔCT glycoproteins and cleavage-defective envelope glycoproteins for any of the ligands tested (Fig. 12). However, one notable difference in the binding profiles was observed. The binding profile of sCD4 on cells expressing cleavage-competent gp160ΔCT glycoproteins had a biphasic shape. As sCD4 is known to induce shedding of gp120 from envelope glycoprotein complexes (67), CD4 binding to the cleavage-competent gp160ΔCT glycoprotein may induce shedding or other conformational changes that result in a biphasic binding profile.

To confirm the processing of the cleavage-competent envelope glycoproteins, 293T cells were transfected with cleavage-competent and –defective envelope glycoprotein constructs. Proteins expressed on the cell surface were iodinated by lactoperoxidase. After detergent lysis and precipitation with the F105 antibody, iodinated proteins were analyzed by SDS-PAGE. The ratio of unprocessed gp160ΔCT precursor proteins to cleaved gp120 was determined to be approximately 1:1 (data not shown).

3.2 Immunogenicity of proteoliposomes

3.2.1 Immunogenicity of proteoliposomes in mice

To initially test the immunogenic properties of M-450 Dynabeads and proteoliposomes and to determine adjuvant requirements for immunization, M-450 Dynabeads conjugated with 1D4 antibody but devoid of YU2 gp160ΔCT envelope glycoprotein and proteoliposomes containing YU2 gp160ΔCT envelope glycoprotein were generated and inoculated into mice as described in Materials and Methods. As control, one group of mice was inoculated with affinity-purified monomeric YU2 gp120 (Fig. 13). The serum was tested for anti-gp120 activity by ELISA and endpoint titers

were determined as the last reciprocal serum dilution at which an at least two-fold higher signal over background signal detected with preimmune serum could be observed.

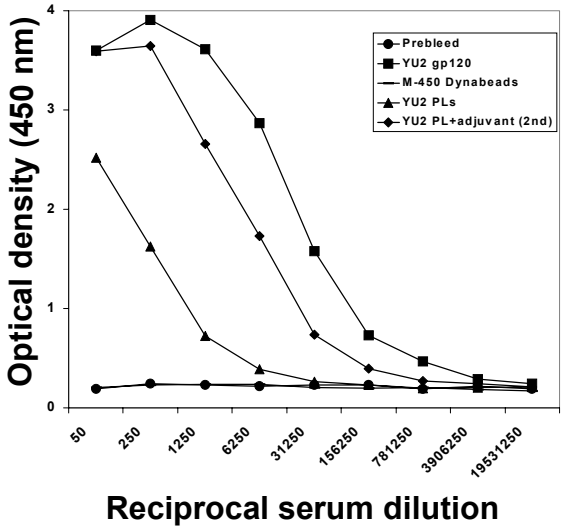

FIG. 13. ELISA of mouse serum. Pooled mouse sera were tested for anti-gp120 activity at the indicated serum dilutions after three inoculations (after two inoculations for the YU2 PL + adjuvant group).

The animals inoculated with M-450 Dynabeads conjugated with the 1D4 antibody did not generate detectable anti-gp120 antibody titers, suggesting that Dynabeads conjugated with the mouse 1D4 antibody do not elicit antibodies crossreactive with gp120 (Fig. 13). The serum of animals inoculated with YU2 gp120 envelope glycoprotein raised an anti-gp120 directed response with endpoint titers of >700,000 after three inoculations. One group of animals was inoculated with MPL+TDM adjuvant the day prior to inoculation with proteoliposomes at the site of injection to limit the exposure of proteoliposomes to the detergent-containing adjuvant. The animals in this group displayed much lower enpoint titers of >1250. The animals inoculated with proteoliposomes in MPL+TDM adjuvant raised anti-gp120 endpoint titers of >30,000 after two inoculations. The enpoint titers after three inoculations were not determined for this group. The effect of the adjuvant on the proteoliposome membrane was determined by incubation of proteoliposomes with either PBS or adjuvant and the integrity of the membrane was monitored at different timepoints by FACS detection of biotinylated lipids incorporated into the membrane (data not shown). A slight loss of Streptavidin signal could be observed over time. However, exposure of the proteoliposomes to adjuvant did not cause greater loss of membrane signal than did incubation with PBS.

Higher antibody titers could be raised with proteoliposomes coadministered with adjuvant rather than inoculation of adjuvant and proteoliposomes on different days. Since no detrimental effect of the adjuvant on the proteoliposome membrane could be detected, proteoliposomes were coadministered with adjuvant in all subsequent proteoliposome inoculations.

These data demonstrated the general feasability of immunizations with these novel reagents. No toxicity could be observed in the animals inoculated with Dynabeads, and anti-gp120 antibody titers similar to those raised against soluble gp120 could be elicited by proteoliposomes. The amount of mouse serum obtained by these experiments was not sufficient to reliably and reproducibly test for neutralizing activity against a panel of viruses.

3.2.2 Immunogenicity of proteoliposomes in rabbits

The immunogenicity of proteoliposomes in different immunization protocols was determined and compared to the immunogenicity of the YU2 gp120 envelope glycoprotein and YU2 gp140-GCN4 envelope glycoprotein. Many reports have shown that monomeric gp120 does not elicit broadly neutralizing antibodies and only after aggressive immunization elicits antibodies capable of neutralizing homologous strains. The YU2 gp140-GCN4 envelope glycoprotein used in this study has previously been shown to elicit neutralizing antibodies with limited breadth in mice (125). These envelope glycoprotein immunogens represent to date the most effective envelope-based immunogens for the elicitation of broadly neutralizing antibodies. Here we tested whether an envelope glycoprotein formulation that possibly recapitulates the trimeric envelope glycoprotein complex more faithfully (proteoliposomes) would better elicit broadly neutralizing antibodies in rabbits.

Slight variations in the amounts of envelope glycoproteins incorporated into proteoliposomes could be detected among the envelope glycoproteins derived from different viral isolates used for proteoliposome preparation. The least efficient incorporation of envelope glycoprotein constructs was observed with the JR-FL-derived construct. The amounts of envelope glycoproteins on proteoliposomes inoculated were

estimated to be approximately 5µg/dose by gel analysis (data not shown). The presence of the lipid membrane around the proteoliposomes was confirmed before each inoculation by FACS analysis detecting biotinylated lipids incorporated into the membrane. Proteoliposomes containing the envelope glycoproteins from the molecular clones HXBc2, 89.6, JR-FL and YU2 were generated. A summary of the immunization protocol is given in Table 1.

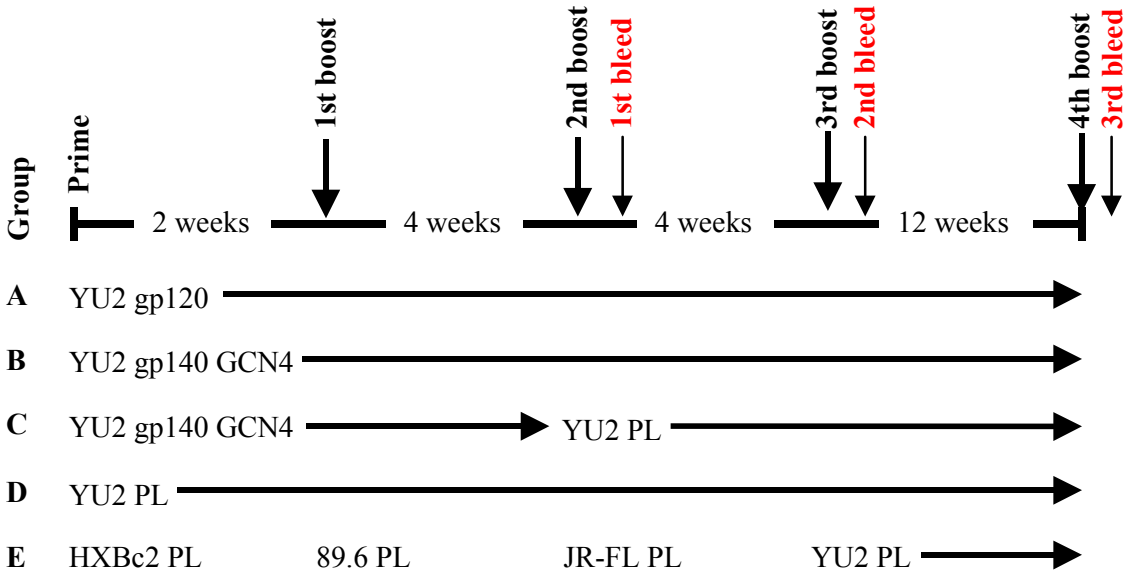

Table 1. Schematic diagram of rabbit immunization. The immunogens and times of inoculation and bleeding are given. Horizontal arrows indicate repeated administration of the same immunogen.

The immunization protocol consisted of a priming inoculation followed by four boosts at two, six, ten, and twenty-two weeks after priming (Table 1). Sera were collected seven days after boosts two, three, and four and assessed for gp120-reactivity and HIV-1 neutralizing reactivity. Reactivity of sera against YU2 gp120 was assessed by ELISA. All groups except group E generated solid anti-gp120 antibody titers with endpoint titers >62,500 (except animal 19, see ELISA data in Table 2). Differences in serum reactivity could be observed between different groups. The highest serum reactivity was detected in sera from rabbits immunized with YU2 gp140-GCN4 envelope glycoprotein, and the lowest reactivity was detected in sera from rabbits immunized sequentially with the four

Immunogen	Animal	Endpoint ELISA	viral isolate HXBc2	SF162	89.6	BaL	ADA	YU2
YU2 gp120	7	+++	*9.8* **36.5**	*11.1* **51.8**	*-35.3* **35.8**	*-42.4* **22.0**	*-46.4* **10.1**	*-4.5* **-3.3**
	8	+++	*32.9* **43.6**	*36.9* **9.9**	*-1.5* **26.1**	*9.3* **-5.2**	*-42.4* **-6.0**	*29.5* **-51.6**
	9	++	*40.2* **36.7**	*59.5* **34.7**	*35.3* **-6.8**	*18.1* **-3.2**	*-23.6* **-6.0**	*13.6* **-35.5**
	10	++	*45.1* **28.9**	*22.8* **36.4**	*10.3* **1.6**	*-3.3* **-3.0**	*-44.7* **-0.8**	*4.5* **-14.3**
	11	+++	*52.4* **12.8**	*20.5* **55.0**	*17.6* **-1.8**	*9.8* **-2.3**	*-41.3* **11.3**	*15.9* **-32.4**
YU2 gp140 GCN4	12	+++	*45.1* **64.4**	*27.5* **72.2**	*8.8* **35.4**	*2.5* **31.6**	*-51.0* **14.4**	*9.1* **71.3**
	14	+++	*47.6* **81.9**	*27.5* **79.7**	*23.5* **13.4**	*12.9* **24.0**	*-28.7* **11.1**	*23.9* **90.1**
	15	+++	*28.0* **83.1**	*33.0* **85.1**	*-5.9* **31.9**	*8.7* **9.7**	*-44.7* **6.3**	*6.8* **64.6**
	16	++	*46.3* **47.7**	*50.2* **28.2**	*19.1* **-9.1**	*10.3* **-19.8**	*-31.6* **-8.3**	*5.7* **-26.5**
YU2 gp140 GCN4/ YU2 PL	17	++	*36.6* **48.1**	*43.1* **16.5**	*17.6* **12.5**	*3.5* **8.1**	*-24.1* **-13.8**	*19.3* **31.0**
	18	++	*45.1* **26.7**	*60.3* **5.9**	*23.5* **0.0**	*14.0* **12.7**	*-29.3* **5.3**	*30.7* **-50.8**
	19	+	*35.4* **35.8**	*40.0* **14.3**	*16.2* **-7.0**	*17.1* **-35.9**	*-25.9* **-3.2**	*19.3* **-43.7**
	20	+++	*54.9* **56.8**	*49.4* **44.8**	*27.9* **-4.1**	*12.9* **18.0**	*-29.9* **4.4**	*26.1* **44.6**
	21	+++	*48.8* **47.6**	*58.8* **64.4**	*22.0* **20.7**	*6.1* **30.6**	*-33.3* **6.0**	*29.5* **96.1**
YU2 PL	22	+++	*46.3* **68.2**	*40.8* **60.7**	*7.3* **22.2**	*21.3* **-11.9**	*-23.6* **9.7**	*28.4* **-31.7**
	23	++	*46.3* **38.6**	*43.9* **75.2**	*30.9* **-2.1**	*19.7* **25.3**	*-28.7* **5.3**	*15.9* **78.4**
	24	+++	*48.8* **64.3**	*-224.7* ***93.5**	*-280.6* ***82.9**	*-54.9* **45.8**	*-36.2* **2.5**	*9.1* **36.3**
	25	++	*36.6* **61.5**	*43.1* **61.8**	*32.3* **8.7**	*18.1* **-14.0**	*-28.2* **2.7**	*1.1* **8.0**
	26	+++	*36.6* **76.9**	*42.4* **61.0**	*13.2* **40.6**	*4.6* **43.2**	*-13.3* **-3.5**	*0.0* **-2.3**
HXBc2 PL/ 89.6 PL/ JR-FL PL/ YU2 PL	27	+	*46.3* **40.9**	*33.0* **38.5**	*11.8* **11.7**	*4.6* **0.5**	*-34.4* **4.7**	*ND* **ND**
	28	+	*46.3* **47.7**	*53.3* **65.2**	*41.1* **-22.5**	*18.1* **3.8**	*-37.9* **10.0**	*31.8* **-61.7**
	29	-	*42.7* **44.7**	*21.3* **36.7**	*11.8* **6.7**	*20.2* **-16.3**	*-34.4* **5.5**	*3.4* **-40.0**
	30	-	*37.8* **68.6**	*43.1* **56.3**	*17.6* **19.6**	*12.9* **19.2**	*-36.7* **25.1**	*19.3* **-62.0**
	31	+	*35.4* **20.8**	*44.7* **50.8**	*7.3* **12.7**	*10.8* **6.4**	*-34.4* **2.1**	*-3.4* **-47.3**

Table 2. HIV-1 single-round *in vitro* neutralization assay using rabbit serum at a 1:5 dilution against a panel of viral isolates. Serum was collected after four inoculations. Numbers indicate % neutralization, which was determined as follows. The effect of individual preimmune serum on infection was determined relative to infection without serum and is given in lower case, italicised numbers. The percent neutralization of immune serum was determined using the reactivity of individual preimmune sera as baseline neutralization and is given in bold numbers. Asterisks indicate neutralization observed against unusually high enhancement of infection with preimmune serum. Endpoint ELISA titers were defined as last reciprocal serum dilution at which a signal of greater than two-fold over the signal of preimmune serum was detected. The symbols represent the following endpoint titers. (+++) 62,500 (++) 12,500 (+) 2500 (+/-) 2500-500 (-) less than 500. ND: Not determined.

different envelope glycoprotein proteoliposomes. The anti-gp120 reactivity of sera raised against YU2 gp120 and YU2 proteoliposomes were similar and in between that of the two other groups.

