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RAPID COMMUNICATION

Development of Bivalent (B/E) Vaccines Able to Neutralize CCR5-Dependent Viruses from the United States and Thailand

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Recombinant envelope glycoproteins prepared from a subtype B (MN) strain and a subtype E (CM244) strain of HIV-1 were combined to create a bivalent vaccine (B/E) effective against viruses circulating in the United States and Asia. Combining the two antigens resulted in formulations that increased the breadth and potency of the inter-subtype neutralizing response. Antibodies to the bivalent vaccine formulation neutralized viruses possessing diverse phenotypes, including syncytia-inducing and non-syncytia-inducing primary isolates, viruses using either the CCR5 or the CXCR4 chemokine receptors, and viruses differing in their sensitivity to soluble CD4. These studies demonstrate for the first time that the magnitude and quality of the immune response to HIV-1 can be improved by combining recombinant envelope glycoproteins from different genetic subtypes.

Key Words: HIV; vaccine; CCR5; CXCR4; antibodies; neutralization.

Introduction. Molecular epidemiologic studies have defined approximately nine genetically distinct subtypes of HIV-1 (*33*). In some geographic regions (e.g., sub-Saharan Africa) multiple subtypes (e.g., A, C, D, G, and O) of HIV-1 are in circulation (*32, 36*), whereas in other geographic regions (e.g., United States, western Europe, Thailand) the representation of genetic subtypes is more restricted, with the majority of infections limited to a single subtype (e.g., B or E) (*19, 22, 33, 40*). Because amino acid sequence variation in the envelope glycoproteins varies from 25 to 30% between subtypes, it has been speculated that multivalent vaccines, containing viral antigens from several genetically distinct viruses, will be required for vaccines to provide protective immunity against all of the genetic subtypes circulating in human populations.

However, the relevance of genetic subtypes to virus neutralization serotypes is unclear (*31*). It has long been known that antisera from individuals infected with temporally and geographically distinct isolates of HIV-1 exhibit a high level of inter-subtype cross-reactivity, as well as significant inter-subtype virus neutralizing activity (*20, 42*). Limited data are available concerning the ability of recombinant HIV-1 envelope glycoproteins to stimulate antibody responses capable of inter-subtype neutralization. It is not known whether the breadth of inter-subtype cross-reactivity seen in some HIV-1$^+$ human sera can be duplicated by immunization with recombinant subunit vaccines or whether this activity occurs as a unique consequence of long-term exposure to the swarm of related virus quasi-species that arise during the course of chronic HIV-1 infection (*9, 28*). Moreover, much of the data that is available regarding inter-subtype neutralization was obtained using T-cell-tropic viruses. 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 are readily neutralized *in vitro,* can be cultured in T-cell lines, induce syncytia formation, and require the CXCR4 chemokine receptor for infection. Macrophage-tropic viruses are difficult to neutralize *in vitro,* are resistant to growth in T-cell lines, are unable to induce syncytia formation, and require the CCR5 chemokine receptor for infection. Based on these differences, it has been speculated that the breadth of virus neutralizing activity elicited by candidate vaccines might be improved by including antigens derived from both macrophage-tropic and T-cell-tropic strains of HIV-1.

In the present study rabbits were immunized with bivalent vaccine formulations containing recombinant HIV-1 envelope glycoproteins from a subtype B, T-cell-

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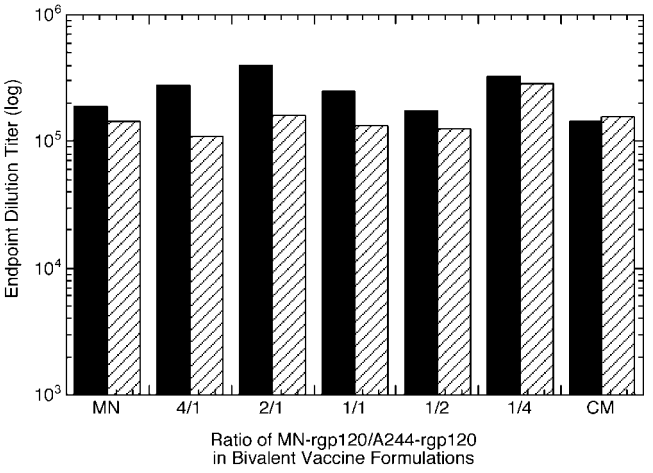

