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Immunization with a Soluble CD4-gp120 Complex Preferentially Induces Neutralizing Anti-Human Immunodeficiency Virus Type 1 Antibodies Directed to Conformation-Dependent Epitopes of gp120

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Preservation of the conformation of recombinant gp120 in an adjuvant, enabling it to elicit conformation-dependent, epitope-specific, broadly neutralizing antibodies, may be critical for the development of any gp120-based human immunodeficiency virus type 1 (HIV-1) vaccine. It was hypothesized that recombinant gp120 complexed with recombinant CD4 could stabilize the conformation-dependent neutralizing epitopes and effectively deliver them to the immune system. Therefore, a soluble CD4-gp120 complex in Syntex adjuvant formulation was tested with mice for its ability to induce neutralizing anti-gp120 antibody responses. Seventeen monoclonal antibodies (MAbs) were generated and characterized. Immunochemical studies, neutralization assays, and mapping studies with gp120 mutants indicated that the 17 MAbs fell into three groups. Four of them were directed to what is probably a conformational epitope involving the C1 domain and did not possess virus-neutralizing activities. Another four MAbs bound to V3 peptide 302-321 and exhibited cross-reactive gp120 binding and relatively weak virus-neutralizing activities. These MAbs were very sensitive to amino acid substitutions, not only in the V3 regions but also in the base of the V1/V2 loop, implying a conformational constraint on the epitope. The last group of nine MAbs recognized conformation-dependent epitopes near the CD4 binding site of gp120 and inhibited the gp120-soluble CD4 interaction. Four of these nine MAbs showed broadly neutralizing activities against multiple laboratory-adapted strains of HIV-1, three of them neutralized only HIV_{IIIB}, and the two lower-affinity MAbs did not neutralize any strain tested. Collectively, the results from this study indicate that immunization with the CD4-gp120 complex can elicit antibodies to conformationally sensitive gp120 epitopes, with some of the antibodies having broadly neutralizing activities. We suggest that immunization with CD4-gp120 complexes may be worth evaluating further for the development of an AIDS vaccine.

It has become increasingly clear that most of the neutralizing activity in the sera of human immunodeficiency virus type 1 (HIV-1)-infected individuals is due to antibodies directed against gp120 (3, 21, 35, 36, 39, 47). Thus, the utility of recombinant gp120 as an HIV-1 vaccine has been studied extensively (5, 6, 11, 14). Several neutralizing domains on gp120 have been characterized. One of these domains has been designated the third hypervariable region (V3). Most of the antibodies elicited against this region, by either vaccination or natural infection, possess strain- or type-specific neutralizing activity (13, 22, 28, 30, 38, 45), although V3 monoclonal antibodies (MAbs) with relatively broadly neutralizing activity for laboratory-adapted HIV-1 isolates have been described (12, 18, 37, 48). Another neutralizing domain resides around the CD4 binding site (CD4 site epitope) of gp120. The epitopes in this region are conformationally dependent and are discontinuous (42, 44). Several reports indicate that antibodies directed to the CD4 binding site can exhibit broadly neutralizing activity against laboratory-adapted HIV-1 strains (8, 17, 21, 34, 40, 46), and they are common in HIV-1-positive sera (8, 15, 24, 40). However, functional and mapping analyses suggest that several distinct epitopes overlap the CD4 binding site (8, 17, 42, 44). Furthermore, some MAbs to this region failed to neutralize certain strains of HIV-1 (17, 34, 46). In addition, the first and second hypervariable regions (V1/V2) have been identified as neutralizing domains (16, 26, 41), although the overall contribution of V1/V2-specific antibodies to the neutralizing activity of the sera of HIV-infected individuals needs to be evaluated (26). Most recently, the existence of a broadly neutralizing gp120 epitope which is better exposed after the attachment of CD4 has been demonstrated (20, 43).

One fundamental problem in developing an AIDS vaccine is antigenic variation of HIV. Despite a substantial induced immune response in gp120-immunized monkeys and humans, high titers of V3-directed type-specific neutralizing antibodies may not be sufficient to neutralize continuously emerging new isolates. Several studies analyzing anti-gp120 antibodies in HIV-infected individuals have clearly indicated that most broadly neutralizing antibodies are directed to conformation-dependent epitopes (15, 24, 40, 46). Therefore, it seems important to evaluate the potential efficacy of candidate gp120

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vaccines at inducing such antibodies that might be potentially protective against multiple HIV strains.

One concern in the development of any recombinant protein as a vaccine is its stability when mixed with an adjuvant. This could be a particularly important factor for recombinant gp120, given the conformational nature of its major, broadly neutralizing epitopes (15, 17, 21, 24, 40, 42). We hypothesized that gp120 complexed with recombinant CD4 could stabilize the conformation-dependent epitopes and effectively deliver these epitopes to the immune system. In this study, a soluble CD4-gp120 complex in Syntex adjuvant formulation was tested with mice to analyze the anti-gp120 antibody response. With the aim of defining the fine specificity and neutralizing activities of the immune response, 17 MAbs were generated and characterized. The studies indicate that the CD4-gp120 complex elicits neutralizing anti-gp120 antibodies, most of which are directed to the conformation-dependent epitopes.

