수용성 CD-gp120 결합체의 면역화로 유도된 항 gp120 항체의 특성에 관한 연구

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Immunization with a soluble CD4-gp120 complex preferentially induces neutralizing anti-Human Immunodeficiency Virus Type 1antibodies directed to conformation-dependent epitopes of gp120

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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. Therefore, it seems important to evaluate the potential efficacy of candidate gp120 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. 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 gp120-CD4 complex in Syntex Adjuvant Formulation was tested in mice to analyze the anti-gp120 antibody response. With the aim of defining the fine specificity and neutalizing activities of the immune response, 17 Mabs were generated and characterized. The studies indicate that the gp120-CD4 complex elicits neutralizing anti-gp120 antibodies, most of which are directed to the conformation-dependent epitopes.

Generation and Characterizations of Monoclonal Anti-gp120 Antibodies.

To characterize the different populations of anti-gp120 antibodies elicited by immunization with a CD4-$gp120_{HxB2}$ complex, murine monoclonal antibodies 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 Ig antibodies; then, ^{125}I labeled $gp120_{HxB2}$ was added. This capture assay was designed to detect antibodies to all possible epitopes on gp120. It is preferential to an assay in which gp120 was coated directly onto the plastic surface, since some of the conformational epitopes on gp120 may be fully or partially denatured by the latter procedure. From two fusions, seventeen anti-gp120 monoclonal antibodies 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 shows that all the anti-gp120 Mabs bound to gp120 IIIB (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 HxB2 and BH10 gp120 probably reflect the influence of amino acid differences in the C4 regions of the two gp120 clones. Seven of the 17 Mabs (MAG 45, 55, 72, 86, 95, 97 and 116) strongly 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 $gp120_{SF2}$. Secondly, 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. Finally, we determined whether the Mabs bound preferentially to native or SDS/DTT denatured forms of BH10 gp120 in an antigen captive ELISA. 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 (data not shown). However, four of the Mabs (MAG 49, 53 and 109) did bind to denatured gp120, albeit with a significant loss of affinity compared to their binding to native gp120. This group of Mabs is represented by MAG 109 (data not shown).

Definition of the Epitopes for Mabs.

To characterize the epitopes for the Mabs, we tested the 13 conformationally-sensitive Mabs for reactivity with a panel of mutant HxB2 gp120 molecules using an ELISA format. No detergent was used in the assay, except when testing the low affinity Mabs 3B, 6B, 12B and MAG 104, where non-ionic detergent was included. Amino acid substitutions eliminating Mab binding, or reducing binding to very low levels, are listed in Table 2. For most Mabs we did not assess rigorously whether other substitutions had minor inhibitory or enhancing effects on Mab binding. Two subclasses of Mabs were identifiable from this analysis; the first class was sensitive to amino acid substitutions at residues 368 and 370; the second class was 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. This is confirmed here; all 9 Mabs sensitive to 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, 262), in C3 (381, 384, 386), in C4 (421) and in C5 (470, 475, 477), although different Mabs were differentially sensitive to

changes at these positions. Other Mabs to epitopes overlapping CD4 binding site on gp120 have been previously reported to be influenced by substitutions at these same residues. 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 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: 88 Asn Æ 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, as other Mabs to C1 epitopes are similarly non-neutralizing. Finally, we screened the four Mabs that were

Table 1. Immunochemical properties of Murine Mabs to gp120s

Mab	Half-Maximal Binding to BH10 gp120 in ELISA (µg/ml)	Binding Activity (cpm)* in RIA			
		$gp120_{SF2}$		$gp120_{IIIB}$	
		without sCD4	with sCD4	without sCD4	with sCD4
MAG 45	0.03	9450	8484	21999	21793
MAG 49	3.0	231	303	9313	8374
MAG 53	3.0	326	405	2551	3003
MAG 55	0.25	2315	1023	8300	2167
MAG 56	1.0	275	589	5883	5834
MAG 72	0.2	2262	1024	7285	1527
MAG 86	0.2	2776	850	9605	2335
MAG 95	0.15	5365	5232	25667	23527
MAG 96	0.75	956	419	1845	586
MAG 97	1.5	3489	3224	21440	17673
MAG 104	5.0	526	483	2379	2443
MAG 109	0.5	310	477	12504	11005
MAG 116	0.2	2962	922	10281	2528
MAG 3B	> 10	795	578	8491	1800
MAG 6B	> 10	574	211	1897	427
MAG 12B	10	665	247	4949	1489
MAG 29B	0.75	674	313	5344	1210

