A new generation of “bivalent” gp120-based vaccines, effective against subtype B and subtype E viruses, has been developed. Antisera from rabbits and humans immunized with these vaccines are able to neutralize macrophage tropic and T-cell tropic viruses grown in activated peripheral blood mononuclear cells (PBMCs). These vaccines are now available for efficacy trials to determine the role of humoral immunity in providing protection against human immunodeficiency virus type 1 (HIV-1) infection.

Rationale for gp120-based HIV-1 vaccine

Many lines of evidence suggest that a strong antibody response to the HIV-1 envelope glycoprotein, gp120, will be an essential feature of any AIDS vaccine. gp120 is the major protein on the surface of the virus and mediates attachment of the virus to the two types of cell surface receptors, CD4 and a chemokine receptor, required for HIV-1 infection.

Immunoadsorption studies have shown that most of the virus-neutralizing antibodies in serum from HIV-1 infected humans bind to gp120, and most of the monoclonal antibodies that neutralize macrophage tropic and T cell tropic viruses bind to gp120. Finally, passive transfer studies with monoclonal and polyclonal antisera have shown that antibodies to gp120 are both necessary and sufficient to protect nonhuman primates from infection by HIV-1 and related lentiviruses. Although several alternative strategies (e.g., bacteria, yeast, insect cells, and recombinant viruses) have been considered for the production of gp120 for use in vaccines, experience has shown that the best way to produce commercially useful quantities that accurately duplicate the protein and carbohydrate structure of gp120 is through the use of genetically engineered mammalian cells (e.g., Chinese hamster ovary cells).

Selection of vaccine antigens

The only viral proteins known to occur on the surface of viruses and HIV-1 infected cells are gp120 and gp41, both derived from the common precursor, gp160. gp41 is an integral membrane protein that mediates cell-to-cell and virion-to-cell fusion, and anchors gp120 to the surface of virions and virus infected cells through noncovalent interactions. Although it has long been recognized that gp120:gp41 complexes occur naturally as oligomeric spike structures, only recently has it been demonstrated that the HIV-1 spikes represent trimers of gp120:gp41 complexes.

Based on analogy with other subunit vaccines (influenza, hepatitis B), where subunit oligomerization is known to be critical for vaccine efficacy, it has long been suspected that oligomeric forms of the HIV-1 envelope glycoprotein complex might represent a more potent vaccine antigen than monomeric forms of gp120 or gp41. Surprisingly, it has been difficult to show that vaccine antigens incorporating gp120 and gp41 (e.g., full-length gp160) or soluble forms of gp160 (e.g., gp140) have any advantage over gp120 with respect to the quality (based on
virus neutralization) or potency of the antibody response. Previously, we reported that soluble gp160 oligomers elicited a different spectrum of antibodies than rgp120\textsuperscript{18} but that that immunization with this antigen failed protect chimpanzees from HIV-1 infection.\textsuperscript{19}

The recognition that HIV-1 exhibits two distinct phenotypes (discussed below), raised the possibility that oligomeric forms of gp160 from macrophage tropic viruses might be more effective than monomeric gp120. To test this possibility we compared the immunogenicity of gp120 and gp160 produced in mammalian cells from T cell tropic and the macrophage tropic strains of HIV-1. We found (unpublished data) that rabbit sera raised against both antigens neutralized the macrophage tropic JRcsf strain of HIV-1 and no consistent advantage in macrophage tropic virus neutralizing activity was seen with gp160-based antigens compared with gp120-derived antigens. Based on these experiments, as well as other considerations (ability to distinguish vaccine-induced immune responses from immune responses due to virus infection) we have selected gp120 as the basis of two new vaccine products, AIDSVAX B/B and AIDSVAX B/E.

Structure of gp120

gp120 is a complex molecule (Fig. 1) containing 18 cysteine residues (that form 9 disulfide bridges) and approximately 25 N-linked glycosylation sites.\textsuperscript{20} Comparison of sequences from different strains of HIV-1\textsuperscript{21} have defined 5 conserved regions (C1–C5) and 5 variable regions (V1–V5) of gp120 (Fig. 1). A number of discrete functions can be attributed to different regions of the molecule. For example the V1, V2, V3, the C4 domains of gp120 have been shown to contain epitopes recognized by virus neutralizing antibodies.\textsuperscript{11,22–27}

Mutagenesis experiments and studies with monoclonal antibodies have shown that the C4 domain, and a discontinuous region spanning the C3 and C4 domains, is involved with CD4 binding.\textsuperscript{28} However, it was surprising that antibodies to the C4 domain that blocked CD4 binding seemed less potent than antibodies to other regions of the molecule, particularly the V3 domain. Within the V3 domain a sequence termed the principle neutralizing determinant (PND) was identified that was critical for the binding of many potently neutralizing antibodies.\textsuperscript{22,25,29} The reason that antibodies to the V3 domain exhibit potent neutralizing activity remained a mystery for many years. However, recent studies have provided a structural basis for their activity. It is now recognized that HIV-1 requires two receptors for infection, CD4 and one of several chemokine receptors.\textsuperscript{30–32} It is now recognized that the C4 domain is located in close proximity to the CD4 binding site, and the V3 domain appears to represent part of the chemokine receptor binding site.\textsuperscript{9,33,34}