MATERIALS AND METHODS

Recombinant proteins. Recombinant $gp120_{IIIB}$, a glycoprotein of HIV_{IIIB} HxB2 clone origin, is secreted from a *Drosophila melanogaster* cell line and was obtained from Smith-Kline Beecham, King of Prussia, Pa. Recombinant gp120 from the BH10 clone of HIV_{IIIB} is secreted from CHO cells and was obtained from Celltech, Ltd., via the United Kingdom Medical Research Council AIDS Directed Programme reagent repository (25). Recombinant $gp120_{SF2}$, an envelope glycoprotein of HIV_{SF2} origin, secreted from CHO cells, was obtained from Chiron, Emeryville, Calif. (14). The quality of recombinant gp120 molecules was assessed by a radioimmunoassay, which revealed high-affinity binding of both types of gp120 to the CD4 molecules on $SupT_1$ cells. Recombinant soluble CD4 (sCD4) was prepared by transfection of a truncated CD4 gene into CHO cells. The sCD4 secreted into the culture fluid of CHO cells (representing 95% of the amino-terminal extracellular portion of the molecule) was purified on an anti-CD4 antibody (1F3)-conjugated Sepharose column (31).

Immunization of mice and the generation of anti-gp120 MAb. BALB/c mice were immunized with recombinant $gp120_{HxB2}$ complexed to sCD4. Each injection was prepared by incubating 10 μg of $gp120_{HxB2}$ with 50 μg of sCD4 for 2 h at room temperature and then mixing the gp120-sCD4 complex with Syntex adjuvant formulation containing 25 μg of *N*-acetyl muramyl-L-alanyl-D-isoglutamine (Syntex, Palo Alto, Calif.). Mice were initially immunized subcutaneously and then were given two intraperitoneal booster injections without *N*-acetyl muramyl-L-alanyl-D-isoglutamine at biweekly intervals. Three days after the last booster injection, the splenocytes were fused with SP-2/0 cells and the resulting hybrids were selected in hypoxanthine-aminopterin-thymidine medium. The culture fluids were tested as described below, and the positive clones were subcloned at least twice. Anti-gp120 MAbs were affinity purified from ascitic fluid by protein A-Sepharose columns.

Radioimmunoassay. Recombinant $gp120_{SF2}$ and $gp120_{HxB2}$ were iodinated with Bolton-Hunter reagent (NEN, Wilmington, Del.). Immulon II microtiter plates (Dynatech Laboratory, Chantilly, Va.) were coated overnight at 4°C with goat anti-murine antibodies (1 μg per well) in phosphate-buffered saline (PBS). Plates were blocked with PBS containing 10% fetal calf serum, and 100 μl of culture supernatants was added per well. Plates were incubated for 2 h at room temperature and washed before addition of ^{125}I-labeled $gp120_{SF2/HxB2}$ (10^5 cpm/100 μl). After another 2-h incubation, the plates were washed and the bound radioactivity was measured.

To determine the effect of sCD4 on the binding of MAb to gp120, microtiter plates were coated with MAb in PBS for 18 h at 4°C and blocked for 1 h with 1% bovine serum albumin in PBS. After blocking, ^{125}I-labeled $gp120_{SF2}$ or $gp120_{HxB2}$ (100,000 cpm/50 μl per well), preincubated for 1 h with or without sCD4 (2.5 μg/50 μl per well), was added. After a 3-h incubation, the plates were washed and the bound radioactivity was measured.

Binding of MAbs to $gp120_{BH10}$ in enzyme-linked immunosorbent assay (ELISA). Soluble gp120 molecules were captured onto a solid phase via absorbed antibody D7324 to the carboxy-terminal 15 amino acids, as described previously (23, 24, 26). For experiments with native gp120, the buffer was Tris-buffered saline supplemented with 10% fetal calf serum. In some experiments, gp120 was denatured by boiling with 1% sodium dodecyl sulfate (SDS)–50 mM dithiothreitol (24). MAbs were bound to gp120 in TMSS buffer (2% nonfat milk powder–20% sheep serum in Tris-buffered saline), which was supplemented with 0.5% Tween 20 for experiments with denatured gp120. Bound murine MAb was detected with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (Dako Diagnostics). The effect of MAbs on the binding of biotin-labeled sCD4 (approximately 1 μg/ml) to D7324-captured gp120 was determined essentially as described previously (23), but with alkaline phosphatase-labeled streptavidin to detect bound sCD4.