To assess neutralizing activity in the sera, 1:5 serum dilutions of preimmune and immune serum obtained after four inoculations were tested in a single-round *in vitro* neutralization assay (59) against the clade B molecular clones HXBc2, SF162, BAL, 89.6, ADA, and YU2 (Table 2). This panel of viruses represents a range of neutralization phenotypes, with increasing resistance from the TCLA isolate HXBc2 to the primary isolate YU2.

In previous experiments, preimmune rabbit serum was found to exhibit a wide range of nonspecific activity on single-round neutralization assays ranging from enhancement of infection to neutralization, often greater than 50% (data not shown). To be able to assess the neutralizing reactivity in individual sera relative to the baseline reactivity of preimmune serum in these animals, sera were tested individually and compared to the corresponding individual preimmune sera. The neutralizing activity was determined as a decrease in infection compared to infection observed with preimmune serum. As observed previously, preimmune sera had a significant effect on viral infection. Compared to data from infections in the absence of serum, enhancement of infection as high as 50% was commonly observed (Table 2, italicised numbers). Most preimmune sera neutralized virus by 2.3 to 60.3%, averaging around 30%. Enhancement of infection was also frequently observed. Given this wide variation, only neutralization greater than 50% was considered significant.

The weakest neutralizing activity was found in the YU2 gp120 group with only two animals generating antibodies neutralizing >50% of infection with the isolate SF162, the most neutralization-sensitive of the primary isolates tested. No homologous neutralization of the YU2 isolate was observed in this group; in contrast, immune serum consistently enhanced infectivity of the YU2 isolate.

The animals inoculated with YU2 gp140-GCN4 exhibited the greatest degree of both homologous and heterologous neutralization (Table 2). Three out of four animals significantly neutralized the isolates HXBc2, SF162, and YU2, with one of the animals (#14) neutralizing both HXBc2 and YU2 and one animal (#15) HXBc2 and SF162 by

more than 80%. By comparison, a high concentration (1mg/ml) of a mixture of IgG purified from sera obtained from HIV-1-infected individuals (HIV-IG) was required in the same experiment to neutralize infection by 96.8% and 94.2%, respectively (Table 3). To achieve neutralization of >90% of the HXBc2 isolate, 0.5 and 5µg/ml of the potent neutralizing monoclonal antibodies 2F5 and 2G12, respectively, were required. To achieve >90% neutralization of SF162, 50µg/ml 2F5 were required; 2G12 did neutralize the SF162 isolate by only 72.8% at the highest antibody concentration tested (50µg/ml) (Table 3). No neutralization greater than 50% was observed in this group (YU2 gp140-GCN4) of the primary isolates 89.6, BAL, and ADA.

		viral isolate					
	µg/ml	HXBc2*	SF162	89.6	BaL	ADA	YU2
HIV-IG	10000	96.8	100.2	99.6	99	97	99.5
	1000	58.5	94.2	80.8	70.8	75.3	64.8
2F5	50	100	93.8	92.5	77.6	85.6	95
	5	95.6	71.3	52.8	40.6	46.7	62.5
2G12	50	96.1	72.8	83.7	81.3	23.9	-1.1
	5	53.7	59.5	61.7	62	8.4	1.1

Table 3. HIV-1 single round *in vitro* neutralization assay using HIV-IG and monoclonal anti-envelope glycoprotein antibodies against a panel of viral isolates. Numbers indicate % neutralization. Data were obtained in the same experiment as data in Table 2. (*) For the neutralization of HXBc2, ten-fold lower concentrations of HIV-IG and monoclonal antibodies than indicated were used.

To overcome the limitations of the amount of protein that could be inoculated into each animal in the proteoliposome format, one group of rabbits received two inoculations of 25µg of YU2 gp140-GCN4 followed by two inoculations of YU2 proteoliposomes. In this group, only two animals displayed neutralizing activity greater than 50% with one and two viruses, respectively (Table 2). Rabbit 21 showed the greatest homologous neutralization of the YU2 isolate of all rabbits with 96.1% neutralization. This neutralizing activity, however, crossed over only to the SF162 isolate, which was neutralized by 64.4%. Overall, the neutralizing reactivity in this group was markedly poorer than that in the group inoculated with YU2 gp140-GCN4 alone.

In the group inoculated with YU2 proteoliposomes, all five animals displayed neutralizing activity greater than 50% against at least two viral isolates. Animal 24 may be the only exception in this regard, since the neutralizing activity of 82.9% and 93.5%

against isolates 89.6 and SF162, respectively, are likely due to the unusual enhancing effect detected in preimmune serum of these animals (280.6% and 224.7%). This enhancement may artificially elevate the calculated % neutralization. Homologous neutralization of the YU2 isolate was weaker in this group than in the animals inoculated with YU2 gp140-GCN4. Only one animal significantly neutralized the YU2 isolate (animal 23 with 78.4% neutralization), while other animals showed little effect or even enhancement of infection (animals 22 and 26). Overall, the neutralization activity of sera from this group, when achieved, was weaker than that observed in the animals inoculated with YU2 gp140-GCN4.

The sequential inoculation with different envelope glycoprotein proteoliposomes (group 5) was designed to focus the immune response on the conserved determinants of the envelope glycoproteins. Here, the hypothesis was tested that antibodies directed against the highly variable loop regions are less efficiently elicited because the respective loops are only presented once to the immune system. The conserved regions, however, are presented four consecutive times, allowing for B-lymphocytes recognizing these regions to be repeatedly stimulated. This group displayed the lowest anti-gp120 titers as determined by ELISA (Table 2). This may be a consequence of a lower envelope glycoprotein dose in this group due to less efficient JR-FL incorporation into proteoliposomes (data not shown). Very limited neutralizing activity was observed in this group with all animals showing enhancement of infection with YU2 and only weak neutralization of SF162 (animals 28, 30, 31) and HXBc2 (animal 30). The poorer neutralization performance of these sera, however, may merely reflect the lower overall titers of anti-gp120 antibodies present in the sera.

To assess if repeated boosting after an extended resting period of three months increases neutralizing antibody titers, the animals were inoculated for a fifth time. No consistent boosting effect could be observed (Table 4). The overall anti-gp120 ELISA endpoint titers in serum after the fifth inoculation were generally equal or lower by one 5-fold dilution than those observed after the fourth inoculation (Table 4). Only some animals displayed better neutralizing activity after the fifth inoculation compared to the fourth inoculation, but this better neutralizing activity was not consistently observed for all viral isolates. Better neutralizing activity after the additional inoculation could, for

example, be observed in serum from animal 12 against HXBc2 and 89.6. Against the viral isolates SF162 and YU2, however, the neutralizing activity decreased (Table 4). No consistent effect of the additional boost on the neutralizing activity in serum could be observed.

		Endpoint	viral isolate					
Immunogen	**Animal**	**ELISA**	**HXBc2**	**SF162**	**89.6**	**BaL**	**ADA**	**YU2**
YU2 gp120	**7**	+++	36.5	51.8	35.8	22.0	10.1	-3.3
		++	32.4	16.2	13.5	-17.5	13.7	-21.3
	8	+++	43.6	9.9	26.1	-5.2	-6.0	-51.6
		+++	21.3	39.0	-1.2	40.8	-51.8	-100.0
	9	++	36.7	34.7	-6.8	-3.2	-6.0	-35.5
		++	-124.1	32.8	-26.9	16.6	-162.0	-150.0
	10	++	28.9	36.4	1.6	-3.0	-0.8	-14.3
		++	7.0	4.7	-71.9	-20.2	-31.3	-59.7
	11	+++	12.8	55.0	-1.8	-2.3	11.3	-32.4
		++	40.6	-31.1	-31.1	-19.1	2.0	-45.8
YU2 gp140 GCN4	**12**	+++	64.4	72.2	35.4	31.6	14.4	71.3
		++	88.3	49.7	72.7	16.9	-21.7	58.1
	14	+++	81.9	79.7	13.4	24.0	11.1	90.1
		++	70.9	54.8	50.3	-16.8	-30.7	77.4
	15	+++	83.1	85.1	31.9	9.7	6.3	64.6
		+++	94.7	95.9	35.5	20.2	-31.7	55.6
	16	++	47.7	28.2	-9.1	-19.8	-8.3	-26.5
		++	74.9	-16.3	-55.0	-63.3	-39.9	-53.8
YU2 gp140 GCN4/ YU2 PL	**17**	++	48.1	16.5	12.5	8.1	-13.8	31.0
		+	27.1	-2.3	-41.8	-59.1	-40.4	13.7
	18	++	26.7	5.9	0.0	12.7	5.3	-50.8
		+	13.6	-15.3	-89.1	-50.9	-39.0	-84.0
	19	+	35.8	14.3	-7.0	-35.9	-3.2	-43.7
		+/-	-11.2	-40.5	-161.4	-117.3	-37.8	-70.2
	20	+++	56.8	44.8	-4.1	18.0	4.4	44.6
		++	36.5	9.9	-7.5	-44.2	-65.4	25.5
	21	+++	47.6	64.4	20.7	30.6	6.0	96.1
		++	ND					
YU2 PL	**22**	+++	68.2	60.7	22.2	-11.9	9.7	-31.7
		++	80.0	32.4	23.8	-44.5	-18.8	-60.0
	23	++	38.6	75.2	-2.1	25.3	5.3	78.4
		+	19	0.0	-103.7	-15.1	-52.3	52.1
	24	+++	64.3	*93.5	*82.9	45.8	2.5	36.3
		++	56.1	74.2	29.8	7.4	-53.8	4.0
	25	++	61.5	61.8	8.7	-14.0	2.7	8.0
		+	40.5	9.9	8.7	-24.9	-16.3	-22.6
	26	+++	76.9	61.0	40.6	43.2	-3.5	-2.3
		++	54.0	14.5	57.7	-19.7	-7.7	-57.6
HXBc2 PL/ 89.6 PL/ JR-FL PL/ YU2 PL	**27**	+	40.9	38.5	11.7	0.5	4.7	ND
		+/-	-5.1	-46.8	6.6	-57.5	-31.9	-57.6
	28	+	47.7	65.2	-22.5	3.8	10.0	-61.7
		+/-	7.4	19.5	-88.2	-52.1	-24.1	-124.4
	29	-	44.7	36.7	6.7	-16.3	5.5	-40.0
		+/-	6.3	18.6	-91.0	-2.8	-37.5	-76.3
	30	-	68.6	56.3	19.6	19.2	25.1	-62.0
					ND			
	31	+	20.8	50.8	12.7	6.4	2.1	-47.3
		+/-	13.6	34.0	-16.5	-49.3	-17.6	-69.5

Table 4. HIV-1 single-round *in vitro* neutralization assay using rabbit serum at a 1:5 dilution against a panel of viral isolates. Numbers indicate % neutralization. Serum was collected after inoculation four (first row) and five (second row) for each animal. Percent neutralization and endpoint ELISA titers were calculated as described for Table 2. ND: Not determined.

Serum from animal 24 that displayed neutralization of >80% of the isolates 89.6 and SF162 after the fourth inoculation, and unusually high enhancement of infection with preimmune serum, showed no significant neutralization against the 89.6 isolate after the fifth inoculation. Thus, the neutralization observed in serum from this animal after inoculation four is most likely an experimental artefact, as it could not be detected after the fifth inoculation. Neutralization of the SF162 isolate in serum from this animal, however, could still be detected after the fifth inoculation (74.2% versus 93.5% after the fourth inoculation). With preimmune serum enhancement in this experiment being 59.5% and within the range of normal variation (data not shown), the neutralizing reactivity against the SF162 isolate in this animal does not appear to be an experimental artefact.

Notably, the neutralization of the SF162 isolate by most sera decreased after the additional fifth boost. In the sera raised against YU2 proteoliposomes in particular, a decrease in neutralizing activity could be observed. This raises the possibility of variation of the viral preparation or variation of the PBMC target cells. This, however, would not explain the decreased ELISA titers after boost five. The neutralization of monoclonal antibody 2F5 and HIV-IG used as internal controls in both assays against SF162, however, were highly reproducable with a variation of neutralization of <5%.

In summary, sera from the group inoculated with YU2 gp140-GCN4 displayed the greatest neutralizing activity. The homologous viral strain YU2 as well as HXBc2 and SF162 were efficiently neutralized by three out of four animals. No neutralization of the isolates ADA and BaL could be observed in any of the groups. Only one animal inoculated with proteoliposomes displayed homologous neutralization, but neutralization of the strains HXBc2 and SF162 was observed in four out of five animals in this group. None of the other groups displayed notable neutralizing activity.