FIG. 1. Antibody binding to MN-rgp120 and A244-rgp120. Two rabbits were immunized with various mixtures of MN-rgp120 and A244-rgp120. Ratios indicate the composition of different vaccine formulations. For example, a 4/1 ratio indicates a formulation consisting of four parts MN-rgp120 and one part A244-rgp120. Sera were collected 2 weeks after the fourth immunization and assayed by ELISA for antibody binding to MN-rgp120 and A244-rgp120. Data represent the mean log end-point dilution titers for pairs of rabbits immunized with each formulation. Black bars indicate antibody binding to MN-rgp120; cross-hatched bars indicate antibody binding to A244rgp120.

tropic virus (MN-rgp120) and a subtype E, macrophage-tropic virus (A244-rgp120). Experience with other recombinant envelope glycoproteins (IIIB-rgp120, MN-rgp120) has shown that antibody responses to gp120 in rabbits (*7*) resemble antibody responses to rgp120 in humans (*3, 39*). We found that antisera from rabbits immunized with rgp120 were effective in neutralizing macrophage-tropic and T-cell-tropic viruses from subtype B and subtype E strains of HIV-1. In principle, a vaccine prepared from the antigens described in this report might be useful in Pacific Rim countries where both subtype B and subtype E viruses are in circulation.

Results. gp120-Binding Studies. Animals were immunized with MN-rgp120 alone, with A244-rgp120 alone, and with various mixtures of these two antigens (Fig. 1). High levels of antibodies (range 5.04 to 5.60 $\log_{10}$ titers) reactive with both MN-rgp120 and A244-rgp120 gp120 were observed after the third booster immunization in the animals immunized with all formulations tested (Fig. 1). Formulation-dependent differences in titers to either antigen were not observed. These data suggested that all of the bivalent formulations were comparably immunogenic and that the major antigenic determinants were conserved between the two different viral proteins.

CM244 is a CCR5-Dependent Macrophage-Tropic Virus. Previous studies have shown that HIV-1_{MN} possessed a syncytia-inducing, CD4-sensitive, CXCR4-dependent phenotype (*11, 12*). Because the phenotype of CM244 virus had not previously been defined, chemokine coreceptor usage and soluble CD4 sensitivity studies were carried out. It was observed that the CM244 isolate was resistant to stromal-derived factor (SDF), a ligand for CXCR4 (*37*), but was sensitive to neutralization by Rantes (*1, 13*), a ligand for CCR5 (Fig. 2A). These results suggested that the CM244 isolate utilized the CCR5 chemokine receptor typically used by macrophage-tropic viruses (*1, 10, 12*) and not the CXCR4 chemokine receptor used by T-cell-tropic viruses (*15*). In other studies, the sensitivity of the CM244 isolate to inhibition by CD4-IgG was measured (Fig. 2B). Like other primary isolates of HIV-1 (*2, 11*), the CM244 isolate was

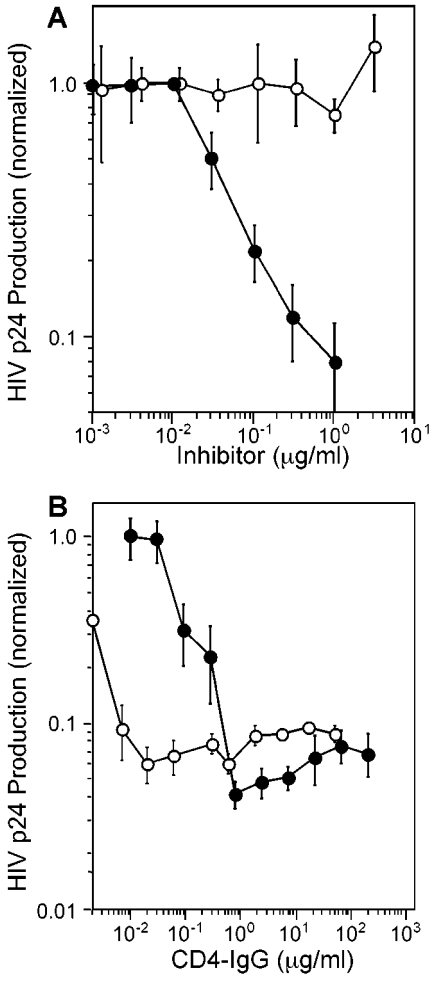

FIG. 2. Chemokine and CD4-IgG sensitivity of the CM244 isolate of HIV-1. (A) Various concentrations of the Rantes and SDF chemokines were added to cultures of PBMCs prior to the addition of CM244 virus. The effect of the CXCR4 ligand SDF on the growth of the CM244 virus (open circles) was compared to the effect of the CCR5 ligand, Rantes (filled circles). (B) The sensitivity of the MN and CM244 isolates of HIV-1 to inhibition by soluble CD4-IgG was compared. Diluted stocks of the CM244 isolate (filled circles) or the MN isolate (open circles) were treated with various concentrations of CD4-IgG before being added to cultures of PHA-activated PBMCs. Virus replication was monitored by measurement of p24 production using a commercial ELISA. The production of p24 antigen in the presence of chemokines was normalized to p24 production in control cultures, without added inhibitors.