Characterizing MAb epitopes with gp120 mutants. The binding of MAbs to mutant gp120 molecules was assessed with culture supernatants (100 μl) from COS-1 cells transfected 48 h previously with 10 μg of pSVIIIenv plasmid expressing either wild-type or mutant $HIV\text{-}1_{HxBc2}$ envelope glycoproteins (33, 42, 44). These were used in an ELISA format, in which the gp120 molecules were captured onto a solid phase via antibody D7324, essentially as described previously (26, 43). No detergent was used at any stage of the assays, except when testing the low-affinity MAbs 3B, 6B, 12B, and MAG 104, when nonionic detergent was included (26, 43). The calculations of binding ratios and definitions of criteria for assessing the significance of the data are described elsewhere (26).

Neutralization assay. A quantitative neutralization assay with HIV-1 laboratory strains was performed as previously described (29). Monolayers of the CEM-SS target cells were cultured with different HIV strains, in the presence or absence of different MAbs, and the number of synctium-forming units was determined 3 to 5 days later. An equivalent amount of virus was used in the assays to allow direct comparison of the various antibody concentrations tested. The assays were reproducible over a virus-surviving fraction range of 1 to 0.001 within a two- to fourfold difference in the concentration of the antibody required for neutralization ($P < 0.001$).

RESULTS

Generation and characterization of anti-gp120 MAbs. To characterize the different populations of anti-gp120 antibodies elicited by immunization with a $CD4\text{-}gp120_{HxB2}$ complex, murine MAbs were generated. Splenocytes from immunized mice were used to generate hybridoma-secreting anti-gp120 MAbs. To screen for anti-gp120 MAbs, antibodies in hybridoma supernatants were captured onto a microtiter plate coated with goat anti-mouse immunoglobulin antibodies and ^{125}I-labeled $gp120_{HxB2}$ was added. This capture assay was designed to detect antibodies to all possible epitopes on gp120; it is preferable to direct coating of gp120 onto the plastic surface, because some of the conformational epitopes on gp120 may be fully or partially denatured by the latter procedure. From two

TABLE 1. Immunochemical properties of murine MAbs

MAb	Half-maximal binding to $gp120_{BH10}$ in ELISA (μg/ml)	Half-maximal inhibition of sCD4 binding to $gp120_{BH10}$ in ELISA (μg/ml)[a]	Binding activity (cpm) in radioimmunoassay of[b]:			
			$gp120_{SF2}$		$gp120_{HxB2}$	
			Without sCD4	With sCD4	Without sCD4	With sCD4
MAG 45	0.03	>>10	9,450	8,484	21,999	21,793
MAG 49	3.0	ND[c]	231	303	9,313	8,374
MAG 53	3.0	ND	326	405	2,551	3,003
MAG 55	0.25	4.0	2,315	1,023	8,300	2,167
MAG 56	1.0	ND	275	589	5,883	5,834
MAG 72	0.2	4.0	2,262	1,024	7,285	1,527
MAG 86	0.2	2.5	2,776	850	9,605	2,335
MAG 95	0.15	ND	5,365	5,232	25,667	23,527
MAG 96	0.75	10	956	419	1,845	586
MAG 97	1.5	>>10	3,489	3,224	21,440	17,673
MAG 104	5.0	>>10	526	483	2,379	2,443
MAG 109	0.5	ND	310	477	12,504	11,005
MAG 116	0.2	1.5	2,962	922	10,281	2,528
MAG 3B	>10	>>10	795	578	8,491	1,800
MAG 6B	>10	10	574	211	1,897	427
MAG 12B	10	10	665	247	4,949	1,489
MAG 29B	0.75	10	674	813	5,344	1,210

[a] Values represent concentrations of MAb giving half-maximal binding to $gp120_{BH10}$ in an antigen-capture ELISA in the absence of detergent.

[b] Plates were coated with 0.1 ml of 4-μg/ml antibody in PBS. After addition of PBS containing 10% fetal calf serum to block the absorbed layer, 10^5 cpm of ^{125}I-$gp120_{SF2}$ or ^{125}I-$gp120_{HxB2}$ was added to each well in the presence or absence of sCD4 at 10 μg/ml. After 3 h, the plates were washed and the radioactivity was measured. Background values (nonspecific binding) were in the range 100 to 200 cpm. Values greater than 2,000 cpm are judged to represent strong binding; those from 2,000 to 500 cpm represent weak binding.

[c] ND, not done.

separate fusions, 17 anti-gp120 MAbs were selected for further analysis.