3.3 Proteoliposomes as antigens

3.3.1 Identification of HIV-1 envelope glycoprotein-binding antibodies from phage-displayed antibody libraries

Seven phage-displayed antibody libraries were panned with proteoliposomes containing envelope glycoproteins, or the chemokine

Panning	Library	Antigen	Blocking	Counterscreen
A	FDA-2 Fab	YU2		1D4 on M-280
B	FDA-2 Fab	89.6, JR-FL, YU2, YU2		1D4 on M-280
C	FDA-2 Fab	YU2	YU2 gp120, α-gp41 cluster II	YU2 gp120
D	FDA-2 Fab	CCR5 PL-YU2 gp140 GCN4 -CD4	X5 scFv, α-gp41 cluster II	CCR5 PLs
E	Unbound phage from D	YU2		CCR5-YU2 gp140 GCN4 -CD4
F	FDA-2 Fab	YU2	Fab b6	1D4 on M-280
G	FDA-2 scFv	YU2	Fab b6	1D4 on M-280
H	FDA-2 Fab	89.6 cleavage +		1D4 on M-280
I	FDA-2 scFv	89.6 cleavage +		1D4 on M-280
J	DS/DL/DM	YU2		1D4 on M-280
K	MT	YU2		1D4 on M-280
L	Naïve scFv library	YU2		1D4 on M-450

Table 5. Summary of phage display panning experiments. The libraries panned, the antigen used, blocking regimens and counterscreens are indicated for each experiment.

receptor CCR5 in complex with YU2 gp140-GCN4 and CD4. A scFv library generated from healthy uninfected donors was obtained from Dr. Wayne Marasco. The FDA-2 Fab- and scFv-libraries (8, 20), as well as the MT, DS, DL, and DA libraries (13) were generated from the bone marrow of asymptomatic HIV-1 infected patients in the laboratory of Dr. Dennis Burton. These libraries contain the IgG1 and IgG3 isotypes and lambda and kappa light chains. A summary of the panning experiments is given in table 5. After four rounds of selection, Fab and scFv antibodies were produced and individual clones were tested by ELISA for their reactivity to ovalbumin, gp41 derived from the viral isolate IIIB, and YU2 gp120 with or without prior incubation with CD4. Clones that

	Clone	Panning	Epitope/Phenotype	HIV-1 neutralization
Fab	Fbb21	A	gp120/CD4-induced	+
	Ia3	A,B	gp120/CD4BS	+
	Fbb4	A	gp41 cluster II	-
	Fbb14	B	gp120/CD4BS	+/-
	C12	A,B,C	gp120/CD4-induced	+++ (Moulard *et al*.)
	D10	D	CD4	ND
	J5	J	gp120/CD4BS	+ (Ditzel *et al.*)
	J10	J	gp120	ND
	J2	J	gp41	+/-
	CG5	A	gp120/CD4BS	+++
	CG9	A,B	gp120/CD4BS	+++
	CG21	B	gp120/CD4BS	++
scFv	1	L	gp120/V3 loop	ND
	6	L	gp120/V3 loop	ND
	7	L	gp120/V3 loop	ND
	10	L	gp120/V3 loop	+/-

Table 6. Summary of Fab- and scFv antibodies identified from phage display pannings.

tested positive with gp41 or gp120 were sequenced and compared to sequences of antibodies that had previously been identified from the same libraries. Novel Fab or scFv antibodies were then produced in larger amounts, purified, and tested for neutralization of HIV-1 infection in a single-round neutralization assay.

In total, fifteen different clones binding to either gp120 or gp41 were identified in the twelve panning experiments. A summary of the antibody clones, the panning experiments they were obtained from, the epitopes and phenotypes as well as neutralizing activity is given in table 6. Whereas the panning of the biased libraries FDA-2, MT, DS, DL, and DA yielded six CD4BS antibodies, two CD4-induced antibodies, two gp41 antibodies and one gp120 binder that is not yet characterized well (pannings A-K), the naïve library yielded four unique V3 loop-binding antibodies (panning L). Pannings E, F, G, H, and I did not yield gp120 or gp41 binding antibodies. Only clones that exhibited significant neutralization of HIV-1 in a preliminary neutralization assay were analyzed further. Of the sixteen clones, four displayed significant HIV-1 neutralizing activity. The Fab C12 was found to be a variant of the recently identified neutralizing Fab fragment, X5, that was retrieved from the FDA-2 library with JR-FL gp120 in complex with CCR5

and CD4 (68). This Fab fragment was not analyzed further. Three more antibodies with neutralizing activity were identified from the FDA-2 Fab library, CG5, CG9, and CG21.

3.3.2 Characterization of CG5, CG9, and CG21

Sequence analysis indicates that the heavy chain complementarity determining regions (CDR 3) af CG5, CG9, and CG21 are 15, 16, and 22 amino acids in length, respectively. The VH genes are from the VH3 (CG5, CG9) and VH1 (CG21) gene families. The heavy chain CDR3 sequences of CG5, CG9, and CG21 were unique, and no significant similarities could be found in GenBank. The Fab fragments CG5, CG9, and CG21 all have gp120-affinities in the nanomolar range as determined by ELISA (data not shown).

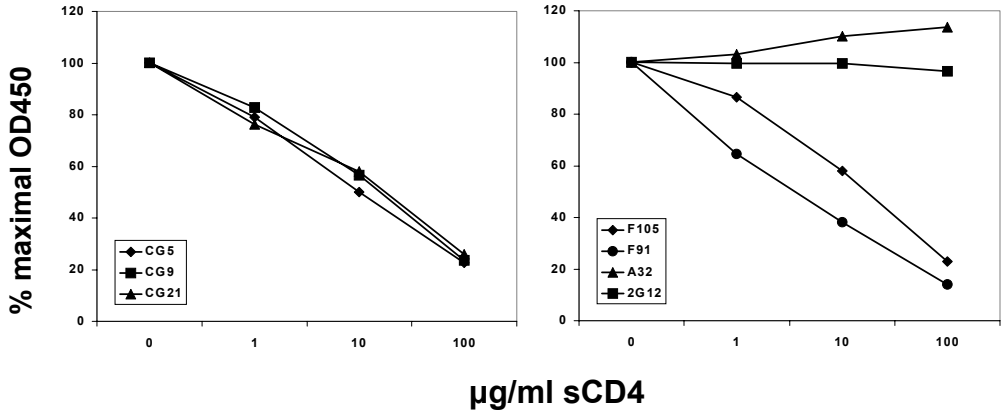

FIG. 14. CD4 competition ELISA. Binding of Fabs CG5, CG9, and CG21 and of control antibodies F105, F91 (CD4BS antibodies), A32 and 2G12 to gp120 after preincubation with increasing concentrations of sCD4 was detected.

Initial epitope mapping of CG5, CG9, and CG21 was performed by competition ELISA with a panel of monoclonal antibodies against different regions on the gp120 envelope glycoprotein and sCD4. CD4BS antibodies and sCD4 inhibited the binding of the CG Fabs to gp120 (data not shown). To further analyze the effect of sCD4 on Fab/gp120 binding by ELISA, gp120 was preincubated with sCD4 at different concentrations and the CG Fabs or control antibodies were incubated with the gp120/sCD4 complexes (Fig. 14). The binding of the CG Fabs to gp120 was inhibited by

sCD4 in a dose-dependent manner. At the highest concentration of sCD4, binding of the Fabs to gp120 was inhibited by approximately 80% (Fig. 14, left panel). As control antibodies, the CD4BS antibodies F105 and F91 were used, as well as the 2G12 and A32 antibodies that bind a cluster of mannose moieties on the outer domain and the C1/C4 region of YU2 gp120, respectively. A similar degree of inhibition of binding to gp120 by sCD4 was observed with the two CD4BS antibodies, whereas binding of the 2G12 and A32 antibodies was either not affected or enhanced by increasing amounts of sCD4 (Fig.14, right panel).

3.3.3 Neutralization of *in vitro* HIV-1 infection by Fab CG5, CG9, and CG21

The ability of the Fab fragments CG5, CG9, and CG21 to neutralize infection of PBMC (A3R5 cells for YU2) by a panel of HIV-1 isolates in a single-round *in vitro* neutralization assay was determined and compared to that of a panel of other human monoclonal Fab fragments of CD4 binding site antibodies. The inhibition of *in vitro* infection of PBMC with a panel of HIV-1 isolates at different antibody concentrations was determined and the concentrations at which the antibodies neutralize 50, 80, or 90% infection (IC_{50}, IC_{80}, and IC_{90}) were derived from the neutralization data. A representative dose response curve for neutralization of the MN viral isolate is shown in Fig. 15. The IC_{50}, IC_{80}, and IC_{90} concentrations are indicated. The Fab fragments of the monoclonal CD4BS antibodies F105, F91, and b12 were tested side by side with CG5, CG9, and CG21 as controls to establish a rank order of neutralizing potency (Table 7).

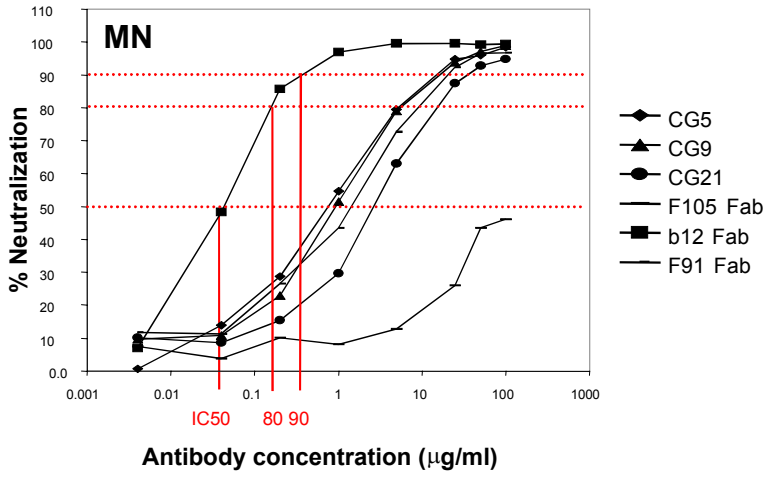

FIG. 15. Representative dose-response curve of the MN viral isolate with a panel of monoclonal antibodies. Approximate IC_{50}, $_{80}$, and $_{90}$ values are indicated for the b12 Fab.

A

viral isolate	Clade	b12 Fab IC50	b12 Fab IC80	b12 Fab IC90	CG5 Fab IC50	CG5 Fab IC80	CG5 Fab IC90
IIIB	B	0.9	3.7	8.8	4.7	>200	>200
MN	B	0.0	0.1	0.1	0.7	4.3	12.6
SF162	B	0.5	1.4	2.8	6.7	139.6	>200
BaL	B	1.7	4.0	6.8	8.1	>200	ND
ADA	B	8.0	53.2	163.0	14.6	>200	>200
89.6	B	0.2	1.1	1.7	>200	ND	ND
JR-CSF	B	1.5	4.0	7.4	>200	>200	ND
JR-FL	B	0.3	1.3	3.1	50.8	>200	>200
YU2	B	9.2	43.6	112.4	ND	ND	ND
ZA012	C	ND	ND	ND	27.6	>200	>200

viral isolate	Clade	F91 Fab IC50	F91 Fab IC80	F91 Fab IC90	CG9 Fab IC50	CG9 Fab IC80	CG9 Fab IC90
IIIB	B	34.8	>200	>200	6.0	>200	>200
MN	B	1.0	6.4	18.9	0.9	4.6	12.0
SF162	B	57.8	>200	>200	10.8	>200	>200
BaL	B	>200	ND	ND	15.6	>200	>200
ADA	B	152.0	>200	>200	38.3	>200	>200
89.6	B	>200	ND	ND	169.1	>200	>200
JR-CSF	B	>200	ND	ND	>200	>200	ND
JR-FL	B	>200	>200	>200	54.7	>200	>200
YU2	B	ND	ND	ND	ND	ND	ND
ZA012	C	ND	ND	ND	24.9	>200	>200

viral isolate	Clade	F105 Fab IC50	F105 Fab IC80	F105 Fab IC90	CG21 Fab IC50	CG21 Fab IC80	CG21 Fab IC90
IIIB	B	99.6	>200	>200	26.1	>200	>200
MN	B	178.5	>200	>200	1.8	12.5	37.7
SF162	B	94.0	>200	>200	35.9	>200	>200
BaL	B	>200	>200	ND	32.9	>200	>200
ADA	B	>200	>200	ND	>200	>200	>200
89.6	B	>200	ND	ND	>200	ND	ND
JR-CSF	B	ND	ND	ND	>200	>200	ND
JR-FL	B	>200	>200	>200	>200	>200	>200
YU2	B	ND	ND	ND	ND	ND	ND
ZA012	C	ND	ND	ND	57.3	>200	>200

B

viral isolate	Clade	4E10 IgG IC50	4E10 IgG IC80	4E10 IgG IC90
IIIB	B	6.4	99.5	>200
MN	B	0.3	2.1	6.5
SF162	B	8.6	103.3	>200
BaL	B	3.8	68.6	>200
ADA	B	8.2	73.2	>200
89.6	B	1.9	14.0	44.0
JR-CSF	B	10.0	167.9	>200
JR-FL	B	2.1	14.9	46.1
YU2	B	5.9	63.3	>200
ZA012	C	11.7	140.7	>200

Color key:

< 1

< 10

<100

Table 7. HIV-1 single round *in vitro* neutralization assay of anti-envelope glycoprotein antibodies against a panel of viral isolates. $IC_{50, 80,}$ and $_{90}$ values represent the concentration of antibody (µg/ml) required for the reduction of infection by 50, 80, or 90 %, respectively. (A) Anti-gp120 Fab antibodies. (B) Anti-gp41 IgG antibody. ND: Not detectable.

The CD4 binding site antibody F105 has previously been shown to neutralize TCLA-isolates with moderate potency (77), but not primary isolate viruses. This phenotype could be reproduced in this neutralization assay, with Fab F105 being able to significantly neutralize (IC_{50} <100µg/ml) only the TCLA-isolate IIIB and the primary isolate that is the most neutralization-sensitive in the viral panel examined here, SF162. The Fab fragment of the F91 antibody exhibited slightly greater breadth and potency of neutralization than Fab F105. Fab F91 neutralized, in addition to IIIB and SF162, the TCLA isolate MN, with an IC_{50} of 1µg/ml. It exhibited generally greater potency than Fab F105 with IC_{50} values for the neutralization of IIIB, MN, and SF162 of 34.8, 1, and 57.8, respectively. Consistent with previous reports, the potent neutralizer Fab b12 displayed the by far greatest breadth and potency of neutralization of all tested antibodies. Fab b12 was able to significantly neutralize all viral isolates except the clade C isolate ZA012. The IC_{50} of Fab b12 for the neutralization of the TCLA isolates IIIB and MN as well as the primary isolates 89.6 and JR-FL were below 1µg/ml, and IC_{90} was achieved at 10µg/ml for all tested clade B isolates but ADA.