TABLE 1

Neutralization of CCR5-Dependent, Macrophage-Tropic HIV-1 Isolates with Rabbit Antisera to Bivalent Subtype B/E Vaccine Formulations

	Viruses		
Ratio MN/A244	CM244 (E)	302031 (E)	301660 (B)
MN	75	15	105
4:1	900	105	225
2:1	290	45	215
1:1[a]	1300	150	110
1:2	225	75	188
1:4	450	40	10
A244[a]	1475	100	<10
HIV^+ Pool (B)	700	<10	30
HIV^+ Pool (E)	900	100	83

Note. Data represent mean neutralization titers obtained from pairs of rabbits immunized with the vaccine formulations indicated. HIV^+ Pool (E) represents pooled sera from individuals infected with subtype viruses. HIV^+ Pool (B) represents pooled sera from individuals infected with subtype B viruses.

[a] Data represent mean neutralization titers obtained from two pairs of rabbits immunized with two different lots of vaccine antigen.

at least 100-fold more resistant to neutralization by CD4-IgG (0.2 μg/ml) than was the MN strain (0.002 μg/ml CD4-IgG) of HIV-1 (Fig. 2B). These results, together with other studies demonstrating that CM244 was unable to grow in cell lines commonly used to propagate CXCR4 viruses, such as H9 and MT4 (data not shown), demonstrate that the CM244 virus possessed a phenotype typical of CCR5-dependent, macrophage-tropic viruses.

Neutralization of Viruses Used to Produce Vaccine Antigens. Initial experiments examined the ability of antisera to MN-rgp120 and A244-rgp120 to neutralize the homologous MN and CM244 viruses. Previous studies (*25, 26, 30*) have shown that T-cell-tropic viruses (e.g., $HIV\text{-}1_{MN}$) are extraordinarily sensitive to *in vitro* neutralization and often exhibit neutralization titers in the range 10^{-3} to 10^{-5}. In contrast, macrophage-tropic viruses have proved to be resistant to neutralization by polyclonal antisera and, at best, give neutralization titers in the range 10^{-1} to $10^{-2.5}$ (*9, 25, 31*). We found that antisera to MN-rgp120 neutralized $HIV\text{-}1_{MN}$ at high dilution and exhibited low levels of neutralizing activity (mean titer of 1:75) against the subtype E, CM244 virus (Table 1, Fig. 3A). Neutralization of CM244 was somewhat unexpected since the amino acid sequence of MN-rgp120 differs from CM244-rgp120 by approximately 30% (*27*) and because others have reported that neutralization of subtype E viruses by antisera to subtype B viruses is subtype specific (*25*).

When antisera to A244-rgp120 were examined (Table 1, Fig. 3B), high levels of neutralizing activity (mean titer 1:1475) against the parental CM244 strain of HIV-1 were observed when assayed in PBMC culture. When antisera to A244-rgp120 were tested for activity against $HIV\text{-}1_{MN}$, one of four sera exhibited significant neutralizing antibodies (titer of 1:270) while the remaining three sera showed no neutralizing activity against this virus (data not shown). In all cases, neutralizing titers to the homologous virus were greater than titers to the heterologous virus. Together, these experiments demonstrated that immunization with rgp120 from either subtype B or subtype E vaccines can elicit antibodies capable of inter-subtype virus neutralization, but that the inter-subtype neutralizing response elicited by monovalent vaccines was sporadic and inconsistent.

To determine whether the relative amount of the subtype B and subtype E antigens in bivalent vaccines affected the potency of the virus neutralizing response, antisera raised against five different mixtures of MN-rgp120 and A244-rgp120 were examined. All antisera to the bivalent formulations were able to neutralize $HIV\text{-}1_{CM244}$ and $HIV\text{-}1_{MN}$ (Table 1, Fig. 3C) with mean titers comparable to those obtained with monovalent vaccines. As would be expected, antisera to the bivalent formulations were more effective in neutralizing the CM244 virus than antisera to the monvalent MN-rgp120 formulation. Similarly, antisera to the bivalent formulations were more effective in neutralizing the MN virus than the monovalent sera to A244-rgp120. No indication of antigenic competition or interference was detected when bivalent vaccines were compared to monovalent vaccines.