The immunochemical properties of the anti-gp120 MAbs were analyzed. First, their binding to recombinant gp120 of two strains, SF2 and IIIB, was determined. The data in Table 1 show that all of the anti-gp120 MAbs bound to $gp120_{IIIB}$, derived either from HxB2 or BH10 clones, in two types of immunoassay. This result was expected, since the MAbs were generated against the CD4-$gp120_{HxB2}$ complex and then selected against $gp120_{HxB2}$. Variations in the relative binding of MAbs to $gp120_{HxB2}$ and $gp120_{BH10}$ may reflect the influence of amino acid differences in the C4 region and other regions of the two gp120 clones (27). Seven of the 17 MAbs (MAG 45, 55, 72, 86, 95, 97, and 116) significantly cross-reacted with $gp120_{SF2}$. Six other MAbs (MAG 96, 104, 3B, 6B, 12B, and 29B) showed weak reactivity against $gp120_{SF2}$, while the remaining four MAbs (MAG 49, 53, 56, and 109) did not bind to $gp120_{SF2}$. Second, the inhibition of binding of the MAbs to $gp120_{HxB2}$ or $gp120_{SF2}$ by sCD4 was investigated. The results in Table 1 show that the binding of nine MAbs (MAG 55, 72, 86, 96, 116, 3B, 6B, 12B, and 29B) to both gp120s was significantly reduced by sCD4, indicating that these MAbs bind to epitopes close to or within the CD4 site of gp120. Twelve of the MAbs were also tested in a separate ELISA (23) for their ability to block biotin-labeled sCD4 (approximately 1 μg/ml) binding to $gp120_{BH10}$. The four MAbs with the highest affinity for $gp120_{BH10}$ (MAG 55, 72, 86, and 116) were the most efficient at inhibiting sCD4 binding to gp120 (Table 1). Finally, we determined whether the MAbs bound preferentially to native or SDS-dithiothreitol-denatured forms of $gp120_{BH10}$ in an antigen-capture ELISA (23, 24, 26). Thirteen of the 17 MAbs were completely unable to bind to denatured gp120, indicating that their epitopes were discontinuous or conformationally sensitive. This group of MAbs was exemplified by MAG 86 (Fig. 1A). However, four of the MAbs (MAG 49, 53, 56, and 109) did bind to denatured gp120, albeit with a significant loss of affinity compared with their binding to native gp120. This group of MAbs is represented by MAG 109 (Fig. 1B).

Definition of the epitopes for MAbs. To characterize the epitopes for the MAbs, we tested MAbs for reactivity with a panel of mutant $gp120_{HxB2}$ molecules (33, 42, 44) by using an ELISA format (26, 43). The purpose of this experiment was to classify the MAbs, so our analysis was qualitative rather than quantitative: we sought to identify amino acid substitutions that completely eliminated MAb binding and those that significantly reduced binding, without rigorously quantitating the extent of the reduction. Our prior studies indicate, however, that those substitutions underlined in Table 2 are likely to cause an approximately 3-fold reduction in affinity, whereas the others listed cause at least a 10-fold reduction. We did not assess which substitutions enhanced MAb reactivities with gp120. These will be the focus of further studies.

Three subclasses of MAbs were identifiable from this analysis. The first class was sensitive to amino acid substitutions at residues 368 and 370; the second and third classes were not. From our previous studies, we are aware that a reduction in binding to gp120 mutants with changes at residues 368 and 370 is characteristic (almost diagnostic) of MAbs that recognize discontinuous epitopes overlapping the CD4 binding site on gp120 (42, 44). This is confirmed here; all nine MAbs sensitive to residue 368 and 370 substitutions showed reduced binding to gp120 in the presence of sCD4 (Table 1). As well as those at residues 368 and 370, other substitutions reducing binding of these MAbs involved amino acids in C2 (256, 257, and 262), C3 (381, 384, and 386), C4 (421), and C5 (470, 475, and 477), although different MAbs were differentially sensitive to changes at these positions. Other MAbs to epitopes overlapping the CD4 binding site on gp120 have been previously reported to be influenced by substitutions at these residues (42, 44). It is not, however, possible to predict subtle differences in the properties of the various MAbs from the amino acid substitutions to which their binding is sensitive.