Compelling evidence now exists to show that virus binding to cell surfaces is a two-step process where initial docking is mediated by CD4, which then causes a conformational change in gp120 that exposes the chemokine receptor binding site.\textsuperscript{9,33,34}

FIG. 1. Amino acid sequence and disulfide structure of MN-rgp120. Circled areas indicate approximate location of epitopes recognized by virus neutralizing antibodies.
Once the CD4 and chemokine receptor binding sites on gp120 are engaged, a conformational change in gp41 is thought to occur that mediates fusion between the lipid envelope of the virus with the plasma membrane of the cell, and the concomitant injection of the viral genome (contained within the nucleocapsid) into the cell. Thus antibodies to the V3 region appear to neutralize virus infectivity by interfering with the binding of gp120 to chemokine coreceptors (CXCR4 or CCR5).

**Virus variation between genetic subtypes**

It is well known that HIV-1 is a highly mutable virus, and that genetic variation represents a major challenge in AIDS vaccine development. Molecular epidemiological analysis has defined approximately nine genetic subtypes of HIV-1, and have shown that different genetic subtypes are present in different regions of the world. 

Like other RNA virus vaccines, it is expected that multivalent vaccines, incorporating gp120 from several different genetic subtypes, will be required to achieve a high level of protection against viruses circulating worldwide. Thus, the first-generation AIDS vaccines are likely to be effective against viruses that predominate in specific geographic regions (e.g., United States, Western Europe, Southeast Asia), and will then be expanded to increase the breadth of protection against viruses in circulation in other geographic regions where multiple virus serotypes are in circulation and no single virus serotype predominates (e.g., sub-Saharan Africa).

**Virus variation within genetic subtypes**

While differences in genetic subtype represent one level of virus variation important for vaccine development, virus variation within a given subtype may also be an important factor in the development of a successful AIDS vaccine. Within each genetic subtype, two types of sequence variation appear to be important for vaccine development: (1) variation that determines virus tropism and (2) variation affecting the structure of epitopes recognized by virus-neutralizing antibodies.

It is now recognized that HIV-1 exhibits two distinct phenotypes related to virus tropism: T-cell tropic viruses and macrophage tropic viruses. T-cell tropic viruses can be cultured in T-cell lines, induce syncytia formation (SI), and require the CXCR4 (fusin) chemokine receptor for infection. Macrophage tropic viruses are unable to grow in T-cell lines, are unable to induce syncytia formation (NSI), and require the CCR5 chemokine receptor for infection. Based on chemokine receptor usage, macrophage tropic viruses have recently been termed R5 viruses, and T-cell tropic viruses are termed X4 viruses. For the purpose of this article, we use the term macrophage tropic viruses to indicate viruses that depend on CCR5 for infection, and the term, T-cell tropic viruses, to indicate CXCR4 dependent viruses.

Macrophage tropic and T-cell tropic viruses differ in their sensitivity to in vitro neutralization. Thus, viruses capable of growth in T-cell lines are typically 10- to 100-fold more sensitive to neutralization by antibodies and soluble CD4. Several studies suggest that virus tropism correlates with clinical disease progression. Thus, macrophage tropic viruses are present in all stages of HIV-1 infection, whereas T-cell tropic viruses are associated with an accelerated course of disease progression.

Based on these differences in virus tropism and pathogenesis it is important for HIV-1 vaccines to stimulate an immune response that is effective against both kinds of virus. The best way to guarantee such responses is to include envelope glycoproteins derived from both types of virus in vaccine products.

**Neutralization sites are polymorphic**

Considerable data from monoclonal antibody binding studies have documented that many of the regions of gp120 recognized by virus-neutralizing antibodies are polymorphic, and that polymorphism at these sites affect antibody binding. The best known example of this occurs in the V3 domain where it has been observed that approximately 66% of virus isolates from the United States and Western Europe possess the consensus sequences: IGPGRA or GPGRAF at the tip of the V3 loop (Fig. 2). These sequences define whether viruses are MN-like or non-MN-like. The importance of sequence variation in this region was demonstrated previously where antisera to the MN strain of HIV-1 neutralized a wide variety of viruses that possessed these sequences, whereas these antisera failed to neutralize viruses with radical amino acid substitutions in this region. Conversely antisera made to strains of viruses (e.g., IIIB, RF) that differed from the MN in this region were essentially strain specific and showed little if any cross-neutralization.