The Fab fragments CG5, CG9, and CG21 were found to be intermediate in neutralization breadth and potency, between that of Fab b12 and the Fab fragments of F91 and F105. The neutralization profiles of CG5 and CG9 were similar, with significant neutralization being observed with the TCLA isolates as well as five primary isolates (SF162, BaL, ADA, JR-FL, and ZA012). CG21 exhibited less potency and breadth than CG5 and CG9 and was not able to significantly neutralize the primary isolates ADA and JR-FL. The IC_{50} values of CG5 for the neutralization of IIIB, MN, SF162, and BaL were below 10µg/ml, whereas IC_{50} values for F91 in this concentration range were obtained only for MN. Notably, CG5, CG9, and CG21 were able to neutralize the clade C isolate ZA012 with IC_{50}s of 27.6, 24.9, and 57.3, respectively, whereas no other tested Fab could significantly neutralize this isolate. Only Fab b12 was able to neutralize the primary isolate YU2, although the Fabs CG5, CG9, and CG21 had been selected against envelope glycoproteins derived from this isolate. All Fabs showed enhancement of infection with YU2, with Fab b12 exhibiting slight enhancement only at concentrations of under 1µg/ml and neutralization at higher antibody concentrations. For the other Fabs, the enhancement of infection generally increased with increasing amounts of Fab at the lower

concentrations of 0.004, 0.04, 0.2 and 1μg/ml and plateaued at approximately 60% enhancement of infection at higher Fab concentrations (data not shown).

The neutralization breadth of the gp41 binding IgG 4E10 was found to be similar to that of Fab b12, with 4E10 also significantly neutralizing the clade C isolate ZA012. However, 4E10 displayed generally lower potency than did Fab b12. The neutralization profiles of the Fabs CG5 and CG9 were found to resemble that of 4E10, although the Fabs displayed less potency and not as broad neutralization as 4E10.

3.3.4 Neutralization of *in vitro* HIV-1 infection by Fabs versus IgGs

Fab fragments of antibodies have previously been shown to exhibit different binding affinities and neutralization potency compared to whole antibodies due to avidity effects. Therefore, the difference in neutralization potency of Fab fragments and their respective whole antibody counterparts was examined. Fab fragments of the human antibodies IgG1b12, F105, and F91 were generated biochemically and tested for their neutralization potency and breadth against the previously used panel of viral isolates (Table 8).

		μg/ml to achieve 50% reduction of infection					
		b12		**F105**		**F91**	
Isolate	**Clade**	Fab	IgG	Fab	IgG	Fab	IgG
IIIB	B	0.9	0.1	99.6	6.9	34.8	35.8
MN	B	0.0	0.0	178.5	11.2	1.0	1.8
SF162	B	0.5	0.2	94.0	10.8	57.8	42.8
BaL	B	1.7	0.3	>200	47.0	>200	>200
ADA	B	8.0	0.9	>200	60.4	152.0	>200
89.6	B	0.2	0.1	>200	46.4	>200	29.7
JR-CSF	B	1.5	0.5	ND	76.3	>200	88.9
JR-FL	B	0.3	0.0	>200	158.5	>200	>200
YU2	B	9.2	0.7	ND	ND	ND	ND
ZA012	C	ND	178.3	ND	31.9	ND	>200

Table 8. HIV-1 single round *in vitro* neutralization assay comparing the neutralizing activity of Fab and IgG antibodies against a panel of viral isolates. The concentration of antibody required to achieve 50% reduction of infection (IC_{50}) are given. ND: Not detectable.

The neutralization potency of b12 and F105 whole antibodies was consistently higher than that of the corresponding Fab fragments. The antibody IgG1b12 inhibited infection more efficiently than did the Fab fragment with between 2- to 13-fold lower IC_{50} values for the neutralization with whole antibody. The gain in neutralization potency of the whole antibody F105 over the Fab fragment was more pronounced, with up to 16-fold lower IC_{50} values for neutralization with the whole antibody. The differences in neutralization by F91 between whole antibody and Fab fragment were less consistent, with better neutralizing activity of the whole antibody over the Fab fragment only observed for the isolates 89.6 and JR-CSF. Compared to projected IC_{50} values (data not shown) for the F91 Fab fragment, these two isolates were neutralized at IC_{50} values 60- and 4600-fold lower than for the whole antibody, respectively. The ADA isolate was neutralized better by the Fab fragment than the IgG, with projected IC_{50} values 9.4-fold lower than for the Fab F91. The projected IC_{50} values, however, should only be interpreted as rough estimates. For all other isolates, where determined, the IC_{50} values for neutralization with F91 Fab and IgG were similar.

3.4 Envelope glycoproteins deficient in T-cell helper epitopes

Viral escape from recognition by $CD8^+$ T-lymphocytes is a well documented phenomenon. It is conceivable that escape of viruses from recognition by $CD4^+$ T-helper cells would also endow a virus with a replicative advantage. Escape from $CD4^+$ T-helper cells recognition, however, has not yet been described for HIV.

In preliminary experiments, gp120 core+V3 envelope glycoproteins (Δ82ΔV1,2ΔC5) (Fig. 16) were generated to test if these glycoproteins would more efficiently elicit neutralizing antibodies against conserved elements maintained in the gp120 core than full-length gp120. BALB/c mice were immunized with core gp120 envelope glycoprotein derived from the TCLA isolate HXBc2 and the primary isolate YU2. Although a detectable anti-gp120 antibody response was elicited by the HXBc2 core gp120, the YU2 core gp120 elicited no detectable antibody response (data not shown). To determine if this nonresponder phenotype could be observed in another mouse strain, and to explore the reasons for the defect of the primary isolate-derived core gp120 to elicit antibodies, the experiment was repeated in C57BL/6 mice.

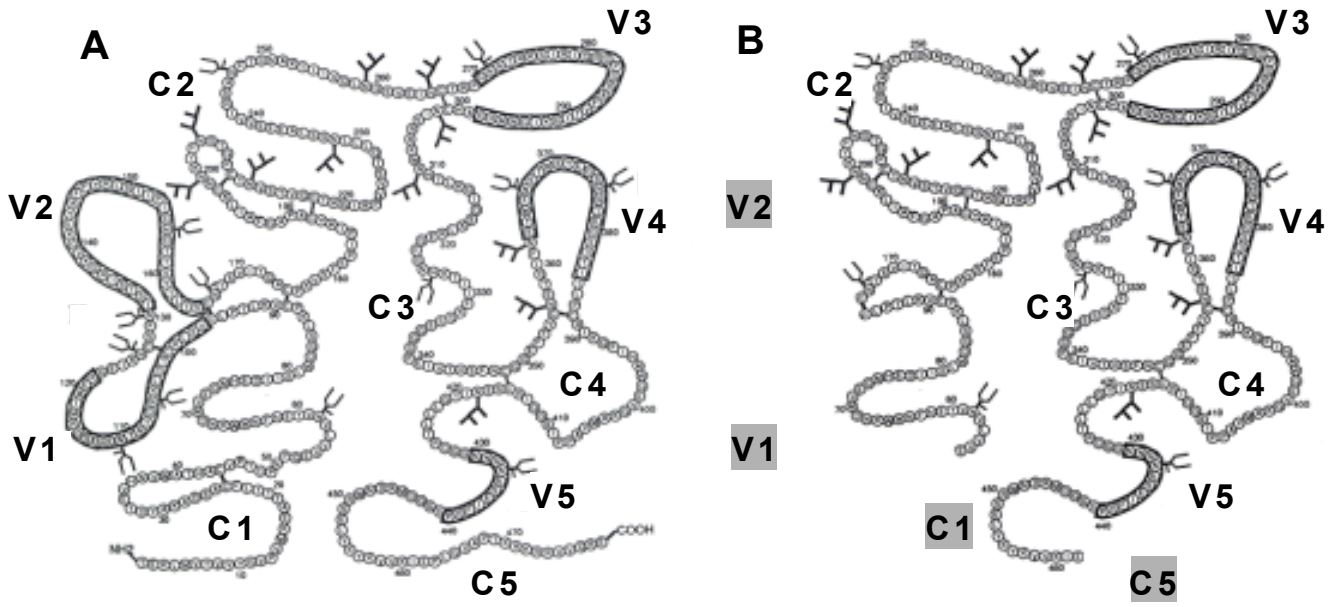

FIG. 16. Schematic representation of full length (A) and core gp120 (B). Glycosylation sites are indicated as branched chains. Constant and variable regions are labeled C1-5 and V1-5. Regions deleted in the core gp120 are shaded (B). Amino acid numbering does not include the signal peptide (30 amino acids).

3.4.1 Anti-gp120 IgG reactivity in mouse sera raised against YU2 core gp120 and HXBc2 core gp120

Groups of six C57BL/6 mice were inoculated four times with 20 μg of YU2 core gp120 and HXBc2 core gp120. The anti-gp120 IgG reactivity in the sera raised against recombinant YU2 or HXBc2 core gp120 was determined by ELISA (Fig. 17). Mice immunized with the TCLA isolate-derived core gp120 (HXBc2) displayed high titers of anti-gp120 reactive IgG with an endpoint titer of greater than 100,000. The anti-gp120 IgG titers detected in sera raised against the primary isolate YU2 core gp120 was only minimally higher than the anti-gp120 reactivity of preimmune serum, even at the lowest serum dilution of 1:100. To explore if the lack of a gp120 IgG response is a general phenomenon that extends to other primary isolate core gp120, core gp120 derived from the primary isolate JR-FL was produced and inoculated into BALB/c mice as described above. A similarly low anti-gp120 IgG response was also observed in the sera of mice immunized with JR-FL gp120 (data not shown).

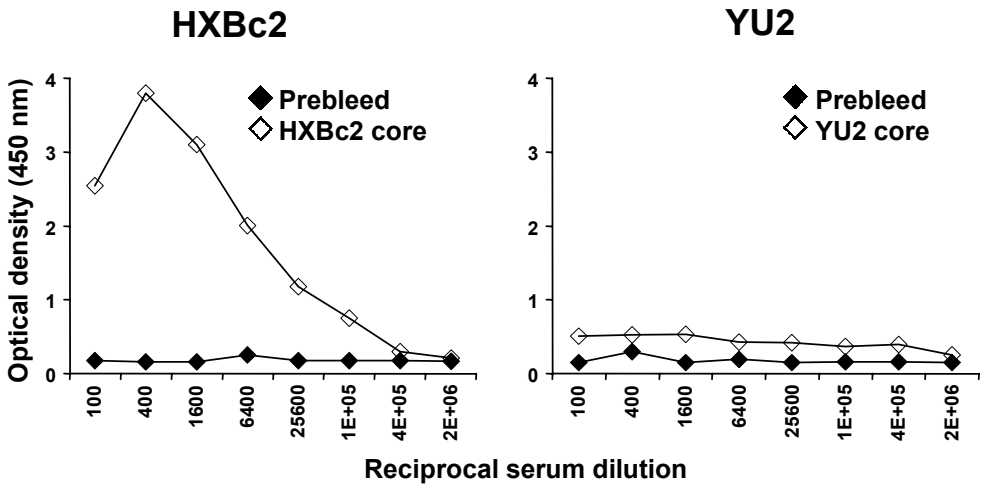

FIG. 17. ELISA of anti-gp120 IgG reactivity of mouse sera raised against HXBc2 core gp120 (left panel) and YU2 core gp120 (right panel). Mice were inoculated four times with HXBc2 core gp120 or YU2 core gp120. Sera were tested against HXBc2 core gp120 and YU2 core gp120 antigen, respectively.

3.4.2 Generation of fusion proteins of core gp120 and the heterologous helper epitope PADRE

To test if the absence of serum IgG reactivity against core gp120 derived from the primary isolate YU2 was due to a lack of T-helper epitopes in this protein, both HXBc2

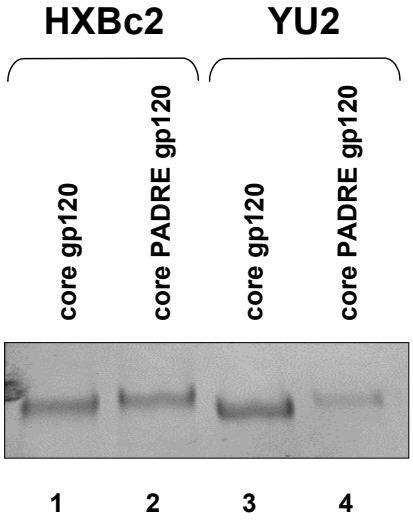

Fig. 18. 12 % SDS-PAGE of Drosophila-expressed core gp120 (lanes 1 and 3) and core-PADRE gp120 (lanes 2 and 4) from HXBc2 and YU2, Coomassie-stained. The core-PADRE gp120 proteins (lanes 2 and 4) migrate slightly slower due to the additional PADRE sequences.

and YU2 core gp120 molecules were expressed as fusion proteins with two repeats of the heterologous helper epitope PADRE, one each appended on the C- and N-terminus. To confirm the presence of the PADRE sequences on the core gp120, the proteins were subjected to 12% SDS-PAGE analysis (Fig. 18). A difference in electrophoretic mobility between the core gp120 and the core-PADRE gp120 could be detected. This difference in mobility corresponded to the difference expected for the core gp120 proteins and the fusion proteins carrying two repeats of PADRE with an additional molecular weight of approximately 2.6 kD.

3.4.3 Anti-gp120 IgG reactivity in mouse sera raised against YU2 and HXBc2 core gp120 and core-PADRE gp120

To test if the addition of heterologous helper epitopes restores the immunogenicity of the primary isolate-derived core gp120, YU2 core gp120 and core-PADRE gp120 were inoculated into groups of six C57BL/6 mice. This strain of mice was chosen because they express the MHC class II I-A^b allele that has been shown to bind the PADRE epitope with high affinity (2). HXBc2 core gp120 and HXBc2 core-PADRE gp120 were inoculated similarly. After four inoculations, anti-gp120 IgG serum titers were determined by ELISA (Fig. 19). The anti-gp120 titers raised against the HXBc2 core gp120 and core-PADRE gp120 were similar, with endpoint titers of greater than 100,000, indicative of no obvious effect on immunogenicity of the PADRE epitope on this core gp120. The titers raised against YU2 core-PADRE gp120, in contrast, were substantially higher than the titers raised against the core gp120. The anti-gp120 titers of YU2 core-PADRE gp120 immune serum exhibited endpoint titers of greater than 100,000 compared to preimmune serum. These titers were similar to those observed for the immune and preimmune sera of the animals inoculated with HXBc2 core gp120, whereas the YU2 core gp120 did not elicit a detectable anti-gp120 antibody response. These data suggest that the addition of the heterologous helper epitope PADRE fully restored the immunogenic potential of the YU2 core gp120.