Four MAbs (MAG 45, 95, 97, and 104) were not sensitive to

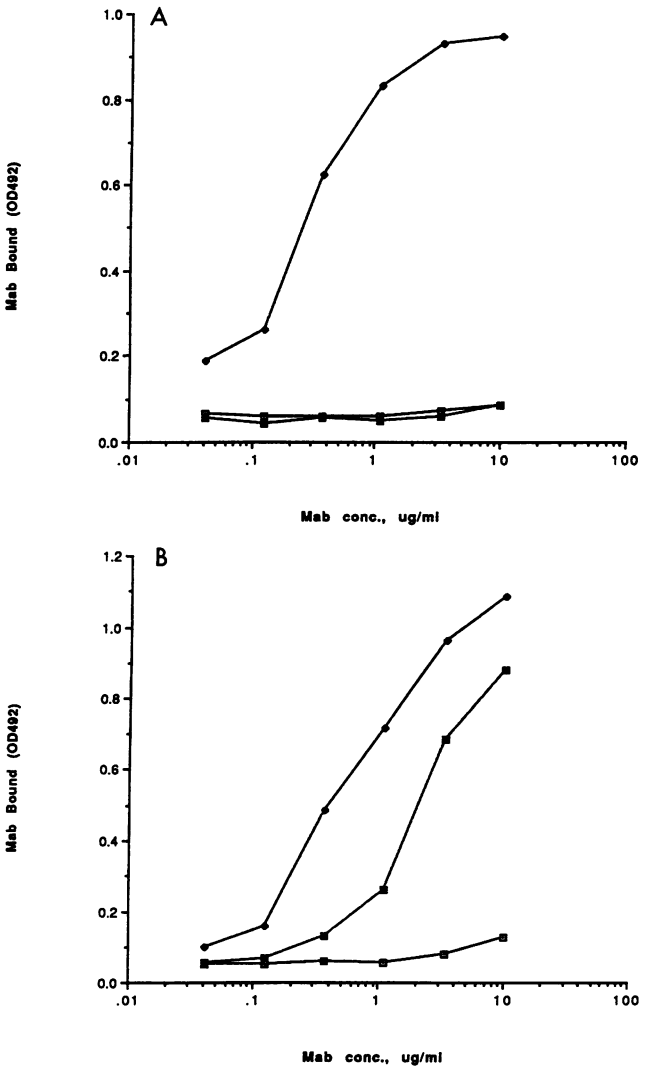

FIG. 1. Binding of MAbs (MAG 86 and MAG 109) to native (◆) and denatured (■) $gp120_{BH10}$. ⊡, no gp120. Recombinant $gp120_{BH10}$ was captured indirectly onto a solid phase with a C-terminal antibody, in the absence of detergent (26) or after denaturation by boiling with SDS and dithiothreitol followed by dilution with 1% Nonidet P-40 nonionic detergent (24). (A) MAG 86. (B) MAG 109. For panel B, MAG 109 was added at the concentration indicated, and bound MAb was detected. Background binding was measured in the absence of added gp120 and the presence of 1% Nonidet P-40. OD492, optical density at 492 nm.

substitutions at residues 368 and 370 (Table 2), and it is notable that the binding of these four MAbs to gp120 was not reduced significantly by sCD4 (Table 1). The only substitution that consistently, but usually weakly, affected the binding of these four MAbs was at residue 88: Asn→Pro. None of these MAbs was able to bind C1-derived 20-mer peptides, however (data not shown). Thus, we tentatively identify the epitope for these MAbs as being a conformationally sensitive one involving the amino-terminal C1 domain of gp120. This would be consistent with the inability of these MAbs to neutralize HIV-1 infectivity, because other MAbs to C1 epitopes are similarly nonneutralizing (9, 28, 32).

Finally, we screened the four MAbs that were significantly reactive with denatured gp120 (MAG 49, 53, 56, and 109) against a set of 20-mer peptides spanning HIV-1 gp120 by using a solid-phase assay (26). All four MAbs (3 to 10 μg/ml) reacted with V3 peptide 302-321 and only with that peptide out of the entire set. The optical density at 492 nm values were as follows: MAG 49, 1.660; MAG 53, 1.779; MAG 56, 1.795; and MAG 109, 1.788 (background, <0.100). We confirmed that the V3 loop was involved in the epitope for these MAbs by testing them against the gp120 mutants. Removal of the V3 loop completely abolished binding of each of the four MAbs to gp120, as did a five-amino-acid insertion (308-310 RIQ/RPE-LIPVQ) in the N-terminal flank and a single-amino-acid substitution at the crown of V3 (313 P/S, 314 G/W). An additional substitution (120/121 VK/LE) in the base of the V1/V2 loop structure also destroyed binding of all four MAbs.

Neutralizing activity of anti-gp120 MAbs. The ability of each anti-gp120 MAb to neutralize three different laboratory strains of HIV-1 was determined (Table 3). In general, the MAbs specific for the CD4 site epitopes exhibited a broad spectrum of neutralizing activities against laboratory-adapted strains of HIV-1 (MN, IIIB, and RF). Four of these MAbs (MAG 55, 72, 86, and 116) neutralized all three strains tested, although their potencies against different strains varied. Three MAbs (MAG 96, 12B, and 29B) weakly neutralized HIV_{IIIB} only. The other two MAbs (MAG 3B and 6B) did not show any neutralizing activity; these two MAbs had the lowest affinity for $gp120_{BH10}$ (Table 1). It was notable that, although MAG 3B bound strongly to the CD4 site of $gp120_{HxB2}$ (Table 1), it did not neutralize HIV_{IIIB}. This may reflect sequence variation within different clones of HIV_{IIIB} that can affect the binding of CD4 site MAbs (27); MAG 3B bound very weakly to $gp120_{BH10}$ compared with $gp120_{HxB2}$ (Table 1). The clonal composition of the IIIB stock used in our neutralization experiments is not known.