Neutralization of Heterologous Primary Macrophage Tropic Viruses by Bivalent Vaccines. Further studies were carried out to examine neutralization of primary isolates of HIV-1 unrelated to the vaccine immunogens. For these studies we employed a subtype B isolate termed 301660 and a subtype E isolate termed 302031. Both viruses had been cultured exclusively in PBMCs and possessed non-syncytium-inducing, CCR5, CD4-resistant phenotypes (Table 2). We observed that antisera to MN-rgp120 neutralized the 301660 virus, with a mean titer of 1:105, whereas antisera to A244-rgp120 failed to exhibit significant neutralizing activity against this virus (Table 1). When sera from rabbits immunized with the five bivalent formulations were examined, mean titers ranging from 1:10 to 1:225 were observed (Table 1, Fig. 3D). As would be expected, the sera to formulations that were predominantly A244-rgp120 (A244-rgp120 alone and the formulation containing four parts A244-rgp120 and one part MN-rgp120) exhibited the lowest neutralizing titers.

Antisera to the bivalent vaccine formulations were similarly tested for the ability to neutralize other Thai, subtype E, primary isolates (Table 1, Fig. 3D). We found that all of the antisera tested possessed virus neutralizing activity against the 302031 isolate (Table 1), though the peak titers to this virus were lower than the peak titers to the CM244 and 301660 strains of HIV-1. As would