Polymorphism at neutralizing epitopes in other regions of gp120 has similarly been documented. We and others have discovered a common polymorphism (K for E) at position 429 in the C4 domain of gp120 that effects the binding of virus-neutralizing monoclonal antibodies. Polymorphisms that affect virus neutralization occur in the V1 and V2 domains of the C4 domain of gp120.
gp120 as well as at discontinuous epitopes. Data from sequence analysis and monoclonal antibody binding experiments have allowed us to develop a proprietary algorithm (Table 1) to define antigenically significant polymorphisms at virus neutralizing epitopes of gp120. Our analysis suggests that polymorphisms at neutralizing sites are finite and often assort independently. Analysis of variation at neutralizing epitopes provides a method for rational selection of envelope glycoproteins for inclusion into multivalent vaccines. Thus if the first component of a multivalent vaccine possesses a linear epitope independently. Analysis of variation at neutralizing epitopes virus neutralizing epitopes of gp120. Our analysis suggests that polymorphisms have allowed us to develop a proprietary algorithm for inclusion into multivalent vaccines. Thus if the first component of a multivalent vaccine possesses a linear epitope pattern of: a1, b2, c3, the we would select second component with complementarity epitope pattern (e.g., a2, b1, c1).

**Molecular epidemiology is required for antigen selection**

Perhaps the most important requirement for the development of an AIDS vaccine is the identification of high-risk cohorts in which vaccine efficacy trials can be conducted. Once such cohorts are identified, epidemiology studies are required to characterize the serotypes of the viruses circulating in those populations, so that vaccines can be produced that match the viruses that are being transmitted.

Historically vaccine serotyping studies have been carried out in animal models. However, in the case of HIV-1, animal models suitable for serotyping studies do not exist. To overcome this limitation, molecular epidemiology studies, aimed at defining polymorphisms affecting humoral and cellular immunity are the most powerful tools currently available. The strength of this approach was demonstrated in 1990 where La Rosa et al. sequenced the V3 domain of 245 virus isolates from the United States and concluded that the sequence of the common IIIB laboratory isolate was unrepresentative of viruses circulating in the United States, and predicted that vaccines that resembles the sequence of the MN strain of HIV-1 were more representative of viruses in circulation. Subsequent studies showed that vaccines possessing V3 domain sequences similar to the MN strain.

<table>
<thead>
<tr>
<th>Table 1. Algorithm to Analyze Sequence Variation at gp120 Neutralizing Epitopes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epitope Loci</strong></td>
</tr>
<tr>
<td><strong>Linear</strong></td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>a1</td>
</tr>
<tr>
<td>a2</td>
</tr>
<tr>
<td>a3</td>
</tr>
<tr>
<td>*</td>
</tr>
<tr>
<td>AN</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>d1</td>
</tr>
<tr>
<td>d2</td>
</tr>
<tr>
<td>d3</td>
</tr>
<tr>
<td>*</td>
</tr>
<tr>
<td>*</td>
</tr>
<tr>
<td>AN</td>
</tr>
</tbody>
</table>

Functionally significant sequence variation occurs at multiple loci (epitopes) recognized by virus neutralizing antibodies (A–F). Such loci include the V1, V2, V3, and C4 domains of gp120, as well as discontinuous epitopes bridging different domains. Antigenic variation at each locus is used to define alleles at each neutralizing site (lower case). Antigenic variation at each epitope is highly restricted and variants can be treated as independently assorting elements.

**Immunogenicity of gp120**

To date, our group has produced rgp120 from approximately 12 diverse strains of HIV-1 for immunization studies, 4 of which (IIIB, MN, A244, and GNE8) have been manufactured for human clinical trials. In addition many other envelope glycoproteins have been expressed for antigen binding and epitope mapping studies. All of the recombinant gp120s tested to date are highly immunogenic in rodents (mice guinea pigs, rabbits) and nonhuman primates (macaques, baboons, and chimpanzees) and typically elicit enzyme-linked immunosorbent assay (ELISA) titers in the $10^{-4}$ to $10^{-6}$ range. Anti-gp120 antisera typically show a high degree of cross-reactivity with gp120s from different genetic subtypes (Fig. 3A) and resemble sera from HIV-1-infected people with respect to the magnitude and cross-reactivity of antibody response.

Many studies have shown that gp120 typically induces antibodies against functionally significant epitopes of gp120. Thus, antisera from rgp120 immunized animals typically contains antibodies to the chemokine receptor binding site (V3 domain) and CD4 binding site (C4 domain). In addition, recombinant gp120 also induces antibodies to regions in the V2 domain known to be recognized by virus-neutralizing monoclonal antibodies.

Studies with IIIB-rgp120 and MN-rgp120 showed that immunization could protect chimpanzees from infection by homologous and heterologous strains of HIV-1 including a primary isolate of HIV-1 SF-2. The heterologous protection was particularly significant because the envelope glycoprotein of this virus differed from MN-rgp120 by approximately 20% in amino acid sequence. This high degree of sequence variation is similar to the degree of variation seen among clinical isolates circulating in populations infected with subtype B viruses. Finally, although on a worldwide basis sexual transmission of HIV-1 is more common than intravenous transmission, all of the available evidence suggests that intravenous transmission is far more efficient than sexual transmission, and may represent a more rigorous virus infection paradigm.