The four MAbs which were directed to a conformational C1 epitope were unable to neutralize any of the three strains tested (Table 3). However, all four V3-specific MAbs exhibited neutralizing activity. MAG 49 weakly neutralized MN, IIIB, and RF strains; MAG 109 neutralized MN and IIIB strains; and MAG 53 and 56 neutralized only the MN strain. It was unexpected that the neutralizing activities of all V3-specific MAbs against HIV_{MN} were more potent than those against HIV_{IIIB}, although these were generated against $gp120_{IIIB}$.

DISCUSSION

This study was conducted to analyze the humoral immune response of mice to a soluble CD4-gp120 complex in Syntex adjuvant formulation. To define the fine specificity and neutralizing activities of the response, 17 MAbs were generated and characterized. The data indicated that the 17 MAbs fell into three groups. Four of them were directed to a conformational epitope, probably centered on the C1 domain, and did not exhibit neutralizing activity. Another four MAbs were specific for the V3 region and exhibited cross-reactive, but weak, neutralizing activity. The last group of nine MAbs recognized conformation-dependent epitopes near the CD4 binding site. Four of these nine MAbs showed broadly neutralizing activities against multiple strains of HIV-1, whereas three of them neutralized only HIV_{IIIB}, and two were not neutralizing for any strains tested.

Early studies showed that gp120 subunit vaccination mainly induced neutralizing antibodies against only the specific isolate from which the immunizing antigens were derived (or closely related isolates). However, recent studies have indicated that recombinant gp120 immunization in adjuvants could elicit broadly neutralizing antibodies in baboons, rabbits, and guinea

TABLE 2. Effect of amino acid substitutions on binding of MAbs to recombinant $gp120_{HxB2}$

MAb	Mutant(s) with substantially impaired binding[a]
CD4 binding site MAbs	
MAG 55	256 S/Y, 257 T/R, 257 T/A, 257 T/G, 368 D/R, 368 D/T, 370 E/R, 370 E/Q, 384 Y/E, 421 K/L, 470 P/L, 475 M/S, 477 D/V[b]
MAG 72	257 T/R, 257 T/A, 257 T/G, 262 N/T, 368 D/R, 368 D/T, 370 E/R, 370 E/Q, 384 Y/E, 421 K/L, 429 K/L, 477 D/V
MAG 86	256 S/Y, 257 T/R, 368 D/R, 368 D/T, 370 E/R, 370 E/Q, 384 Y/E, 421 K/L, 470 P/L, 477 D/V
MAG 96	256 S/Y, 257 T/R, 257 T/G, 262 N/T, 368 D/R, 368 D/T, 370 E/R, 475 M/S, 477 D/V
MAG 116	256 S/Y, 257 T/R, 257 T/A, 368 D/R, 368 D/T, 370 E/R, 370 E/Q, 384 Y/E, 386 N/Q, 421 K/L, 470 P/G, 477 D/V
MAG 3B	106 E/A, 113 D/R, 256 S/Y, 257 T/R, 257 T/A, 257 T/G, 262 N/T, 368 D/R, 368 D/T, 370 E/R, 370 E/Q, 381 E/P, 384 Y/E, 421 K/L, 470 P/G, 475 M/S, 477 D/V
MAG 12B	257 T/R, 257 T/G, 262 N/T, 314 G/W, 368 D/R, 368 D/T, 370 E/R, 370 E/Q, 384 Y/E, 386 N/Q, 421 K/L, 477 D/V
MAG 29B	102 E/L, 257 T/R, 257 T/G, 314 G/W, 356 N/I, 368 D/R, 368 D/T, 370 E/R, 370 E/Q, 384 Y/E, 386 N/Q, 421 K/L, 470 P/G, 477 D/V
MAG 6B	256 S/Y, 257 T/R, 257 T/G, 257 T/A, 262 N/T, 314 G/W, 368 D/R, 368 D/T, 370 E/R, 370 E/Q, 381 E/P, 384 Y/E, 421 K/L, 470 P/L, 470 P/G, 475 M/S, 477 D/V
NH_2-terminal MAbs	
MAG 45	88 N/P
MAG 95	88 N/P
MAG 97	88 N/P
MAG 104	88 N/P, 106 E/A
V3-specific MAbs	
MAG 49	120/121 VK/LE, 313 P/S, 314 G/W, 308-310 RIQ/RPELIPVQ, ΔV3, ΔV1/2/3, 477 D/V
MAG 53	120/121 VK/LE, 313 P/S, 314 G/W, 308-310 RIQ/RPELIPVQ, ΔV3, ΔV1/2/3
MAG 56	120/121 VK/LE, 313 P/S, 314 G/W, 308-310 RIQ/RPELIPVQ, ΔV3, ΔV1/2/3
MAG 109	120/121 VK/LE, 313 P/S, 314 G/W, 308-310 RIQ/RPELIPVQ, ΔV3, ΔV1/2/3

[a] Mutants underlined cause an approximate 3-fold reduction in the affinity for binding to MAG MAbs, whereas the others cause at least a 10-fold reduction in affinity for binding.