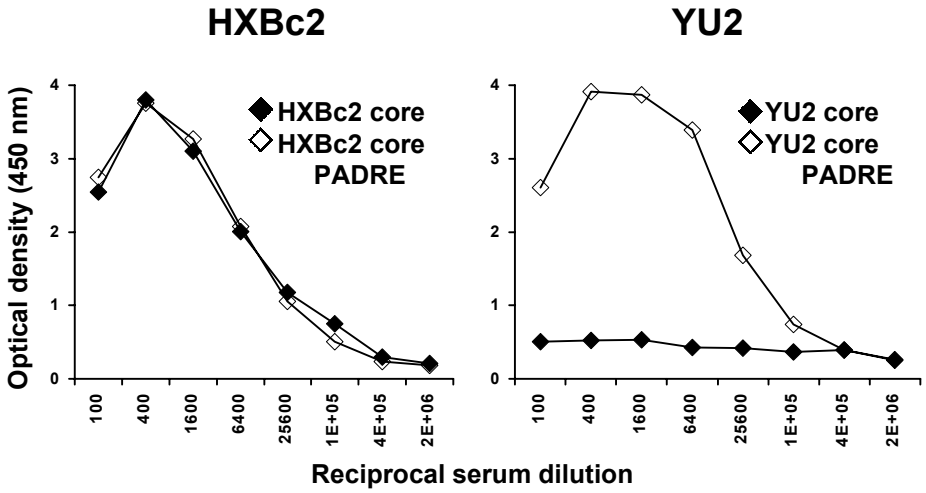

FIG. 19. ELISA of anti-gp120 IgG reactivity of mouse sera raised against core gp120 and core-PADRE gp120 from HXBc2 and YU2. Mice were inoculated four times with HXBc2 core gp120 and core-PADRE gp120 (left panel) or YU2 core gp120 and core-PADRE gp120 (right panel). Sera were tested against HXBc2 core gp120 and YU2 core gp120 antigen, respectively.

Although the titer of anti-gp120 IgG raised in mice inoculated four times with HXBc2 core gp120 and core-PADRE gp120 appeared to be unaffected by the addition of PADRE, a clear difference in titers raised against the two gp120 could be observed after

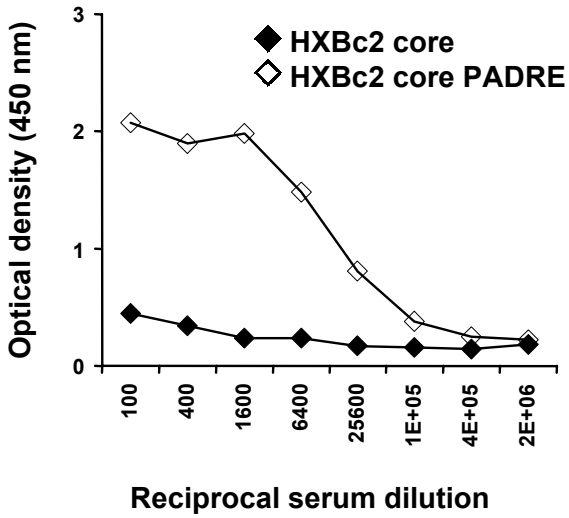

FIG. 20. ELISA of anti-gp120 IgG reactivity of mouse sera raised against HXBc2 core gp120 and core-PADRE gp120 after two inoculations. Mice were inoculated two times with HXBc2 core gp120 and core-PADRE gp120. Sera were tested against HXBc2 core gp120 antigen.

one inoculation and, most pronounced, after two inoculations (Fig. 20). When pooled serum from the two groups were analysed, the endpoint titer generated by the HXBc2

core-PADRE gp120 envelope glycoprotein were greater than 100,000, whereas the enpoint titer of the HXBc2 core gp120 was 400.

3.4.4 Serum reactivity against PADRE, neoepitopes, and carbohydrate moieties

To rule out the possibility that the increase in serum reactivity observed in sera raised against YU2 core-PADRE gp120 compared to YU2 core gp120 was due to reactivity of these sera against the PADRE epitope itself or neoepitopes created by the fusion of PADRE with the core gp120, reactivity of serum raised against YU2 core-PADRE gp120 was tested by ELISA for reactivity to YU2 core gp120 and YU2 core-PADRE gp120 antigen. The reactivity of serum raised against YU2 core-PADRE gp120 to YU2 core gp120 antigen was roughly equivalent to the reactivity to the YU2 core-PADRE gp120 antigen (Fig. 21). Thus, the increased anti-gp120 reactivity observed in serum raised against YU2 core-PADRE gp120 is not due to reactivity against PADRE or neoepitopes spanning the PADRE-core junction.

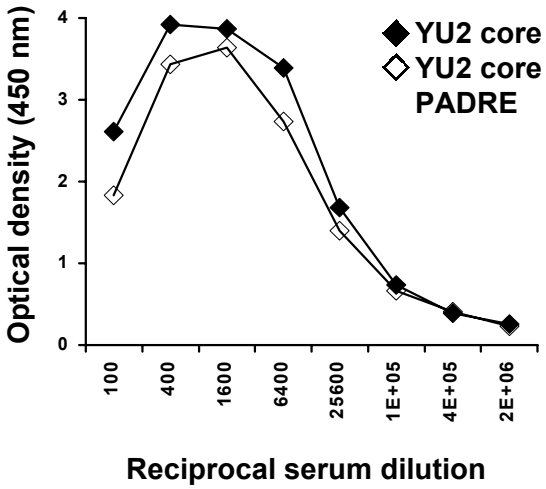

FIG. 21. ELISA of IgG reactivity directed against YU2 core gp120 and core-PADRE gp120 antigen in sera raised against YU2 core-PADRE gp120 antigen in mice.

Heterologous helper epitopes are capable of breaking natural immunologic tolerance against self-determinants (32). The gp120 envelope glycoprotein is heavily glycosylated with about half of its molecular weight being contributed by carbohydrate moieties. Since this glycosylation is catalyzed by the host glycosylation system, the carbohydrate moieties are likely considered "self" by the human immune system.

Breaking tolerance to these moieties may generate a substantial anti-carbohydrate antibody response that would represent a trivial explanation for the effects of the heterologous helper epitope observed here. Anti-self carbohydrate reactivities would not be selectively directed against virus and therefore would be highly undesirable in a vaccine setting. Consequently, we tested the possibility that the addition of PADRE

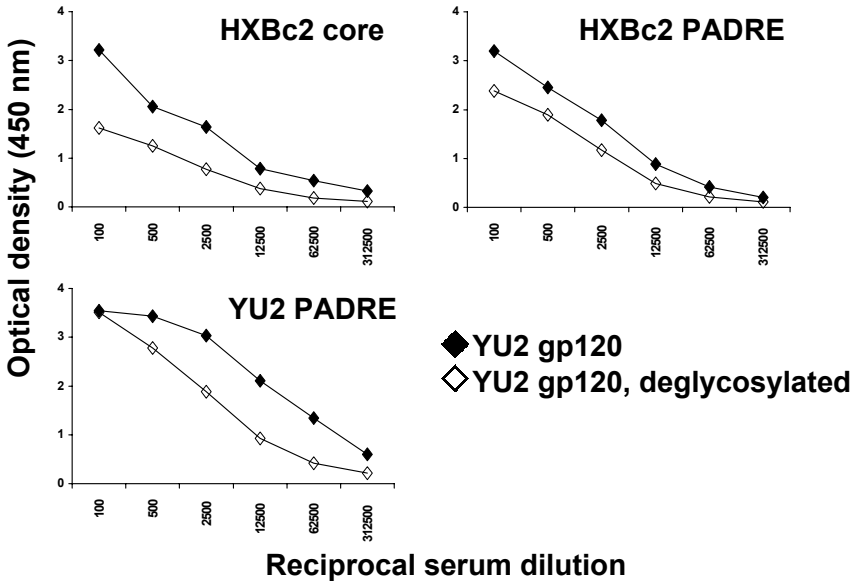

FIG. 22. ELISA of IgG reactivity against wild-type (WT) and deglycosylated core gp120 antigen. Mouse serum was collected after four inoculations with HXBc2 core gp120, HXBc2 core-PADRE gp120, and YU2 core-PADRE gp120, respectively, and tested for reactivity with WT and enzymatically deglycosylated homologous core gp120 antigen.

sequences increases the generation of carbohydrate-directed antibodies rather than antibodies directed against viral protein determinants. Sera raised in mice against HXBc2 core gp120, HXBc2 core-PADRE gp120 and YU2 core-PADRE gp120 were tested for IgG reactivity against wild-type (WT) gp120 and enzymatically deglycosylated gp120 derived from the same strain by ELISA (Fig. 22). Some degree of carbohydrate reactivity to the glycosylated gp120 when compared to the deglycosylated gp120 could be observed in all sera, even those elicited by the HXBc2 cores not containing PADRE. Since all sera exhibited carbohydrate reactivity, this may represent a baseline carbohydrate reactivity generally raised by gp120, or a quantitative difference in the amounts of WT and deglycosylated input gp120 antigen.

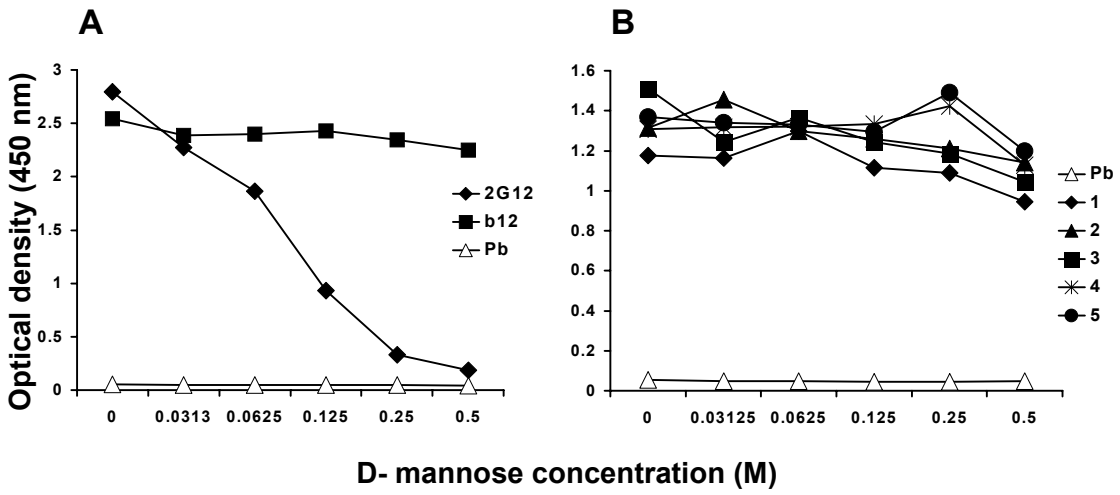

FIG. 23. D-mannose inhibition ELISA. (A) Binding of the monoclonal antibodies 2G12, IgG1b12 (5µg/ml), and preimmune rabbit serum (Pb, 1:1000 serum dilution) to *Drosophila* gp120 in the presence of increasing amounts of D-mannose. (B) Binding of rabbit sera raised against core-PADRE gp120 (animals 1-5, 1:1000 serum dilution) in the presence of increasing amounts of D-mannose.

To further examine carbohydrate reactivity, sera raised in rabbits against core-PADRE gp120 were tested in a competition ELISA using monomeric D-mannose. The 2G12 antibody was used as a control. This antibody has recently been shown to bind to a cluster of D-mannose moieties in the proximity of the C4/V4 region of gp120 (94, 98, 112). D-mannose competed with 2G12 for binding to gp120 expressed in *Drosophila* S2 cells in a dose-dependent manner (Fig. 23A). At a concentration of 0.5M D-mannose, 2G12-binding to gp120 was completely inhibited. No D-mannose competition for binding of gp120 was observed with the CD4BS antibody IgG1b12 that recognizes gp120 in a carbohydrate-independent manner. Binding of sera raised against core-PADRE gp120 was only minimally inhibited by D-mannose at the highest concentration tested, suggesting low carbohydrate reactivity elicited by the *Drosophila* high-mannose glycans on gp120 (Fig. 23B). Thus, it is likely that no or only slight additional carbohydrate reactivity was generated by the addition of PADRE sequences.

3.4.5 Anti-gp120 IgG and IgM reactivity in rabbit serum

A strong deficiency of the core gp120 of the primary isolate YU2 and JR-FL to elicit anti-gp120 IgG in mice was observed. This lack of an IgG response could be restored by the addition of heterologous T-helper epitopes. Both strains of mice, however, possess a very limited number of MHC II alleles; I-A^b in the C57BL/6 mice and I-A^d and I-E^d in the BALB/c mice.