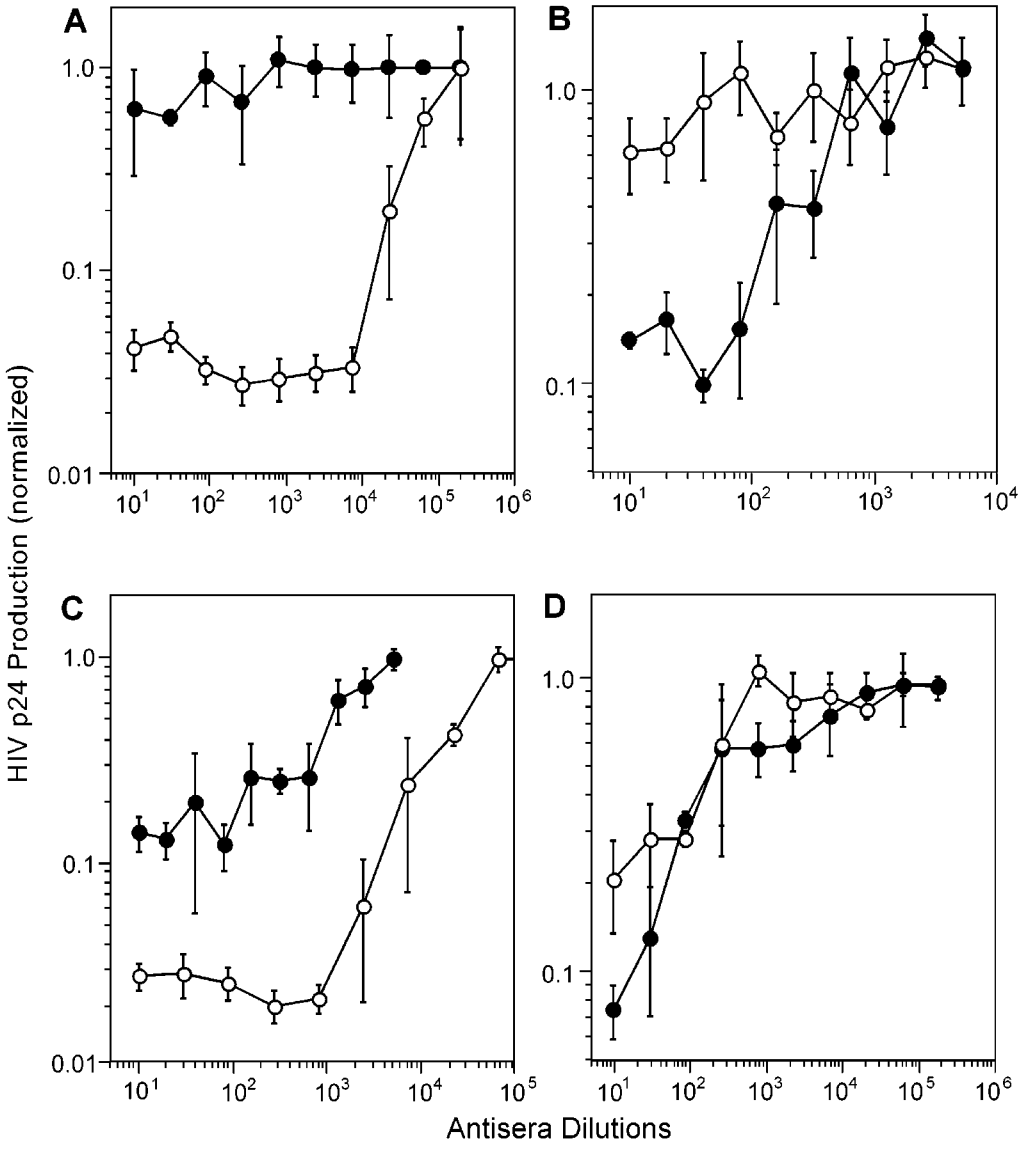

FIG. 3. Neutralization of subtype B and subtype E viruses by antisera to monovalent and bivalent subtype B/E vaccine formulations. (A) Neutralization of the MN strain of HIV-1 by rabbit antisera to MN-rgp120 (open circles) or antisera to A244-rgp120 (filled circles). (B) Neutralization of the CM244 strain of HIV-1 by antisera to MN-rgp120 (open circles) or A244-rgp120 (filled circles). (C) Neutralization of the MN strain of HIV-1 (open circles) and the CM244 strain of HIV-1 (filled circles) by antisera from a rabbit immunized with a mixture of MN-rgp120 and A244-rgp120. (D) Neutralization of the subtype B, CCR5-dependent, 301660 strain of HIV-1 (filled circles) and the subtype E, CCR5-dependent 302031 strain of HIV-1 (open circles) by the same antisera shown in C. Virus replication was monitored by measurement of p24 production using a commercial ELISA. The production of p24 antigen in the presence of antibodies was normalized to p24 production in control cultures in the presence of preimmune or normal control sera.

be expected, the lowest virus neutralization titers were observed in rabbits that received MN-rgp120 alone, whereas all of the sera to the bivalent formulations were able to neutralize this virus, with mean titers ranging from 1:40 to 1:150 (Table 1).

Subtype-specific neutralizing activity was rare in these studies and was seen only with antisera raised against formulations containing a high percentage of A244-rgp120 and a low percentage of MN-rgp120. Thus, antisera from animals immunized with the monovalent A244-rgp120 vaccine exhibited little or no neutralizing activity against the 301660 virus. Similarly, the antisera raised against the bivalent formulation consisting of one part MN-rgp120 and four parts A244-rgp120 also exhibited low virus neutralizing titers. These results (Table 1) suggested that the potency of bivalent B/E vaccines was relatively insensitive to the relative amount of each antigen, provided that the preparations contained at least 33% MN-rgp120.

To gain perspective on the magnitude of the virus neutralizing response elicited by the recombinant envelope glycoproteins, neutralization experiments were car-

TABLE 2

Neutralization of Primary and Macrophage-Tropic Isolates by Rabbit Antisera to Bivalent B/E Vaccine[a]

Virus	Subtype	Origin	SI/NSI	Chemokine receptor	B/E Neut. titer	HIV+ Neut. titer	V3 Crown[b]
301660	B	USA	NSI	R5	150	30	GPGRAF
JRcsf	B	USA	NSI	R5	30	60	GPGRAF
BZ167	B	Brazil	SI	X4	100	100	GPGRTF
TH92014	B′	Thailand	NSI	R5/X4	20	100	GPGRAW
TH92009	E	Thailand	NSI	R5	20	<10	GPGQVF
CM244	E	Thailand	NSI	R5	1000	700	GPGQVF
302051	E	Thailand	SI	X4	50	<10	GPGRVY
302031	E	Thailand	NSI	R5	150	<10	GPGQVF

[a] SI/NSI indicates syncytia-inducing or non-syncytia-inducing phenotype. Chemokine receptor usage was defined by sensitivity to inhibition by SDF, the CXCR4 (R4) ligand, or Rantes, the CCR5 (R5) ligand. B/E Neut. titer indicates the dilution of rabbit sera to a mixture of A244-rgp120 and MN-rgp120 able to inhibit virus growth by 50%. HIV^+ Neut. titer indicates the dilution of pooled HIV-1^+ human sera able to inhibit virus growth by 50%.