[b] MAG 56 and MAG 109 were not tested against the 477D/V mutant.

pigs, although the neutralizing titers were relatively low (5, 6, 14). This phenomenon was also observed in the clinical vaccine trials with the same recombinant gp120s (2, 19). However, whether the titer of broadly neutralizing antibodies is sufficient to prevent heterologous virus infection is still in question. Several studies analyzing anti-gp120 antibodies in HIV-infected asymptomatic individuals have clearly indicated the existence of high titers of broadly neutralizing anti-gp120 antibodies in the sera (15, 24, 35, 47). Most of these are directed to conformation-dependent epitopes (15, 24, 39). Therefore, an important question should be answered—what causes the discrepancy between HIV infections and recombinant gp120 vaccinations in inducing anti-gp120 antibody responses? Since most broadly neutralizing antibodies are known to be directed to conformation-dependent epitopes, we speculated that the deficiency in eliciting broadly neutralizing antibodies by immunization with recombinant gp120 antibodies may be due to an unstable conformation of recombinant gp120 when mixed with an adjuvant or may be due to a structural instability when the molecule is delivered to the immune system. We reasoned that CD4 could complex with recombinant gp120 and stabilize the native structure of the gp120 monomer. Subsequently, the complex may dissociate to a certain extent as it interacts with the cells of the immune system. Thus, all epitopes in gp120, including epitopes near the CD4 binding site of gp120, could be recognized by antigen-presenting cells. Our results strongly support these assumptions. First, 13 (possibly all 17) of the 17 MAbs elicited by the complex were directed against conformation-sensitive epitopes, indicating that the complex as an immunogen successfully preserves the conformational structure of gp120.

TABLE 3. Neutralizing activity of gp120-specific murine MAbs

MAb	Virus neutralization in[a]:		
	HIV_{MN}	HIV_{IIIB}	HIV_{RF}
CD4 binding site MAbs			
MAG 55	+	+++	+
MAG 72	+	+++	++
MAG 86	+++	+++	++
MAG 96	−	++	−
MAG 116	+	+++	++
MAG 3B	−	−	−
MAG 12B	−	+	−
MAG 29B	−	++	−
MAG 6B	−	−	−
NH_2-terminal MAbs			
MAG 45	−	−	−
MAG 95	−	−	−
MAG 97	−	−	−
MAG 104	−	−	−
V3-specific MAbs			
MAG 49	++	+	+
MAG 53	+	−	−
MAG 56	+++	−	−
MAG 109	+++	+	−

[a] +++, 10 μg/ml > 50% inhibitory concentration (IC_{50}); ++, 10 μg/ml < IC_{50} < 50 μg/ml; +, 50 μg/ml < IC_{50} < 200 μg/ml; −, no detectable neutralizing activity below 200 μg/ml.

TABLE 4. Immunobiological properties of CD4 binding site MAbs

MAb	Affinity (μg/ml) to monomeric gp120[a]	Virus neutralization[b]			Knockout mutations at gp120 constant region[c]:				
		IIIB	MN	RF	C1	C2	C3	C4	C5
MAG 86	0.2	+++	+++	++		256 257	368 370 384	421	470 477
MAG 72	0.2	+++	+	++		257 262	368 370 384	421	477
MAG 116	0.2	+++	+	++		256 257 256	368 370 384	421	470
MAG 55	0.25	+++	+	+		257	368 370 384	421	475 477
MAG 96	0.75	++	−	−		256 257	368 370		
MAG 29B	0.75	++	−	−		257 256	368 370 384 386 368	421 421	475
MAG 3B	>10	−	−	−		257 262 262	370 381 384		477
MAG 6B	>10	−	−	−		256 257 262	368 370 381 384	421	470 475 477

[a] Concentration of antibody at which half-maximal binding to $gp120_{BH10}$ in ELISA was observed.
[b] Quantitative neutralization assays of HIV were performed as described in the text, and the results were expressed as follows: +++, 10 μg/ml > 50% inhibitory concentration (IC_{50}); ++, 10 μg/ml < IC_{50} < 50 μg/ml; +, 50 μg/ml < IC_{50} < 200 μg/ml; −, no detectable neutralizing activity below 200 μg/ml.
[c] Mutation at different constant regions (C1, C2, C3, C4, and C5) of gp120.

Second, 9 of the 17 MAbs recognized epitopes near the CD4 binding site. This suggests that free gp120 dissociated from the complex could expose the CD4 binding site epitopes.