To exlude the possibility that the observed deficiency in T-helper epitopes of the primary isolate YU2 core gp120 is an effect only manifest in the context of a limited MHC class II background, YU2 core gp120 and YU2 core-PADRE gp120 were inoculated into outbred rabbits. Groups of five New Zealand White rabbits were inoculated with 20 μg of gp120 and serum was collected after each inoculation following the priming. The sera were tested for anti-gp120 IgG-reactivity by ELISA (Fig. 24). Generally, the same poor elicitation of an anti-gp120 antibody response by the YU2 core gp120 was observed as before in C57BL/6 and BALB/c mice. The serum raised against YU2 core-PADRE gp120 showed consistently higher anti-gp120 reactivity than did the YU2 core gp120 serum. The YU2 core gp120, however, did elicit slightly higher antibody titers in rabbits compared to mice; the helper epitope deficiency appeared less pronounced in this particular MHC class II context. As previously observed with the HXBc2 core gp120 and HXBc2 core-PADRE gp120, the difference between titers raised against YU2 core gp120 and YU2 core-PADRE gp120 diminished slightly from the third inoculation to the fourth inoculation. When endpoint titers were determined relative to prebleed serum of the respective animals after the third and fourth inoculation, sera raised against YU2 core-PADRE gp120 displayed endpoint titers of >62,500 compared to endpoint titers raised with the YU2 core gp120 at >2500 (Fig. 24A). In the group immunized with YU2 core gp120, animal # 54 displayed strong anti-gp120 IgG reactivity that was almost as high as the anti-gp120 serum reactivity observed in the YU2 core-PADRE gp120 group. The particular MHC class II repertoire of this animals may bind and present endogenous gp120 helper epitopes that the other four animals in this group may not recognize. The fact that the #54 rabbit immunized with YU2 core gp120 exhibits titers similar to the rabbits immunized with YU2 core-PADRE indicates that the pronounced differences observed with core gp120 and core-PADRE gp120 are not

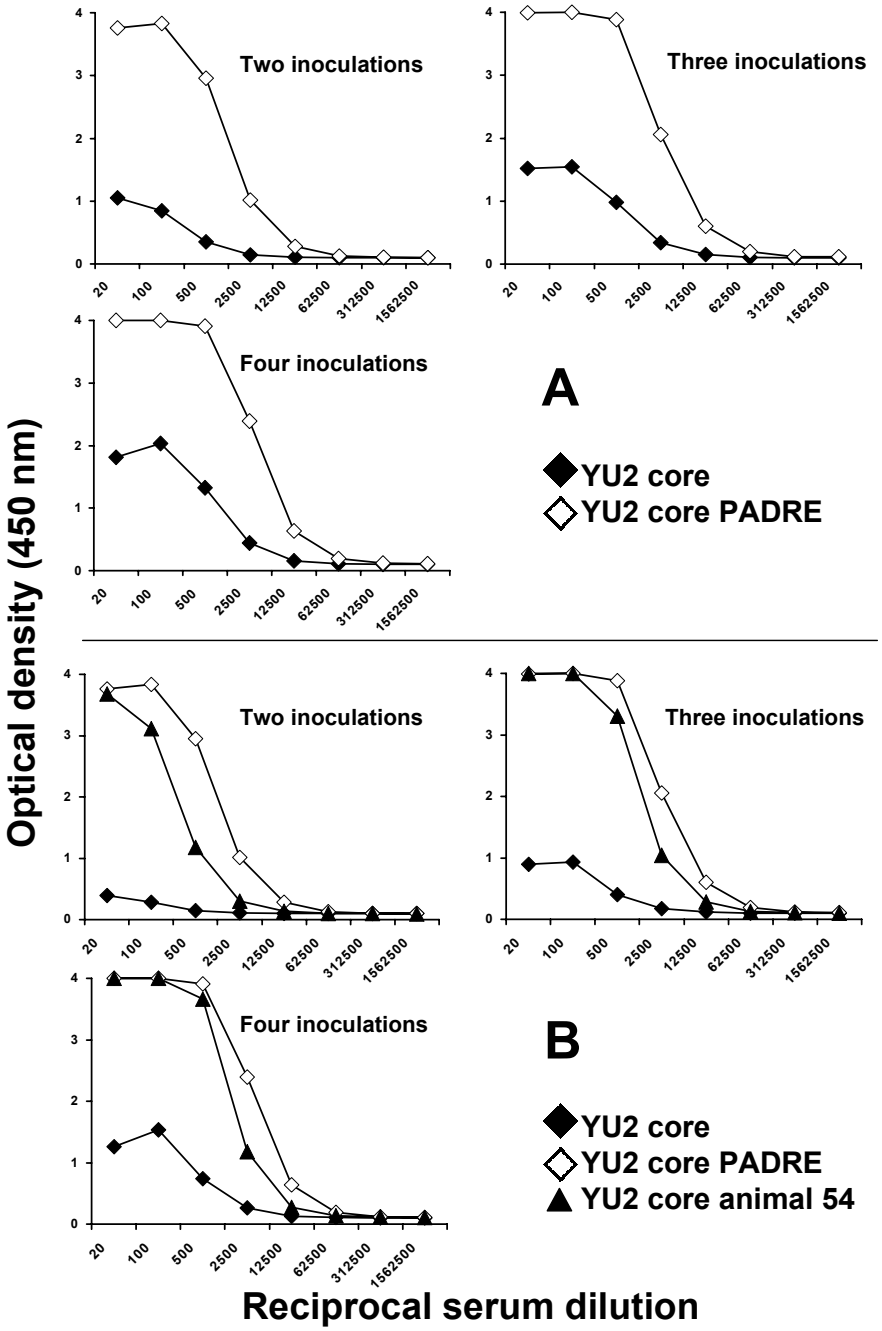

FIG. 24. ELISA of IgG reactivity against YU2 gp120 in rabbit sera. Serum was collected after two, three, and four inoculations with YU2 core gp120 and YU2 core-PADRE gp120, respectively, and anti-gp120 reactivity was tested against YU2 gp120 antigen. Panel A: The average of anti-YU2 gp120 IgG reactivity of five rabbits inoculated with YU2 core gp120 and core-PADRE gp120 are shown. Panel B: The anti-gp120 IgG reactivity of the responder animal #54 from the group inoculated with YU2 core gp120 is plotted separately.

merely due to dose differences. Rather, they are indicative of a difference in the MHC II haplotype. When the responder animal #54 was individually plotted and compared to the core gp120 and core-PADRE gp120 groups, the difference between the two groups became more pronounced (Fig. 24B). After inoculation three, the endpoint titer for the core-PADRE gp120 group was >62,500, whereas the endpoint titers in the core gp120 group were >2500 with and >500 without the responder animal #54.

To assess the anti-gp120 IgM reactivity in sera raised against the core gp120, serum of rabbits inoculated with YU2 core gp120 and YU2 core-PADRE gp120 was tested by ELISA. Generally, similar low titers of anti-gp120 IgM could be detected in all animals (data not shown).

4 Discussion

The inability of HIV-1 gp120 envelope glycoproteins to either elicit broadly neutralizing antibodies or to efficiently select inhibitory ligands from libraries has hampered efforts to develop reagents that intervene in viral envelope glycoprotein-receptor interactions. Recent reports have demonstrated that soluble oligomeric envelope glycoprotein constructs possess characteristics consistent with the trimeric structures found on the surface of virus or infected cells (122). An immunogenicity study with one particular construct, trimeric YU2 gp140-GCN4 glycoproteins, demonstrated that the trimeric construct better elicits neutralizing antibodies when compared to monomeric gp120 (125). These results support the notion that proteins that more faithfully mimic the structure of the viral envelope glycoprotein complex may more efficiently elicit neutralizing antibodies and may also be more efficient in identifying neutralizing antibodies from libraries.

4.1 Generation of proteoliposomes

Because of these possible advantages as an immunogen, an oligomeric envelope glycoprotein formulation has been designed, solid-phase proteoliposomes, in an attempt to most closely mimic the trimeric envelope glycoprotein complex found on the surface of the virus or on the surface of envelope glycoprotein-expressing cells. The approach pursued here was to reduce the mutational changes introduced into the envelope glycoproteins to a minimum and to retain the homologous trimerization and transmembrane domains. This could be achieved by reconstitution of a lipid bilayer around the proteoliposomes.

The proteoliposomes described here possess native gp160ΔCT glycoproteins, as assessed by extensive FACS analysis with conformationally-sensitive ligands. The gp160ΔCT glycoproteins captured on the proteoliposomes are cleavage-defective and thereby possess a covalent linkage between gp120 and gp41 subunits, which enhances spike stability. The envelope glycoproteins exist primarily as trimers on the proteoliposome surface as determined by elution and size-exclusion chromatography under non-denaturing conditions. As opposed to soluble trimeric GCN4-stablized

constructs (122, 123), the native gp41 oligomerization domain in the context of the lipid bilayer is sufficient to maintain the trimeric conformation and no heterologous trimerization motifs need to be appended to the gp160ΔCT glycoprotein. The reconstituted lipid bilayer may provide advantages over the soluble gp140 glycoproteins by both stabilizing known gp41 neutralizing determinants as well as limiting access by antibody to non-neutralizing envelope glycoprotein determinants.

Other approaches, such as the use of virus-like particles (VLPs), also allow for the formulation of envelope glycoproteins as membrane-associated, native entities. However, as VLPs bud from the cell surface, cellular proteins other than envelope glycoproteins are incorporated into VLPs, limiting the purity of these preparations. The well-defined protein content of the proteoliposomes described here provides an advance over traditional VLPs, as the affinity capture-purification method renders the proteoliposomes practically devoid of other cellular components. Also, since the bead core of the proteoliposomes is optimized for binding of protein at high density, the protein density on the surface of the proteoliposomes is likely much greater than that found on VLPs. The proteoliposomes have properties that allow for their use in probing libraries of biological or chemical compounds, including phage display libraries. Their paramagnetic properties, although not an absolute requirement for their composition, allow for the fast and complete recovery of the proteoliposomes from complex mixtures.

Antigenically, proteoliposomes are virtually indistinguishable from envelope glycoproteins present on the cell surface of gp160ΔCT-expressing cells. For some anti-gp120 ligands, namely IgG1b12 and sCD4, the affinity towards envelope glycoproteins on proteoliposomes is marginally but reproducibly higher than that to envelope glycoproteins expressed on the cell surface. This might be due to better accessibility of the envelope glycoproteins on proteoliposomes as a consequence of the lack of other masking proteins and glycocalix components.

The *de novo* generation of a lipid bilayer around the individual beads has previously been shown to be an effective method to retain functionality and conformational integrity of the seven-transmembrane proteins CCR5 (64) and CXCR4 (7). When formulated as proteoliposomes, the seven-transmembrane CCR5 molecule retained native structure and could withstand changes in salt and pH that denatured

detergent-solubilized CCR5 (64). For the gp160ΔCT proteoliposomes, reconstitution of the membrane was dependent on the presence of gp160ΔCT glycoprotein on the bead, suggesting that the lipid does not bind to the bead non-specifically but rather forms in a coordinated fashion. The hydrophobic transmembrane regions of the envelope glycoprotein likely nucleate membrane formation during the reconstitution.

The binding of a secondary antibody to the 1D4 capture-antibody was significantly reduced by membrane reconstitution. This indicates that, at least in part, the lipid bilayer was reconstituted around the protein constituents captured on the bead. That the membrane interacts with elements in the gp160ΔCT transmembrane region is supported by the 2F5 antibody binding profiles on proteoliposomes with reconstituted membranes versus proteoliposomes without membrane. The neutralizing antibody 2F5 recognizes a linear epitope in the gp41 ectodomain (ELDKWAS) directly adjacent to the transmembrane region (80). The comparison of binding of 2F5 to gp160ΔCT on proteoliposomes versus beads without a reconstituted membrane shows a roughly ten-fold higher affinity towards the epitope on the membrane-embedded envelope glycoprotein. Such an affinity difference was not seen with the F105 or 2G12, two gp120-directed antibodies (Fig. 10). Although the 2F5 epitope appears to be linear, it might exhibit some dependence upon conformation. These requirements of the important but elusive 2F5 epitope seem to be better retained by reconstitution of the lipid environment.

In conclusion, the data presented strongly suggest that the gp160ΔCT proteoliposomes provide a native conformation of HIV-1 envelope glycoproteins in a suitable format to test these unique reagents as immunogens or to select for novel ligands from phage display antibody libraries. The gp160ΔCT proteoliposomes should also be useful for studies of purified HIV-1 envelope glycoproteins in a natural context.

4.2 Proteoliposomes as immunogens

The elicitation of broadly neutralizing antibodies against the HIV-1 envelope glycoproteins remains a major challenge. Monomeric gp120 has proven to be a poor immunogen. To date, only few envelope glycoprotein formulations have been shown to more effectively elicit broadly neutralizing antibodies than monomeric gp120 (125). A

recent study suggests that complexes of monomeric gp120 and soluble CD4 better elicit broadly neutralizing antibodies than does monomeric gp120 in a primate model (40). However, part of the antibody response generated by these complexes is directed against CD4. Anti-CD4 antibodies have been shown to be highly efficient in broadly neutralizing HIV-1 (47, 96). The elicitation of such reactivities in vaccinated individuals, however, is not desirable.

The complex and labile organization of the functional trimeric envelope glycoprotein complex poses difficulties for the design of faithful antigenic mimics of these complexes. Here, we tested proteoliposomes as novel immunogens for their ability to elicit broadly neutralizing antibodies in outbred rabbits. A study by Yang *et al*. demonstrated an ability of a YU2 gp140-GCN4 construct to elicit neutralizing antibodies with limited breadth in mice. Therefore, we tested this construct side-by-side with proteoliposomes and monomeric YU2 gp120 in a comparative immunogenicity study.

The molecular clone YU2 was chosen for the preparation of proteoliposomes for immunization studies because it exhibits one of the most neutralization-resistant phenotypes. This molecular clone was not passaged in tissue culture prior to molecular cloning (55) and can only poorly be neutralized by antibodies and soluble forms of the CD4 receptor. In contrast, YU2 often exhibits significant enhancement of infection by these ligands (104, 106). It was hypothesized that breadth of neutralization would most likely be exhibited by antibodies that are raised against neutralization-resistant molecular clones, with neutralization extending from there to more neutralization-sensitive clones.

By deriving the envelope glycoproteins for the generation of proteoliposomes from the YU2 molecular clone, the immunogenicity of proteoliposomes could be directly compared to that of the previously tested immunogen YU2gp140-GCN4. Thus, it could be assessed if the proteoliposomes represent an advance over existing envelope glycoprotein constructs. There is, however, one caveat to this direct comparison. The dose of envelope glycoprotein that could be inoculated into rabbits with soluble YU2gp140-GCN4 constructs was approximately 5-fold greater than the amount of envelope glycoproteins formulated as proteoliposomes.