**Immunogenicity of gp120 from T cell tropic and macrophage tropic isolates of HIV-1**

The difference in growth characteristics and virus-neutralization sensitivity between macrophage tropic and T cell tropic viruses (discussed above) suggested that there might be significant differences in the structure of envelope glycoproteins from...
macrophage tropic and T-cell tropic viruses. To explore this possibility we examined the ability of antibodies to elicited in humans against gp120 from the S1, T-cell tropic, MN strain of HIV-1 to bind to recombinant gp120 cloned from seven primary, macrophage tropic strains of HIV-1. We found (Fig. 3B), that total antibody binding to MN was similar to that of the primary, macrophage tropic viruses. These results showed the antigenic structure of gp120 from macrophage tropic viruses is not grossly different from the antigenic structure of gp120 from T-cell tropic viruses.

Finally, studies with monoclonal antibodies have failed to detect a significant difference in the epitopes present on macrophage tropic and T-cell tropic viruses. Thus, most of the monoclonal antibodies capable of neutralizing macrophage tropic isolates of HIV-1 are able to bind to recombinant gp120.11-13 By this criteria, MN-gp120 appears to possess the epitopes responsible for neutralization of macrophage tropic viruses. These results suggest that the problem in making an effective vaccine is not finding a new antigen, but rather optimizing the immunogenicity of important antigenic sites on gp120.

Virus-neutralization experiments

While not proven, most investigators agree that in vitro virus neutralization provides a potential correlate of vaccine efficacy. However, there are conflicting data concerning which virus neutralization assay represents the best indicator of protective immunity. Many laboratories have shown that T-cell line adapted viruses can be neutralized by sera from HIV-1 infected humans and volunteers immunized with experimental HIV-1 vaccines.53-55 Moreover there is a good correlation between protection from virus challenge and the presence of neutralizing antibodies has been established in animal models of HIV-1 infection. Thus, protection of chimpanzees from HIV-1 infection, and rhesus macaques from infection by chimeric SIV/HIV viruses (SHIV), both correlate with levels of neutralizing antibodies and suggest a threshold titer of approximately 1:200 provides protection from intravenous virus challenge.19,56

However, the relevance of these assays was called into question by the discovery of macrophage tropic, R5 viruses. These viruses cannot be grown in most T-cell lines, are not infectious in chimpanzees, and show poor infectivity when incorporated into SHIV constructs. Although initial reports suggested that macrophage tropic, R5 viruses were readily neutralized by sera from HIV-1 infected humans, it is now recognized that most sera from infected individuals shows weak and sporadic neutralizing activity against macrophage tropic R5 viruses, and of these, few exhibit broad neutralizing activity.57 Macrophage tropic, R5 viruses are sensitive to neutralization by pooled and concentrated immunoglobulin from HIV-1 infected people (HIVIG),58 by a few rare, highly selected monoclonal antibodies, and by selected sera from HIV-1 infected individuals.13-57

Studies in our laboratory examined the ability of human and animal sera to recombinant gp120 to neutralize macrophage tropic isolates. We found that many sera possessed activity, but that macrophage tropic NSI isolates of HIV-1 were more difficult to neutralize in vitro than T-cell tropic isolates. Thus, antisera from HIV-1 infected humans neutralize most T-cell tropic isolates at high dilution (10^-2 to 10^-4). In contrast only about 50% of HIV-1-positive sera neutralizes macrophage tropic viruses grown in peripheral blood mononuclear cells (PBMCs) and those that do rarely exhibit neutralization titers greater than 3 x 10^-2.

What is the relevance of antibodies able to neutralize macrophage tropic viruses to protection from HIV-1 infection? Because macrophage tropic, R5 viruses are present throughout the course of HIV-1 infection, they clearly represent an important class of virus. However, because of difficulties involved in the culture of these viruses (discussed below) the relevance of in vitro neutralization assays using these viruses is subject to
debate. Because vaccine efficacy trials have not been carried out, there is no direct evidence to support the relevance of either T-cell tropic neutralization assays or macrophage tropic neutralization assays to protection from HIV-1 infection. However, results from chimpanzee infectivity studies raised serious doubts as to the validity of neutralization assays carried out in activated PBMCs.

Two independent studies\(^\text{59,69}\) reported that sera from chimpanzees protected from HIV-1 infection by immunization with candidate vaccines failed to neutralize the HIV-1 challenge stock grown in activated PBMCs. However these sera were able to neutralize a T-cell line adapted (TCLA) variant of the challenge virus grown in T-cell lines. This lack of correlation between protection and in vivo and neutralization in vitro (PBMC assay) suggested that the conditions of infection used in the PBMC assay might not be relevant to those that occur during the course of natural infection (discussed below).