Comparison of the immune responses induced by CD4-gp120 with the natural immune response elicited after HIV-1 infection indicated a major similarity and some differences. The similarity was that, in both cases, a significant proportion of the anti-gp120 antibodies induced were directed against the CD4 binding site and were broadly neutralizing. This is encouraging, since we believe these kind of antibodies may be important in protection from HIV infection. The immunobiological properties of CD4 binding site antibodies are summarized in Table 4. We observed a good correlation between the affinity and the neutralization by CD4 binding site antibodies specific to different constant regions. However, differences were still observed in the CD4 binding site antibodies. Most of the CD4 binding site MAbs elicited by the complex preferentially neutralized IIIB strains, whereas human CD4 binding site antibodies showed comparable neutralization of different strains (8, 15, 17, 21, 46). Some of the CD4 binding site MAbs elicited by the complex exhibited low-affinity binding and low or no neutralization potency. We speculate that these differences could be due to two reasons. First, natural HIV infection with multiple HIV isolates could present various gp120s to the immune system and skew the immune response toward a few epitopes common among the various gp120s. Thus, antibodies induced by the natural infection could interact equally well with multiple isolates compared with those antibodies elicited by immunization with a particular type of gp120. The strong similarities in gp120 amino acids critical for antibody binding between the MAbs elicited here and those derived from HIV-1-infected humans would appear to argue against this explanation. Second, natural infection could be more persistent in presenting antigens to the immune system to drive the affinity maturation of antibodies than conventional immunization. Furthermore, we also observed a difference in the V3-specific antibodies. The majority of V3-specific antibodies elicited by natural infection exhibit highly potent type-specific

neutralizing activities (21). In contrast, the MAbs elicited by our complex were more cross-reactive but less potent at neutralization. We interpret this as follows: bound CD4 molecules on gp120 may influence the V3 region structure and preferentially expose certain epitopes not presented efficiently on polymeric forms of gp120. It was surprising that we did not find any antibodies specific for an epitope which is better exposed after the attachment of CD4 to gp120 (10, 22, 43). This might occur for two reasons. First, these antibodies bind relatively weakly with free gp120 and we did not include sCD4 in our screening assay; rescreening against CD4-gp120 complex might enable those antibodies to be found. Alternatively, the epitope may be much less immunogenic than others. Thus, antibodies specific for this epitope may be induced with much lower frequency.

It was observed that the binding of each of the four V3-specific MAbs (MAG 49, 53, 56, and 109) to gp120 was destroyed by the substitution 120/121 VK→LE in the base of the V1/V2 loop structure. We believe that this substitution has an indirect effect on the presentation of a complex, conformational epitope located within the V3 loop. The observation that a deletion of the V1/V2 loop structure, which eliminates residues 120 and 121, did not affect the binding of the V3-specific MAbs supports this interpretation. It should be noted that the 120/121 VK/LE substitution is not generally disruptive of gp120 conformation; it does not, for example, affect the epitopes for any of the other 13 MAbs tested (Table 2), the binding site for CD4 (33), or the epitope for any other CD4 binding site MAb we have mapped previously (42, 44). The 120/121 VK/LE substitution does, however, abolish the binding of the murine V2 MAb CRA-3 (ADP 324) (26) and that of the human MAb 48d, which recognizes a CD4-enhanced epitope (43). These MAbs are not, however, sensitive to changes in V3. The 120/121 VK/LE substitution also abolishes the binding of two other MAbs (1, 4) that are, like our four V3-specific MAbs, sensitive to substitutions in V3 (unpublished data). Thus, the epitopes for a certain category of conformationally sensitive V3 MAbs are affected by amino acid changes at the base of the V1/V2 stem. This may be related mechanistically to the linkage between these domains that can be inferred from studies by Willey et al. (49, 50).

The neutralizing activities of all V3-specific MAbs were more potent against MN than against IIIB. One possible explanation is that the MAbs recognize preferentially the clone of $gp120_{IIIB}$, $gp120_{HxB2}$, used as immunogen. This clone may be poorly represented in our uncloned neutralization stock. A single-amino-acid polymorphism at residue 306 in the V3 loop of HIV_{IIIB} can have dramatic effects on the binding of MAbs to gp120 from different clones (27). An alternative explanation is that the MAbs recognize a conformational epitope involving the V3 loop that is better presented on $gp120_{MN}$ than on $gp120_{IIIB}$; the MAbs may be heteroclitic.

The CD4-gp120 immunization could induce not only anti-gp120 antibodies but also anti-CD4 antibodies in mice, as described by others (7). Since anti-CD4 antibodies exhibit potent neutralizing activities against HIV, it was expected to be difficult to compare the immune response of the complex with that of gp120 alone by utilizing whole immune sera, especially to assess the neutralizing activities and the fine specificity. Instead, we have generated and characterized anti-gp120 MAbs to evaluate the immune response of the complex. Interpretation of our results depends on the assumption that the MAbs studied here are representative of the diverse population of anti-gp120 antibodies induced by the complex. We believe CD4 molecules in the complex may not be problematic for the development of a human vaccine, since the molecules should be tolerogenic in humans. Therefore, we suggest that immunization with CD4-gp120 complex may be worth evaluating further for the development of an AIDS vaccine.

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