To assess the total amount of IgG antibody elicited by the different immunogens, sera was tested by anti-gp120 ELISA. Interestingly, although the dose of envelope

glycoproteins administered into animals receiving proteoliposomes was approximately 5-fold lower, these animals generated similar endpoint ELISA titers compared to the animals inoculated with soluble envelope glycoproteins (Table 2). The particulate presentation of envelope glycoprotein as proteoliposomes to the immune system may be more effective for the elicitation of antibodies than the administration of soluble protein. One possible explanation for this may be a depot effect of the proteoliposome formulation that better prevents the dilution and clearance of protein immunogen by physically immobilizing and sequestering the immunogen in the tissue at the site of inoculation. This depot effect is also facilitated by the addition of detergent-containing adjuvant, such as the Ribi adjuvant used here, but may not be as efficient as particulate formulation of the immunogen. Also, there may be more efficient uptake of immunogen by antigen-presenting cells if the immunogen is particulate rather than soluble.

Overall, the oligomeric envelope glycoprotein formulations displayed greater neutralizing activity than did monomeric gp120. The neutralizing activity of sera in each group was very diverse although trends could be observed. Homologous neutralization of the YU2 virus generally corresponded with neutralization of the SF162 isolate and, in most cases, the HXBc2 isolate. Conversely, neutralization of HXBc2 or SF162 did not necessarily correspond with homologous YU2 neutralization. This observation is consistent with the initial rationale for choosing YU2 for the generation of immunogens. Neutralizing antibodies raised against a neutralization-resistant viral isolate appear to have a greater propensity to neutralize other viral isolates than antibodies raised against a more neutralization-sensitive viral isolate (125).

There may be several reasons for the better ability of the trimeric glycoprotein formulations (YU2 gp140-GCN4 and proteoliposomes) to elicit neutralizing antibodies when compared to monomeric gp120. The first reason may be that these oligomeric molecules were derived from a primary isolate (YU2), which is highly resistant to most neutralizing antibodies and in particular to strain-restricted V3 loop-directed antibodies. The second possibility is that the trimeric molecules may reduce the conformational flexibility seen with monomeric gp120 and thereby more efficiently elicit antibodies that recognize the functional trimer. By titration microcalorimetry, it was previously shown that gp120 exhibits a large loss of entropy following interaction with CD4 (70). As this

entropy loss is not dependent on the major gp120 variable loops V1, V2 or V3, it is likely that the flexibility detected by the thermodynamic analysis involves the core domains of gp120. This flexibility may contribute to the elicitation of many antibodies that do not cross react with the native trimer. In fact, most antibodies elicited in infected individuals or vaccinated subjects bind with high affinity to monomeric gp120 but not to oligomeric gp120 found on the surface of infected cells (41, 83, 97). Thirdly, the inclusion of gp41 protein sequences might provide additional T-helper epitopes that might quantitatively increase anti-envelope antibody levels. Finally, oligomeric antigens may more effectively trigger immune responses than monomers as they might better be able to crosslink antigen receptors and thus provide better activation signals.

An enhancing effect of immune sera on YU2 infection could be readily observed in all groups. Enhancement of HIV-1 infection by antibodies binding at subsaturating concentrations has been well described (49, 56, 87, 104, 105, 108). Antibody binding to virus can lead to Fc-receptor mediated endocytosis and infection. Also, antibody binding to Fc-receptors may stabilize the interaction of the virus and the target cell's receptor, providing a nonspecific binding component. Complement-mediated modes of enhancement have also been described. These mechanisms, however, cannot easily explain the envelope-specific enhancement observed, especially since enhancement of infection observed here and elsewhere (104) could also be mediated by Fab fragments. Another model suggests that antibodies at subsaturating concentrations may induce conformational changes that may allow for more efficient binding to the cellular receptors.

Neutralization of YU2 greater than 90% was achieved by only two animals at the serum dilution tested. The isolates 89.6, BAL, and ADA could not be neutralized by any of the sera tested (with animal 24 neutralizing 89.6, but the unusual enhancement with preimmune serum calls this result into question). Interestingly, only one animal inoculated with proteoliposomes neutralized the homologous isolate YU2, while the isolates HXBc2 and SF162 were readily neutralized by four out of five and five out of five animals in this group, respectively. Homologous neutralization is likely mediated by variable loop-directed antibodies, whereas cross-neutralization that extends to heterologous strains is usually associated with antibodies directed against conserved

epitopes such as the CD4 binding site. This raises the possibility that proteoliposomes are more efficient in raising antibodies that bind conserved envelope glycoprotein structures than variable loop-directed antibodies. Thus, homologous neutralization may not be achieved in these animals due to a lack of variable loop-directed antibodies and titers of CD4BS antibodies too low to neutralize the neutralization-resistant YU2 isolate. This possibility will be tested by analysis of the specificity of monoclonal antibodies generated from individual animals from each group.

When the animals were inoculated for a fifth time after an extended resting period, no consistent boosting effect could be observed (Table 4). In general, higher nonspecific neutralization of preimmune serum could be observed after the fifth inoculation (data not shown) and may be indicative of a generally elevated immune status in these animals. Some animals that had been inoculated with PLs developed signs of a general inflammatory response and some hematomas. The higher neutralization by preimmune serum, however, was also observed in the groups inoculated with soluble protein. Whereas some animals displayed better neutralization after the fifth inoculation compared to the fourth inoculation, some animals displayed decreasing neutralizing activity. Since the quality of the immunogen was monitored before each inoculation and was not found to deteriorate during the course of the immunization experiment, other factors may account for this effect. In the course of an antibody response, affinity maturation selects for the antibodies with the highest affinity for the immunogen (1, 51). This selection process is strictly affinity-driven. There is no selection for antibodies with neutralizing activity. With progressive maturation of the antibody response, high-affinity antibodies are elicited. Neutralizing antibodies, however, may not necessarily be of high affinity and might become less abundant. Therefore, an immunization protocol that limits the number of boosts may be more effective. This hypothesis will be tested by boosting the animals for a sixth time to see if a general trend towards less neutralizing activity becomes apparent.

Although increasing the number of inoculations does not seem to be consistently beneficial to raise neutralizing antibody titers, increasing the dose of immunogen at each inoculation may very well be. The less efficient elicitation of neutralizing antibodies observed with proteoliposomes compared to YU2 gp140-GCN4 may be due to the

smaller dose of envelope glycoproteins inoculated. The optimization of the proteoliposome formulation towards an increased amount of envelope glycoproteins incorporated per volume unit, for example by decreasing bead size, would address this limitation.

The sera generated in the different immunizations were tested for neutralization of HIV-1 in *in vitro* single-round neutralization assays. These assays are useful as initial screens for the evaluation of immunogens. There are, however, inherent problems of this kind of assay that limit the predictive value of the obtained data. Animal serum is a highly heterogenous mixture containing immune effector molecules such as complement and cytokines that impact on the readout of the assay. These nonspecific components of serum can cause a broad range of effects, from enhancement of HIV-1 infection to significant neutralization, depending on the genetic variation and immune status of individual animals. These effects can mask specific, antibody-mediated effects and therefore limit the fidelity of the assay. It is therefore necessary to carefully control for nonspecific serum effects and to monitor the influence of individual preimmune serum. Here, neutralization of immune serum was monitored against the background of nonspecific enhancement or neutralization of infection observed with preimmune serum from individual animals. To limit nonspecific effects of serum and yet retaining high enough concentrations of specific antibodies, serum was tested in neutralization assays at a dilution of 1:5. Although the data obtained were reproducible and internal controls were included, due to inherent variation from animal to animal, these data need to be interpreted with caution.

This study adds strong evidence in support of the concept that trimeric envelope glycoprotein formulations are more efficient in eliciting broadly neutralizing antibodies. The data presented here demonstrate that two trimeric envelope glycoprotein formulations, proteoliposomes and soluble YU2 gp140-GCN4 ectodomain constructs, better elicit broadly neutralizing antibodies than monomeric gp120. The proteoliposome format holds promise to be an advance in immunogen design if the current dose-related limitations can be overcome.

In a previous study, the YU2 gp140-GCN4 construct was shown to better elicit broadly neutralizing antibodies than gp120 in mice (125). However, to be able to predict

the efficacy of this immunogen in humans, it is of vital importance to extend and validate these findings to different animal models with a more diverse MHC background. In this study, we show that the improved elicitation of broadly neutralizing antibodies with YU2 gp140-GCN4 constructs also translates to outbred animals. Thus, this study supports and extends the previous immunogenicity data by reproducing the neutralization data obtained in a mouse model and extending these data to a more diverse MHC context. These findings document that trimeric envelope glycoprotein formulations are a promising step towards designing an HIV-1 subunit vaccine.

4.3 Proteoliposomes as antigens

Broadly neutralizing antibodies have proven to be a powerful tool for the study and design of immunogens, understanding the mechanisms of neutralization, and the mechanisms by which viruses evolve to evade antibody-mediated neutralization. Also, broadly neutralizing antibodies have helped to elucidate the conditions for protection against HIV-1 infection (5, 29, 52, 58, 62, 74). By defining neutralizing epitopes on the HIV-1 envelope glycoproteins, broadly neutralizing antibodies can serve as templates for the design of subunit immunogens and help reveal the most vulnerable targets for neutralization (95). Probing the antigenicity of vaccine candidates with broadly neutralizing antibodies may have predictive value and provides a means for evaluating the potential of candidate immunogens to in turn elicit neutralizing antibodies. Also, broadly neutralizing antibodies have the potential to be used as therapeutic molecules, for example, for the prevention of HIV- transmission from mother to infant.

Although in many HIV-infected patients, broadly neutralizing antibodies emerge in the course of infection, the isolation and characterization of these reactivities remains a major challenge. To date, only four broadly neutralizing antibodies have been identified: The two gp120-directed antibodies IgG1b12 (21) and 2G12 (112), and the two gp41-directed antibodies 2F5 (69) and 4E10 (18).

Two general strategies have been employed to identify novel broadly neutralizing antibodies. The generation of monoclonal antibodies from fusions of B-cells derived from HIV-1-infected patients has yielded the broadly neutralizing antibodies 2F5, 4E10, and 2G12. The broadly neutralizing antibody IgG1b12 has been identified from a phage-

displayed antibody library that was generated from the bone marrow of a 31-year old long-term nonprogressor with exceptionally high neutralizing antibody serum titers. Antibody libraries generated from HIV-1-infected individuals have the advantage of being enriched, or biased, for anti-HIV-1 directed antibodies. However, during any given antibody response, a number of antigen-specific B-cell clones are selected and driven to proliferate. In the course of this selection process, some antigen-specific reactivities might become relatively underrepresented. Since the parameters selecting for B-cells to mature and proliferate are affinity to the immunogen, not neutralizing potency, valuable reactivities might be poorly represented in a biased library. Naïve libraries generated from healthy uninfected donors, in contrast, are more likely to contain the full antibody repertoire of the donor, although with anti-HIV reactivities at much lower frequencies. To exploit the advantages of both types of libraries, one naïve and six biased libraries were panned in an attempt to identify novel broadly neutralizing antibodies.

A number of different panning strategies was employed to more effectively select for desired reactivities (Table 5). As a modification of panning A in which proteoliposomes from only one viral isolate were used, panning B was performed with envelope glycoprotein constructs derived from three different viral isolates. Thus, the selection was intended to be focused on epitopes conserved among all different strains while selecting against variable regions such as the variable loops V1, V2, and V3. The sequence of envelope glycoproteins used in the subsequent rounds of panning was chosen to be in the order of increasing neutralization-resistance (89.6, JR-FL, YU2). The rationale was consistent with the hypothesis that neutralization-sensitivity correlates with a more open conformation of the envelope glycoproteins.

For some panning experiments, epitope-masking was employed to selectively block unwanted reactivities (33). In pannings C and D, an immunodominant gp41 cluster II antibody that had previously been retrieved from the FDA-2 library was added to the library during the selection to prevent the enrichment of this particular and other cluster II reactivities. In panning C, monomeric YU2 gp120 was used to preabsorb the FDA-2 library and also added in excess to the library during selection. This blocking strategy was aimed at the identification of antibodies specific for the trimeric envelope complex. Other pannings involving epitope masking were panning F and G. For these experiments,

a blocking Fab antibody, b6, was used at a concentration of 50µg/ml. This antibody binds a very similar epitope on the CD4-binding site of envelope glycoprotein as does the broadly neutralizing antibody b12 (Pantophlet *et al*., submitted). However, b6 does not neutralize primary isolates efficiently. It is a longstanding puzzle which properties confer the neutralizing phenotype of b12 when the epitopes of these two antibodies are so similar. One hypothesis is that b6 may be able to bind two of the three envelope glycoprotein spikes with high affinity, but is not able (possibly due to steric constraints) to bind the third spike, leaving one spike to mediate viral binding and fusion. The b12 antibody, in contrast, may also be able to bind the third spike, thus rendering the envelope glycoprotein nonfunctional. This hypothesis was supported by preliminary experiments showing that there is a slight increase of binding when b12 is added to envelope glycoproteins that have been saturated with b6 (data not shown) and provided the rationale for this b6-blocking experiment.

There is some evidence suggesting that there are subtle antigenic differences in the unprocessed gp160 envelope glycoprotein (cleavage-defective envelope glycoproteins were used for pannings A-G and K) and the mature envelope glycoprotein. In pannings H and I, proteoliposomes derived from the primary isolate 89.6 containing fully cleaved envelope glycoprotein was used for selection. Only in the context of this particular viral strain was the labile interaction of gp120 and gp41 subunits stable enough to produce proteoliposomes containing about 60% cleaved envelope glycoprotein (data not shown). Here we tested the hypothesis that mature envelope glycoprotein may identify other reactivities than unprocessed envelope glycoprotein. Such antibodies would help in the further elucidation of the antigenic differences of cleaved and uncleaved envelope glycoprotein.

For the pannings F,G and H,I, a Fab version and a scFv version of the FDA-2 library were used for the panning with each antigen. Previous experiments have shown that although both Fab and scFv libraries were generated from bone marrow of the same patient, different clones can be selected with the same antigen. The two libraries may therefore represent slightly different antibody repertoires. Pannings F, G, H, and I did not yield any gp120 or gp41 binding reactivities. Possibly, the counterselection during pannings F and G also competed with other CD4BS reactivities.