**The conditions of infection in virus infectivity assays fail to replicate the conditions of infection during natural transmission**

Many lines of evidence suggest that the conditions of infection in natural transmission of HIV-1 differ markedly from the conditions of infection in experimental HIV-1 infection. A summary of these differences is provided in Table 2. One major difference between natural infection and experimental infection relates to the probability that virus binding results in productive infection. Although both resting T-cells, and activated T-cells, can be infected by HIV-1, only the activated T-cells can sustain a productive infection.\(^\text{61-63}\) Infection of resting T-cells results in abortive infection.\(^\text{64,65}\) Because activated T-cells normally represent a small fraction of T-cells in peripheral blood, only a small percentage of virus-cell interactions normally result in productive infection. In contrast, most models of experimental infection depend on T-cell lines or activated PBMCs where virtually all of the cells in culture can sustain a productive infection. Besides providing a cellular substrate that can sustain HIV-1 replication, the use of potent mitogens such as phytohemagglutinin (PHA) and interleukin-2 (IL-2) results in the synthesis of cytokines (e.g., tumor necrosis factor [TNF]), transcription factors (e.g., NFκB), and adhesion molecules (e.g., ICAM-1) able to enhance replication and infectivity above and beyond physiological levels.\(^\text{66}\)

A second major difference between experimental HIV-1 infection and natural HIV-1 infection is that HIV-1 infection in humans appears to take place under conditions where the viral inoculum is small and the probability of infection per exposure is low. In contrast experimental infection involves conditions where the virus inoculum is high (typically 10-50% tissue culture infective dose [TCID\(_{50}\)]) and the probability of infection is high. Epidemiological data suggest that the probability of infection per virus exposure is comparatively low (e.g., 1:100-1:1000 depending on the mode of transmission).\(^\text{67,68}\) This conclusion is consistent with DNA sequence data showing that virus variation during acute infection is extremely limited and suggest that HIV-1 infection often results from transmission of a single infectious unit.\(^\text{69-71}\) One consequence of a small virus inoculum relates to the creation of a genetic bottleneck. Studies with other RNA viruses (Duarte et al., 1994) have provided evidence that under conditions where mutation rates are high, viruses transmitted through a genetic bottleneck tend to have mutations resulting in a lowered fitness.

With these considerations in mind, it is likely that experimental models of HIV-1 infection overestimate the difficulty in neutralizing HIV-1 during natural transmission, and at best, are insensitive indicators of vaccine efficacy in humans. These facts suggest that the standard culture procedure used in most macrophage tropic virus neutralization assays provides viruses with significant, nonphysiological growth advantages that are unlikely to exist during natural HIV-1 infection.\(^\text{59,72}\) New generation assays, using more selective means to activate T-cells,\(^\text{73}\) or using “resting T-cells,”\(^\text{72}\) promise to provide data more relevant to the conditions of natural infection.

**High titer human sera to MN-rgp120 neutralize macrophage tropic viruses**

After analyzing the conditions of infection used in neutralization assays, we reasoned that increasing the effective antibody concentration might counteract some of the nonphysiological growth advantages provided to macrophage tropic viruses cultured in vitro. Mindful of the observation that hyperimmune globulin prepared from HIV-1 infected individuals exhibited virus neutralizing activity in this assay, we tested sera from MN-rgp120 infected volunteers concentrated by a procedure used to test hyperimmune globulin. The results of this study are shown in Figure 4A. We found that concentrated sera from MN-rgp120 immunized serum donors was able to neutralize several macrophage tropic viruses (JRcsf, 301660, and

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**Table 2. Conditions of Infection in Assays to Evaluate HIV-1 Vaccine Efficacy**

<table>
<thead>
<tr>
<th>Conditions of infection</th>
<th>Human</th>
<th>Chimpanzee</th>
<th>T cells</th>
<th>PBMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input virus (TCID(_{50}))</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>CD4(^{+}) cells that sustain infection (activated)</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>CD4(^{+}) cells unable to sustain infection (unactivated)</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Genetic bottleneck</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Physical and biological barriers to virus dissemination</td>
<td>Many</td>
<td>Few</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Virus selected for high infectivity and rapid growth</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Unphysiological induction of genes facilitating virus replication</td>
<td>No</td>
<td>No</td>
<td>Some</td>
<td>Yes</td>
</tr>
</tbody>
</table>
BIVALENT rgp120 VACCINES

Neutralization of macrophage tropic viruses by high-titer serum from MN-rgp120 immunized volunteers

Based on the results obtained with hyperimmune globulin, we examined high-titer sera from individuals immunized with MN-rgp120 for neutralizing activity against the macrophage tropic JRcsf strain of HIV-1 grown in activated PBMCs. We found that the highest neutralizing titers were from a phase 1 trial of MN-rgp120 carried out in Thailand, and that sera collected after the 12-month booster injection had significantly better neutralizing activity than sera collected after the 6-month injection. These results (Fig. 4B) confirmed that MN-rgp120 can stimulate an antibody response able to neutralize macrophage tropic viruses, and suggests that an affinity maturation step may be required to elicit such activity.

Extensive analysis of results from macrophage tropic virus neutralization assays has shown that the assay is subject to many variables (including antibody titer), and that once the variables are controlled and the assay optimized for working at low-serum dilutions, consistent neutralization of macrophage tropic NSI and T-cell tropic SI isolates can be reproducibly demonstrated with high titer antisera to recombinant gp120. Based on these experiments, we have concluded that the activated PBMC neutralization assays are relatively insensitive indicators of virus neutralization. Our results suggest that there is a systematic error in such assays, and that results from this kind of experiment greatly underestimate the potency and virus neutralizing activity elicited by rgp120.