[b] GenBank accession numbers and or references for the sequences listed are provided under Materials and Methods.

ried out with sera from HIV-1-infected humans. We found that the antibody response elicited in humans infected with subtype E viruses was more effective against the subtype E viruses (CM244 and 302031) than against the subtype B viruses (Table 1). For example, the titer to HIV-1_{MN} was only 1:67 (data not shown), whereas the titer to 301660 was 1:83. Surprisingly, pooled sera from individuals infected with subtype B viruses showed strong activity (1:700) against the CM244 virus, but weak activity against the 301660 virus. This result may reflect the fact that 301660 is known to be a difficult virus to neutralize (*14*) and that the monoclonal antibody (2G12) best able to neutralize this virus recognizes an epitope that is conserved between different genetic subtypes (e.g., subtype A viruses) (*41*). The present result suggests that subtype E viruses may be able to elicit antibodies to a similar neutralizing epitope that is conserved across genetic subtypes.

Neutralization of Diverse Primary Isolates with Antisera to Bivalent B/E Vaccine. Additional virus neutralization studies were carried out using antiserum from a B/E vaccine immunized rabbit that gave good neutralizing response against the viruses in Table 2. In these studies, virus neutralization activity was measured for five additional primary isolates. The viruses used in this experiment possessed a number of interesting genotypic and phenotypic properties (Table 2). Neutralization of HIV-1_{JRcsf} demonstrated that the B/E vaccine could neutralize a prototypic CD4-resistant, CCR5-dependent, macrophage-tropic virus. Neutralization of the Brazilian isolate BZ167 demonstrated that the bivalent formulation could elicit antibodies able to neutralize a syncytium-inducing, T-cell-tropic, subtype B, primary isolate that differed from MN in the V3 domain subtype B consensus PND sequence (i.e., IRIGPGRTF rather than IHIGPGRAF) (*22*). Similar results were obtained with the Thai B′ isolate, TH92014, and the T-cell-tropic Thai E isolate, 302051, both of which differed from MN-rgp120 and A244-rgp120 at the V3 domain crown sequence (Table 2) yet were neutralized, albeit at low titers, by the bivalent B/E antisera. Finally, neutralization of the Thai TH92009, CM244, and 302031 viruses demonstrated that bivalent B/E subunit vaccines can neutralize a variety of CCR5-dependent, subtype E, primary isolates that contain the consensus Thai E V3 domain crown sequence GPGQVF sequence (Table 2). For the purpose of comparison we tested all of the viruses in this panel for sensitivity to neutralization by pooled sera from randomly selected HIV-1-infected individuals collected in the United States (HIV^+ neutralization titers). We found that the magnitude of the inter-subtype neutralizing activity of the B/E rabbit serum was similar to that of our pooled HIV^+ sera and that the titers measured in our assay were similar to those reported for sera from long-term survivors (*9*) and other sera from HIV-1-infected individuals (*25, 26, 30*).

Discussion. These studies resulted in three important observations: (i) mixing antigens from different HIV-1 subtypes can expand the breadth of virus neutralizing activity compared with monovalent vaccines, (ii) the quality and magnitude of the antibody response elicited by bivalent gp120-based vaccines are similar to those observed in sera from HIV-1-infected humans, and (iii) antisera to recombinant gp120 can elicit antibodies able to neutralize both T-cell-tropic (CXCR4-dependent) and macrophage-tropic (CCR5-dependent) viruses.

We found that both MN-rgp120 and A244-rgp120 are immunogenic in rabbits and elicit high titers of cross-reactive antibodies. Antibody binding to gp120 suggested that the immunodominant domains of gp120 are conserved between subtype B and subtype E viruses. The magnitude of the antibody responses (end-point dilution titer) to bivalent gp120 formulations measured in

rabbits was similar (10^{-4} to 10^{-5}) to that measured in sera from HIV-1-infected humans (M. Peterson, VaxGen, Inc., personal communication). Experiments with the monovalent MN-rgp120 and the A244-rgp120 vaccines demonstrated that inter-subtype virus neutralizing antibodies could be elicited by either antigen. However, the inter-subtype neutralizing responses obtained with monovalent vaccines were often weak and varied from rabbit to rabbit. Studies with bivalent B/E vaccine formulations demonstrated that more potent and consistent, inter-subtype, virus neutralizing responses could be elicited by vaccine formulations when antigens from genetic subtypes were combined. Experiments in which the relative proportion of subtype B and subtype E antigens were varied demonstrated that the MN-rgp120 and A244-rgp120 antigens were similarly immunogenic and suggested that a broad range of formulations are effective in eliciting inter-subtype neutralizing responses.