To test if envelope glycoproteins complexed to their receptors CD4 and CCR5 expose distinct, neutralizing epitopes that are recognized by antidodies, a panning was performed with ternary complexes (panning D). Proteoliposomes were prepared containing the second receptor CCR5. To these proteoliposomes, soluble trimeric envelope glycoprotein complexed with soluble CD4 was bound. In this panning, only a CD4-binding antibody could be retrieved. The most gp120 and gp41 binding reactivities were identified in the pannings A and B. Of the broadly neutralizing Fabs CG5, CG9, and CG21, CG5 was identified in panning A, CG9 was identified in pannings A and B, and CG21 was identified in panning B (Table 6). It appears unlikely that there is a propensity of any of the different panning strategies to selectively retrieve broadly neutralizing antibodies better than any other. In particular, the experiments involving epitope masking generally decreased the yield of gp120- and gp41-positive binders without identifying different reactivities than the less complex pannings.

Interestingly, the panning of a naïve scFv library yielded only V3-loop directed antibodies. The fact that no loop-directed reactivities were retrieved from the biased libraries and only loop-directed reactivities from the naïve library reflects the general dynamics of an antibody response during the course of HIV-1 infection. In the early phases, when the virus encounters a naïve antibody repertoire, mostly strain-restricted, loop-directed antibodies appear. This may be due to a higher precursor frequency of B-cells capable of binding the charged, surface-exposed V3 loop structures. Only later in the course of infection does this pattern change and mostly antibodies directed against more conserved regions such as the CD4BS emerge. This is consistent with the observation, based on sequence analysis of the neutralizing monoclonal antibodies that recognize conserved HIV-1 epitopes, that antigen-driven affinity maturation is required to generate these antibodies.

The Fab fragments CG5, CG9, and CG21 were found to exhibit broadly neutralizing activity in HIV-1 single-round infection assays. The neutralization potency and breadth of these Fabs is intermediate between that of the potent Fab b12 and the two Fabs F91 and F105. When the neutralization efficiency of Fabs was compared to that of the corresponding whole antibodies, a significantly higher neutralization potency of the whole antibodies was consistently observed for b12 and F105, and with some viral

isolates with F91. The antibody input into the neutralization assay was normalized by mass. Thus, more antigen-binding moieties per unit mass (at a ratio of 3:2) were added with Fab than with IgG. And yet, the whole antibodies compensated this disadvantage by, most likely, higher avidity. The Fab fragments CG5, CG9, and CG21 may therefore, too, become more potent in neutralizing HIV-1 as whole antibodies. The IC_{50}s defined here might be underestimates of the neutralization potency of the physiologic whole antibodies.

To date, the broad and potent neutralizing activity in the serum of the FDA-2 patient is unaccounted for. Identifying the nature of this activity would help to understand the correlates of protection from progression to AIDS in general and in this particular patient. The engineering of the three broadly neutralizing antibodies identified here is in progress and may help to elucidate the broadly neutralizing reactivity in this patient.

4.4 Envelope glycoproteins deficient in T-helper epitopes

Escape of viruses from recognition by $CD8^+$ T-cells has been well documented (63). The effect of $CD4^+$ T-helper cells on viral evolution, however, has been less well studied. While CTLs directly exert immune pressure by killing virus-infected cells, the selective pressure exerted by $CD4^+$ T-helper cells by providing help necessary for both CTL and antibody responses is less direct. For HIV-1, the impaired $CD4^+$ T-helper cell reponses due to progressive depletion has been suggested to be a major mechanism of HIV-1 escape from an efficient host immune response (34, 88). It could be shown in a transgenic system for LCMV infection that viruses also evade immune recognition by $CD4^+$ T-helper cells (27). Also, the emergence of altered peptide ligands in Gag that fail to stimulate $CD4^+$ T-helper cells have been described in asymptomatic HIV-1-infected patients (46). Here, we present data that suggests the escape from T-helper cell recognition in the context of the HIV-1 envelope glycoprotein gp120.

Core gp120 derived from two HIV-1 primary isolates, but not from TCLA isolates, are defective in their ability to raise envelope glycoprotein-directed antibodies. Since this effect was not readily observable in TCLA isolate-derived core gp120 envelope glycoproteins, it is likely a direct consequence of the antibody-mediated immune pressure exerted on the primary isolate *in vivo*. The addition of the heterologous

T-helper epitope PADRE to the YU2 primary isolate core gp120 fully restored its immunogenicity as determined by antibody generation, suggesting that the observed defect of core gp120 to elicit antibodies was due to a lack of T-helper epitopes. The T-helper epitope deficiency of primary isolate core gp120s was initially observed in two strains of mice. The limited MHC class II diversity in these animals, however, warranted the extension of these experiments to outbred animals. A general mechanism of viral escape should manifest itself in an outbred population with a diverse MHC class II repertoire. The phenomenon previously observed in mice also translated into outbred rabbits. As expected in the more diverse array of MHC class II alleles present in the outbred rabbits, the lack of antibody elicitation by the YU2 core gp120 was observable but not as absolute a phenomenon as observed in mice. Interestingly, one animal in five inoculated with YU2 core gp120 generated anti-gp120 IgG titers that were similar to the titers detected in rabbits inoculated with the YU2 core-PADRE gp120. This finding indicates that the absence of an anti-gp120 response in the other animals of this group is most likely due to a difference in their MHC class II alleles, not to a quantitative difference in the core gp120 and core-PADRE gp120 inoculated into the two experimental groups. The similar titers of T-cell-independent anti-gp120 IgM responses raised with core gp120 and core-PADRE gp120 also indicate that the input protein was similar for the two groups. Although T-helper epitope deficiency was observed in the context of mouse and rabbit MHC, it suggests that viral evolution of the gp120 envelope glycoprotein to diminish help in the context of human MHC may have occurred.

The T-helper epitope deficiency of primary isolates was observed in the context of core gp120 envelope glycoproteins deleted of variable loops V1 and V2, and parts of the C1 and C5 regions. These cores represent an approximation of the gp120 determinants that are minimally required for basic function, that is the binding of receptor and coreceptor. The variable loops V1 and V2 are dispensible for viral entry and replication. The V3 loop, however, is required for the binding of coreceptors during the entry process. The core gp120 format was chosen for this study initially to test whether envelope glycoproteins deleted of the variable loops V1 and V2 would better generate broadly neutralizing antibodies by focusing the immune response on the conserved receptor binding sites and removing steric barriers that may impede access of B-cell

receptors to these regions. For the study of T-helper epitope deficiencies, this system has advantages over the use of full-length gp120. Limiting the protein determinants to conserved, functionally important regions allowed us to investigate specifically the protein regions that are shared by most viral isolates. These conserved regions have encountered and adapted to a large array of host MHC class II alleles during repeated evolution of virus and immune system in multiple hosts. Any attempt of the virus to evade recognition by the host's polymorphic immune presentation system, therefore, most likely manifests itself in the core gp120. In the core, functional constraints limit the ability of the virus to mutate in response to the host's MHC repertoire, whereas the variable loops V1/2 and parts of V3 can tolerate extensive variation. Therefore, effects seen in the full-length gp120 context might simply reflect adaptation of one particular viral isolate to one host MHC class II allele through changes in the variable loops. Although such changes in the variable loops would also strongly indicate the escape from T-helper cell recognition, they would be difficult to detect unless they were observed in a matched virus/MHC class II system in which the virus sequence as well as the MHC class II alleles the virus encountered are known. For the YU2 and JR-FL envelope glycoproteins, the MHC background of the respective host has not been determined.

A number of studies have focused on the identity and localization of T-helper epitopes in HIV-1 proteins (28, 100, 107, 114). For the primary clade B isolate 1007 and the primary clade C isolate UG, three hotspots of H2-IA^b-restricted helper epitopes in the gp120 envelope glycoproteins have been described in the same animal model that was used here (C57BL/6) (107). These hotspots locate to V2-C2, V3, and V4-C4, with most of the epitopes found in C2 and V3 (22 of 26 total). If these data are interpreted in the context of the core gp120 used here, deletion of the V1 and V2 loop in these viral isolates would have removed only two of 26 helper epitopes. Assuming that the distribution of helper epitopes along hotspots is similar in other primary isolates, the core gp120 molecules used here would not have been rendered help-deficient through deletion of the V1 and V2 loop alone. Helper deficiency brought about by simply deleting the V1 and V2 loops, therefore, appears unlikely to account for the profound deficiency in T-helper epitopes in the primary isolate core gp120s used here. In the study by Surman (107), gp120 T-helper epitopes were suggested to be limited by the exposure on the three

dimensional structure of the protein. A number of T-helper epitopes identified in this study do not appear to function in the primary core gp120 molecules used in our study. As reported, the YU2 and JR-FL core gp120 appear to be profoundly deficient in T-helper epitopes. The differences between our results and the Surman study may be accounted for by several factors. The immunization scheme in the Surman study utilizes DNA, vaccinia virus, and protein in Freund's adjuvant, whereas our study utilizes protein in the nondenaturing Ribi adjuvant. The *in vitro* stimulation of $CD4^+$ T-helper cells may not fully recapitulate the *in vivo* situation. In addition, the Surman study does not discriminate helper responses of the T_H1 and T_H2 subtype, whereas the antibody readout of our study is biased toward detecting T-helper responses of the T_H2 type. Also, the observed T-helper epitope deficiency in primary isolate core gp120s observed here may be more apparent in our study as it was observed relative to TCLA-derived envelopes.

One obvious caveat to our study is that the core gp120s used here were selected against human MHC alleles whereas the T-helper epitope deficiency was observed in a non-human background. Although these findings could be extended to outbred animals, this system may yet not be the most faithful to study viral evolution against a human MHC background. However, we observed the same helper epitope deficiency of primary isolate core gp120 in outbred animals, suggesting that the reduced immunogenicity may have been influenced by viral evolution in the context of human host MHC. For a chronic virus such as HIV-1, virtually any means that reduces the viral immunogenicity will be of selective advantage for the virus.

Escape from recognition by $CD4^+$ T-helper cells of primary isolate HIV-1 envelope glycoproteins has important implications for the design of subunit vaccines. It is becoming increasingly clear that TCLA-derived envelope glycoproteins are not optimal as subunit vaccines for the generation of neutralizing antibodies. Based upon our observations, however, the use of primary isolate-derived envelope glycoproteins as subunit vaccines may be complicated by the relative deficiency in T-cell helper epitopes. The addition of heterologous helper epitopes, as described here, may be necessary to elicit titers high enough for the efficient neutralization of challenge virus. A heterologous helper epitope such as PADRE might be highly effective in generating persistent, circulating antibodies in a large population with diverse MHC class II alleles. To

facilitate the elicitation of a recall response when the vaccinated individual encounters challenge virus, however, the use of additional homologous HIV-1-derived helper epitopes would be warranted.

5 Summary

Solid-phase proteoliposomes containing HIV-1 envelope glycoproteins faithfully mimic the trimeric envelope glycoprotein complex as it is found on the virus. These novel envelope glycoprotein formulations contain high concentrations of affinity-purified envelope glycoprotein in a physiologic membrane setting. The changes in the envelope glycoproteins necessary for retaining native trimeric structure in this format are minimal.

These proteoliposomes have improved immunogenic properties and elicit titers of anti-envelope glycoprotein antibodies in mice and rabbits comparable to those elicited by a 5-fold greater amount of soluble envelope glycoproteins. Two different trimeric envelope glycoprotein formulations (PLs and YU2gp140-GCN4) elicit neutralizing antibodies better than monomeric gp120. Proteoliposomes are more efficient in eliciting neutralizing antibodies with some breadth than monomeric gp120 in rabbits. If dose limitations in this format can be overcome, proteoliposomes may prove to be an advance over current envelope-based immunogens. Soluble trimeric envelope glycoprotein ectodomain constructs (YU2 gp140-GCN4) elicit broadly neutralizing antibodies in rabbits, extending previous findings in mice to a more diverse MHC background.

Three Fab antibody fragments binding to the CD4BS of gp120 have been identified from phage-displayed antibody libraries with proteoliposomes. These antibodies display broad and potent neutralization of a number of HIV-1 molecular clones. Comparison of the neutralizing potency of other CD4BS Fab fragments with the corresponding whole antibodies suggests that the three identified CD4BS Fabs might significantly gain neutralization potency as full antibodies. They may represent new members of the small and elusive family of HIV-1 broadly neutralizing antibodies.

Core gp120 envelope glycoproteins derived from primary isolates are deficient in T-helper epitopes. Viral evolution may have selected for envelope glycoproteins that elicit suboptimal helper responses. Escape from $CD4^+$ T-helper cell recognition may be yet another immune escape mechanism of HIV-1.

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Abbreviations

α	anti
Δ	delta
µg	microgram
µl	microliter
µM	micromolar
AIDS	Acquired Immunodeficiency Syndrome
BME	beta mercaptoethanol
BSA	bovine serum albumin
CD4	cluster of differentiation
CD4BS	CD4 binding-site
CD4i	CD4-induced
CDR	complementarity determining region
CMV	cytomegalovirus
CT	cytoplasmic tail
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immuno-sorbent assay
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gram
GFP	green fluorescent protein
gp	glycoprotein
h	hour
HAART	highly active anti-retroviral therapy
HIV	Human Immunodeficiency Virus
IC	inhibitory concentration
IPTG	isopropyl β-D-thiogalactopyranoside
kDa	kilodalton
kb	kilobase
l	liter
LTNP	long-term nonprogressor
LTR	long terminal repeat
m	milli
M	molar
MHC	major histocompatibility complex
Mg	milligramm
min	minutes
ml	milliliter
MOI	multiplicity of infection

MOPS	3-N-morpholino-propansulfonic acid
MPL	monophosphoryl lipid A
OD	optical density
O/N	over night
PADRE	pan-DR helper epitope
PAGE	polyacrylamide gelelectrophoresis
PBMC	peripheral blood mononuclear cells
PMSF	phenylmethylsulfonylfluoride
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PLs	proteoliposomes
RT	room temperature
s	second
SB	super broth
sCD4	soluble CD4
scFv	single-chain Fv
SDS	sodium-dodecylsulfate
SIV	Simian Immunodeficiency Virus
TCID	tissue culture infectious dose
TCLA	T-cell line adapted
TCR	T-cell receptor
TDM	trehalose dicorynomycolate
VLP	virus-like particle
WT	wild-type