Selection and development AIDSVAX B/E: a vaccine for the Pacific Rim

Vaccine efficacy trials require the identification of high-risk cohorts, extensive characterization of the viruses circulating in the cohort, a well-developed infrastructure to support both the medical and ethical issues involved in AIDS vaccine trials. Moreover, chances of success are greatly improved if the first-generation vaccines can be tested in cohorts where virus heterogeneity is minimal. Based on these considerations, one location where HIV-1 vaccines can be tested is in Southeast Asia. Although national education programs in countries such as Thailand have greatly reduced the incidence of new infections in the general population, selected cohorts still have high infection rates despite counseling and prevention programs. Extensive molecular epidemiological analysis has shown that the virus that is most often transmitted in Thailand, subtype E, is much more homogeneous that the transmitted in any other population that has been described. Thus intrapatient variation in gp120 sequences ranges from 10-12% among subtype E virus infections in Thailand, whereas intrapatient variation ranges from 15-20% among subtype B virus infections in the United States. Because sequence variation is limited, we believe that the vaccine efficacy trials should have a better chance of success in Thailand than anywhere else in the world.

Our group has cloned and expressed several subtype E envelope glycoproteins from Thailand. The sequence of the envelope glycoprotein from the CM244 strain of HIV-1, collected in Chiang Mai Thailand in 1990 is typical of subtype E viruses currently circulating in Thailand. A molecular clone of the envelope glycoprotein (A244) was selected for expression and preclinical immunogenicity studies. Antisera to A244-rgp120 RGVL05) grown in PBMCs, but not the subtype B, BZ167 strain of HIV-1 that has an unusual V3 loop sequence. These results demonstrated that immunization with MN-rgp120 can elicit antibodies able to neutralize macrophage tropic, R5 strains of virus, but the concentration of antibodies to these sites in un fractionated serum was low.

FIG. 4. Human antiserum to MN-rgp120 neutralizes macrophage tropic isolates of HIV-1. A, hyperimmune globulin was prepared from serum donors immunized with MN-rgp120, and the resulting preparation was tested for neutralizing activity against three macrophage tropic viruses: JRcsf, shaded circles; 301660, open circles; RVL05, shaded squares; and one T-cell tropic primary isolate, BZ167, open squares. B, unconcentrated serum from an MN-rgp120 immunized individual (collected at the 12-month time point after four immunizations) was tested for neutralizing activity against the prototypic macrophage tropic virus, JRcsf (shaded circles). p24 antigen production from control cultures is indicated by open squares. All neutralization assays were carried out in PHA activated PBMCs using methods similar to those described by Wrinn et al.65
produced in rabbits neutralized the parental macrophage tropic virus (Fig. 5A), but showed poor activity against the MN strain of HIV-1 (data not shown). When A244-rgp120 was combined with MN-rgp120, bivalent formulations neutralized a variety of subtype B and subtype E macrophage and T cell tropic viruses (Fig. 5B). Based on the A244 immunogenicity profile, we have developed a bivalent vaccine termed AIDSVAX B/E designed to be effective against both subtype B viruses and subtype E viruses. A summary of the characteristics of the two antigens (MN-rgp120 and A244-rgp120) contained in AIDSVAX B/E is provided in Table 3. Phase 1/2 immunogenicity studies with this product are currently in progress. Preliminary results suggest that the immunogenicity and reactogenicity of this vaccine is similar to monovalent gp120 vaccines. Our results demonstrate for the first time that combining antigens from two different subtypes greatly expands the breadth of antibody response and suggest that multivalent vaccines can be produced that will be effective against viruses circulating in different regions in the world.

FIG. 5. Neutralization of subtype B and E macrophage tropic viruses by rabbit antisera to A244-rgp120 or AIDSVAX B/B. A, rabbit antisera to A244-rgp120 (shaded circles) was tested for the ability to neutralize the parental, subtype E, CM-244 strain of HIV-1. p24 antigen production from control cultures is indicated by open squares. B, rabbit antisera to AIDSVAX B/E (containing MN-rgp120 + A244-rgp120) was tested for the ability to neutralize two subtype B primary isolates: Jrcsf (shaded circles), 301660 (open squares), and one subtype E macrophage tropic virus, Th92009 (open circles). All neutralization assays were carried out in PHA activated PBMCs using methods similar to those described by Wrinn et al.55

TABLE 3. CHARACTERISTICS OF BIVALENT AIDSVAX B/E VACCINE

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>MN-rgp120</th>
<th>A244-rgp120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tropism</td>
<td>T-cell</td>
<td>Macrophage</td>
</tr>
<tr>
<td>Chemokine receptor</td>
<td>CXCR4</td>
<td>CCR5</td>
</tr>
<tr>
<td>Syncytia formation</td>
<td>SI</td>
<td>NSI</td>
</tr>
<tr>
<td>V2 epitope</td>
<td>SIGDK</td>
<td>SIRDK</td>
</tr>
<tr>
<td>V3 PND</td>
<td>GPGRAF</td>
<td>GPGQYF</td>
</tr>
<tr>
<td>V3 flanking sequence</td>
<td>RIHI...YTTKNIK</td>
<td>SITI...YRTGDII</td>
</tr>
<tr>
<td>C4 Epitope (429–432)</td>
<td>KVGK</td>
<td>GAGQ</td>
</tr>
</tbody>
</table>