A significant result of these studies was the observation that both subtype E and subtype B viruses, exhibiting phenotypic properties associated with macrophage-tropic viruses (i.e., NSI, CD4-resistant, CCR5-dependent), could be neutralized by antisera to MN-rgp120, antisera to A244-rgp120, or antisera to a bivalent vaccine consisting of a mixture of A244-rgp120 and MN-rgp120. This observation demonstrates that recombinant gp120 is able to elicit antibodies that neutralize CCR5-dependent (macrophage-tropic) isolates of HIV-1 and is consistent with studies showing that most monoclonal antibodies able to neutralize macrophage-tropic, primary isolates of HIV-1 bind to MN-rgp120 (*16, 18, 30*). Several previous studies have failed to detect antibodies capable of neutralizing macrophage-tropic viruses with sera of animals or human volunteers immunized with rgp120 (*17, 26, 43*). The difference between the present studies and the previous studies appears attributable to quantitative differences in antibody titers, controlling assay variables (e.g., PBMCs from a single donor), and improvements to the assay (maintaining antisera in the virus culture for 3 days prior to wash-out).

These studies document that the magnitude and specificity of the virus neutralizing response to CXCR4-dependent (T-cell-tropic) and CCR5-dependent (macrophage-tropic) viruses elicited by recombinant antigens described in this paper are similar to those seen in sera from humans chronically infected with HIV-1 (*25, 26, 31*). Thus, like HIV-1^+ serum, neutralization titers in the range of 10^{-3} to 10^{-5} were obtained with CXCR4-dependent (T-cell-tropic) viruses (e.g., MN), and neutralization titers in the range of 10^{-1} to $10^{-2.5}$ were obtained with CCR5-dependent (macrophage-tropic) viruses.

Recently, some investigators have suggested that a 90% virus neutralization end-point may be more informative for measuring neutralization of macrophage-tropic isolates than a 50% end-point (used in the present studies). We disagree with this view, particularly with respect to the analysis of polyclonal antisera. While many randomly selected sera from HIV-1-infected humans neutralize primary macrophage tropic isolates using a 50% cut-off, few exhibit robust neutralization with a 90% cut-off (*31*). Thus significantly less information can be expected from a 90% end-point compared to a 50% end-point. In addition, even when it is possible to detect neutralization at a 90% cut-off with polyclonal sera, it is usually only at low dilution (i.e., $<$1:10) where nonspecific inhibition is most prone to occur. Finally, a recent NIH study suggests that only a few of the most highly selected and potent monoclonal antibodies could consistently achieve 90% neutralization of a panel of primary CCR5-dependent viruses and that results varied considerably between laboratories (*14*). Thus, while the 90% cut-off may be useful for documenting the neutralizing activity of a few unusually potent polyclonal (*9*) and monoclonal antibodies (*41*), the assay is difficult to reproduce and is insensitive to the magnitude and specificity of the immune response induced by natural HIV-1 infection.

Previous studies suggested that recombinant gp120 induces antibodies able to neutralize CCR5-dependent viruses, but that the concentration of these antibodies varies from serum to serum. Thus, when antibodies were concentrated from volunteers immunized with MN-rgp120 in a manner similar to that used in the production of hyperimmune IgG, we observed neutralization of CCR5-dependent viruses (*5*). Recent data from a Phase I human clinical trial in Thailand (v0633g) with a monovalent vaccine suggest that antibodies capable of neutralizing CCR5-dependent viruses can be elicited in humans, but do not appear until after the 12-month booster injection when immunized according to a 0-, 1-, 6-, and 12-month immunization regime (*5, 29*). These results suggest that an affinity maturation step may be required for the development of antibodies able to neutralize CCR5-dependent, NSI strains of HIV-1.

In conclusion, we found that combining antigens representing two distinct genetic subtypes could increase the reproducibility and potency of the inter-subtype, virus neutralizing antibody response. Antisera raised against bivalent vaccines exhibited neutralizing activity against viruses that varied in cellular tropism, chemokine receptor usage, CD4 sensitivity, and syncytium-inducing capacity. However, the extent to which *in vitro* neutralization assay results correlate with protection from virus infection *in vivo* is unknown. Ultimately the success or failure of any vaccine depends on the conditions of virus infection during natural transmission. Because the conditions of infection in most *in vitro* neutralization assays provide viruses with many growth advantages unlikely to exist *in vivo* (*4, 5, 8, 45*), it is likely that these assays underestimate the magnitude of the protective immune response that can be achieved in humans. Until experimental paradigms that accurately duplicate the conditions of natu-

ral infection can be established, the only way to establish HIV-1 vaccine efficacy is through human clinical trials.