Because none of the currently available in vivo or in vitro neutralization assays accurately replicate the conditions of infection during natural HIV-1 transmission, it is impossible to serotype HIV-1 using currently available assays. While molecular epidemiology studies represent powerful approach to select antigens for use in candidate vaccines, another potential source of information is provided by analysis of “breakthrough viruses” that arise during the course of human vaccine efficacy trials (sieve analysis). Breakthrough infections (infections occurring in people immunized with candidate vaccines) are expected in all vaccine trials and can occur for a variety of reasons. These include incomplete immunization, infection by viruses that differ from the vaccine antigen, or decay of the immune response. Because of the high degree of sequence variation seen with HIV-1, few people expect that the first-generation vaccines will provide complete protection. Rather it is expected that the first generation vaccines will have a high degree of efficacy against some, but not all strains in circulation. Once vaccine efficacy has been established with a first-generation product, it will be improved by addition of antigens from different virus serotypes to increase the breadth of protective immunity.

Our group has been interested in analyzing breakthrough infections from phase 1 and phase 2 clinical trials with the goal of selecting new antigens to include in candidate HIV-1 vaccines. This strategy for new antigen selection primarily depends on immunologic and DNA sequence analysis of breakthrough infections encountered in the course of clinical trials. A diagram of this strategy is provided in Figure 6. Given the variability of HIV-1, we think it unlikely that one viral antigen will stimulate immunity against all strains of HIV-1 that are in circulation. However, if a vaccine has efficacy it should provide protection against viruses that resemble the vaccine antigen at important neutralizing sites. In the case of subtype B viruses from the US approximately 66% (Fig. 2) possess a sequence at the tip of the V3 domain (principal neutralizing determinant) that is identical to that found in the prototypic MN isolate of HIV-1. Subtype B viruses that possess the MN PND sequence...
are termed MN-like-viruses (see Fig. 2). Viruses possessing radical amino acid substitutions in the V3 domain are termed "non-MN-like" viruses. Of the 33% of non-MN-like viruses circulating in the United States, no single PND sequence accounts for more than 5% of the population. Based on previous experience, we expect vaccines made from MN-like viruses to be effective against MN-like viruses and lower efficacy against non-MN-like viruses, such as IIIB. Sequence analysis of breakthrough infections from clinical trials of MN-rgp120 have provided reason to be optimistic about this approach. Based on sequence data, we would expect approximately 66% of virus infections in the United States to be caused by MN-like viruses. When the sequences from the seven infections in people that received three injections of MN-rgp120 were analyzed, we found that only 2 of 7 infections (29%) could be attributed to MN-like viruses (Table 4). In contrast 23 of 32 infections in case matched control infections (72%) were MN-like viruses. In addition 4 of 5 infections (80%) in people immunized with another candidate vaccine could be attributed to MN-like viruses. These results demonstrate that the frequency of MN-like virus infections was 40% lower than that expected, and suggested that immunization with MN-rgp120 prevented infection by MN-like viruses. Statistical analysis by several groups have calculated p values in the 0.03 to 0.06 range depending on the method of analysis. Because the overall number of infections was small, and there were fewer placebo-immunized recipients that vaccine recipients, it is not possible to draw conclusions regarding vaccine efficacy from these studies. However, the difference in frequency of MN-like and non-MN like infections observed "sieving" is what would be expected if vaccination was exerting a protective effect.

**Development of AIDSVAX B/B: a vaccine for subtype B viruses**

Having obtained sequence data consistent with a sieving effect, the breakthrough virus envelope glycoproteins were cloned and expressed and tested for immunologic reactivity. Studies with monoclonal antibodies confirmed the sequence data and demonstrated that the antigenic structure of the break-
TABLE 4. PREVALENCE OF VIRUSES WITH MN-LIKE V3 SEQUENCES IN MN-rgp120 VACCINATED INDIVIDUALS COMPARED TO OTHER COHORTS

<table>
<thead>
<tr>
<th>Cohort</th>
<th>n</th>
<th>MN-like V3 Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN-rgp120 immunized</td>
<td>7</td>
<td>29% (2/7)</td>
</tr>
<tr>
<td>Other vaccine immunized</td>
<td>5</td>
<td>80% (4/5)</td>
</tr>
<tr>
<td>Case-matched control</td>
<td>32</td>
<td>72% (23/32)</td>
</tr>
<tr>
<td>Random HIV-positive population</td>
<td>160</td>
<td>66% (106/160)</td>
</tr>
</tbody>
</table>

Data obtained from AVEG protocols 201 and 401.

through viruses differed from that of the vaccine strain. For example human sera to MN-rgp120 showed poor reactivity with synthetic peptides derived from breakthrough virus V3 domains. This difference in reactivity provided a criteria we could use to identify a second envelope glycoprotein to add to MN-rgp120 in a bivalent HIV-1 vaccine. For this purpose antisera were raised against a number of randomly cloned macrophage tropic R5 viruses and tested for reactivity with breakthrough virus envelope glycoproteins. One antisera, from an isolate designated GNE8- gave better reactivity with breakthrough virus V3 sequences than did antisera to other R5 isolates. GNE8-rgp120 has a sequence typical of many NSI subtype B macrophage tropic isolates and it sequence complements MN-rgp120 with respect to common polymorphisms at the V2 and C4 domain neutralizing epitopes (Table 5). Preclinical immunogenicity studies in rabbits have shown that antisera to GNE8-rgp120 alone and mixtures of GNE8 and MN-rgp120 can neutralize T cell tropic (data not shown) and macrophage tropic subtype B viruses (Fig. 7).

Preliminary results showed that the immunogenicity and reactivity of AIDSVAX B/B is comparable to MN-rgp120, and that antigen-specific immune responses can be detected 6 weeks after immunization. These results demonstrated that the breadth of the antibody response can be expanded to include common polymorphisms at neutralizing epitopes of combining envelope glycoproteins from the same genetic subtype.

**Issues related to the production of gp120**

Because comparatively small amounts of gp120 are synthesized by virus-infected cells, and because inadvertent contamination with virus or nucleic acid from virus-infected cells represents a significant bihazard, the most practical way to produce HIV-1 envelope proteins is through the use of recombinant DNA technology. The use of recombinant DNA technology to produce recombinant gp120 (rgp120) not only provides an abundant source of antigen, it allows for generic production and purification methods that can accelerate the development time and lower the cost of multivalent vaccines containing mixtures of rgp120s from different genetic subtypes. Because gp120 is a complex molecule, experience has shown that the best way to produce rgp120 is by expression in mammalian cells. Although it has been possible to express gp120 in other production substrates (e.g., yeast, insect cells), these systems fail to incorporate the type of terminal carbohydrate modifications (e.g., sialic acid) normally found on gp120 produced in virus-infected cells. Like other recombinant proteins, gp120

**TABLE 5. CHARACTERISTICS OF BIVALENT AIDSVAX B/B VACCINE**

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>MN-rgp120</th>
<th>A244-rgp120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular tropism</td>
<td>T-cell tropic</td>
<td>Macrophage tropic</td>
</tr>
<tr>
<td>Chemokine receptor</td>
<td>CXCR4</td>
<td>CCR5</td>
</tr>
<tr>
<td>Syncytia formation</td>
<td>SI</td>
<td>NSI</td>
</tr>
<tr>
<td>V2 epitope</td>
<td>SIGDK</td>
<td>ELRDK</td>
</tr>
<tr>
<td>V3 PND</td>
<td>GPGRAF</td>
<td>GPGRAF</td>
</tr>
<tr>
<td>V3 flanking sequence</td>
<td>RIHL...YTTKNIK</td>
<td>SIHL...YATGEII</td>
</tr>
<tr>
<td>C4 Epitope (429-432)</td>
<td>K429</td>
<td>E429</td>
</tr>
</tbody>
</table>
is subject to proteolysis and other alterations that arise during the manufacturing process. A variety of quality control procedures have been developed to ensure the consistency, reproducibility, and stability of the final product.

In order to facilitate the rapid introduction of vaccines effective against diverse virus isolates and regulatory approval, a generic gp120 vaccine production strategy has been developed that enables gp120s from different strains of virus to be produced and purified by a common production and purification process. The strategy entails the production of rgp120 in stable Chinese hamster ovary cell lines, followed by an immunoaffinity purification using monoclonal antibodies directed against a "flag epitope" that is engineered into every gp120 that is produced. Compared with other recombinant glycoproteins, gp120 is a difficult protein to produce, and extraordinary methods are required to achieve high levels of expression and product quality.

CONCLUSIONS

HIV-1 vaccines based on gp120 vaccines exhibit all of the characteristics historically associated with successful vaccines (e.g., broad cross-reactivity, chimpanzee protection, in vitro neutralization). The new generation bivalent vaccines (AIDS-VAX B/E and AIDS-VAX B/B) appear safe and immunogenic, eliciting neutralizing antibody effective against macrophage tropic and T cell tropic strains of HIV-1, and are effective in stimulating antibodies reactive with common polymorphisms at major neutralizing sites.

History has shown that the ultimate success of a vaccine depends more on the biology of virus infection rather than on imperfect experimental models. Because natural HIV-1 infection is comparatively inefficient, an effective vaccine to prevent transmission of HIV-1 may need only to lower the probability of infection by an order of magnitude (i.e., 10-fold). For example, a vaccine that reduced the probability of HIV-1 infection from a frequency of 1:100-1:1000 in unimmunized individuals (depending on risk factor) to 1:1000-1:10,000 in vaccinated individuals would, for all intents and purposes, halt the transmission of HIV-1. The urgency of the HIV-1 pandemic requires us to aggressively pursue all potential sources of information. Based on the safety, immunogenicity, animal protection studies, and neutralizing activity of gp120 based vaccines, the question is no longer: how can we afford not to test these products? Rather the question is: how can we afford not to test these products?

REFERENCES


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