Materials and Methods. Animals. Sixteen New Zealand white rabbits (2–3 months of age, 2.5–4.5 kg) were randomly distributed into seven groups of two rabbits each. For each immunization, the rabbits were injected at multiple sites (intramuscular and subcutaneous) as described previously (*7*). Antigens were emulsified in 50% complete Freund's complete adjuvant for the first immunization and incomplete Freund's adjuvant for subsequent booster injections. The animals were immunized and bled at 14-day intervals. Serum was prepared by standard techniques and was stored frozen at −20°C until needed.

Vaccine Antigens. Recombinant HIV-1 envelope glycoproteins (MN-rgp120 and A244-rgp120) were purified from transfected CHO cell lines by immunoaffinity chromatography as described previously (*7, 23*). Seven different formulations of vaccine antigen were prepared by mixing different amounts of MN-rgp120 with A244-rgp120 while keeping the total amount of rgp120 contained in each injection constant at 300 μg/dose. The gp120s used in this study were at least 90% pure as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Analysis of Antisera. Sera were analyzed for the relative concentration of antibodies to each of the rgp120 antigens (MN-rgp120 and A244-rgp120) by enzyme linked immunoadsorbtion assay (ELISA) as described previously (*34, 35*).

Virus Culture and Neutralization Assays. Antisera were tested for virus neutralization using methods similar to those described previously (*43*). Viruses were classified by their suppliers as exhibiting syncytium-inducing or non-syncytium-inducing phenotypes (*21*). The sensitivity of viruses to inhibition by CD4-IgG was measured using assays similar to those described by Daar *et al.* (*11*; see also Ref. *2*). The chemokine coreceptor used by each virus was determined by assessing its sensitivity to Rantes, indicating CCR5 receptor usage (*10, 15*), or to SDF, indicating CXCR4 receptor usage (*12*).

Virus neutralization assays using the MN strain of HIV-1 were carried out in PBMCs using a virus stock grown in H9 cells as described previously (*43*). Virus neutralization assays for all other viruses described were carried out in PHA-activated PBMCs, from a single donor, using a method similar to that described by Wrin *et al.* (*43*). All virus stocks were prepared from cultures of PHA-activated PBMC cells, with the exception of the CM244 stock that was produced in PM-1 cells expressing the CXCR4 and CCR5 chemokine receptors (*24*). The 301660 (*14*), 302051 (CMV08) (*44*), and 302031 (CMV01) (*44*) strains of HIV-1 were obtained from the AIDS Reagent Program (National Institute of Allergy and Infectious Diseases). The BZ167 (GenBank Accession No. L22087), TH92009 (GenBank Accession No. U08830), and TH92014 (GenBank Accession No. U08801) viruses were obtained from the World Health Organization. Cultures of CM 244 (GenBank Accession No. L03704) were obtained from F. McCutchan (Henry Jackson Foundation) and M. Norcross (U.S. Food and Drug Administration). The JRcsf strain (GenBank Accession No. M38429) of HIV-1 (GenBank Accession No. M38429) was provided by I. S. Y. Chen (University of California, Los Angeles). For virus neutralization assays, quadruplicate aliquots of virus were incubated with serial dilutions of test antiserum (37°C for 1 h) and PHA-activated PBMCs were then added (2.5×10^5 cells per 250 μl per well). The culture was continued for 3 days, at which time the cells were washed to remove virus and antiserum. Cultures were then washed three times by centrifugation in microtiter plates (300 *g* for 7 min) and resuspended in growth medium containing interleukin 2. Extensive washing was critical in control experiments containing HIV-1^+ human serum to ensure that antibody directed against HIV-1 p24 would not interfere with the ultimate detection of the p24 antigen. The washed cultures were incubated for an additional day. Accumulated p24 antigen was subsequently detected by enzyme-linked immunosorbent assay (Coulter Corp., Hialeah, FL). Maximal p24 antigen accumulation was determined from cultures incubated in the absence of antiserum (virus control), and test cultures were subsequently diluted for analysis of neutralization. Neutralization titers reported represent the reciprocal of antibody dilutions giving 50% inhibition of p24 production. In control experiments, baseline values for virus replication were determined by measurement of p24 production in cultures without added antibody. All sera tested were prescreened for cell toxicity in a MTT dye conversion assay. Control experiments involving immunoadsorbtion with Protein A–Sepharose (Pharmacia, Piscataway, NJ) were carried out to verify that neutralizing activity could be attributed to IgG.

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