* Plates were coated with 0.1 µg/well (4 ug/ml) of antibody in PBS. After addition of PBS containing 10% FCS to block the absorbed layer, 10^5 cpm of ^{125}I-$gp120_{SF2}$ or ^{125}I-$gp120_{IIIB}$ were added to each well in the presence or absence of sCD4 at 10 ug/ml. After 3 hours, the plates were washed and the radioactivity was measured. Background values (non-specific 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 CPM to 500 CPM, represent weak binding. Values in column 2 represent concentrations of Mab giving half maximal binding to BH10 gp120 in an antigen-captive ELISA in the absence of detergent.

significanly reactive with denatured gp120 (MAG 49, 53, 56, and 109) against a set of 20-mer peptides spanning HIV-1 gp120, using a solid phase assay. All four Mabs (3 to 10μg/ml) reacted with V3-peptide 302-321, and only with that single peptide out of the entire set. The OD_{492} values were: MAG 49 1.660; MAG 53 1.779; MAG 56 1.795; MAG 109 1.788 (background = <0.100). We confirmed that the V3 loop was involved in the epitope for two of the Mabs by testing these against the gp120 mutants (MAG 56 and MAG 109 were not tested). Removal of the V3 loop completely abolished binding of MAG 49 and MAG 53 to gp120, as did two single amino acid substitutions at the crown of V3 (313 P/S, 314 G/W) and a 5 amino acid insertion (308-310 RIQ/RIPLIPVQ) in the N-terminal flank. An additional substitution (120/121 VK/LE) in C1 at the base of the V1/V2 loop structure also destroyed binding of MAG 49 and MAG 53 to gp120. We believe that the effect of this substitution is an indirect one on the presentation of a complex, conformational epitope located within the V3 loop. It should be noted that the 120/121 VK/LE substitution is not generally disruptive to gp120 conformation; it does not, for example, affect the epitopes for any of the other 13 Mabs tested (Table 2) at the binding site for CD4 or the epitope for any other CD4 binding site Mab we have mapped previously. The 120/121 VK/LE substitution does, however, abolish the binding of the V2 Mab CRA-3 (ADP 324) and of the Mab 48d, which recognizes a CD4-enhanced epitope. These Mabs are not, however, sensitive to changes in V3. The 120/121 VK/LE substitution also abolishes the binding of two other Mabs that are, like MAG 49 and MAG 53, sensitive to substitutions in V3 (unpublished data). Thus, the epitopes for a certain category of conformationally sensitive V3 Mabs is affected by amino acid changes in the C1 region. This may be related mechanistically to the linkage between these domains that can be inferred from studies by others.

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. 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, 12 B 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 $gp120_{BH10}$ (Table1) It was notable that although MAG3B 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. Thus, it is notable that MAG 3B bound very weakly to $gp120_{BH10}$ compared to $gp120_{HxB2}$ (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. However, all four V3-specific Mabs exhibited neutralizing activity. MAG 49 weakly neutralized MN, IIIB and RF strains; MAG 116 neutralized MN and IIIB strains; MAG 53 and 56 only neutralized 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 $gp120_{IIIB}$. One possible explanation is that the Mabs recognize preferentially the clone of IIIB gp120, HxB2, used as immunogen. This clone weight is 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. It is notable that MAG 49 has by far the highest affinity for BH10 gp120 of the four V3 Mabs, but gives the second highest extent of binding to HxB2 gp120 (Table 1). This would be consistent with the identity of residue 306 influencing in some way its epitope. An alternative explanation is that the Mabs recognize a conformational epitope involving the V3 loop that is better presented on MN gp120 than on IIIB; the Mabs may be heteroclitic.

Conclusion and Discussion

This study was conducted to analyze the immune response of soluble gp120-CD4 complex in SAF in mice. To define the fine specificity and neutralizing activities of the immune responses, seventeen Mabs were generated and characterized. The data indicated that the seventeen Mabs fell into three groups. Four of them were directed to a conformational epitope and did not exhibit neutralizing activity. Another four Mabs were specific for the V3 region and exhibited strain restricted neutralizing activity. The two of these were very sensitive to amino acid substitutions in the V3 region and C1 region at the base of 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. Four of these nine Mabs showed broadly neutralizing activities against multiple strains of HIV-1. Three of them only neutralized HIV_{IIIB}. Early studies showed that gp120 subunit vaccination mainly induced neutralizing antibodies against only the specific isolate or closely related isolates, from which the immunizing antigens originate . However, recent studies have indicated that recombinant gp120 immunization in adjuvants could elicit not only isolate restricted but also broadly neutralizing antibodies in baboons, rabbits and guinea pigs, although the broadly neutralizing titer was relatively low . This phenomenon was also observed in the clinical vaccine trials with the same recombinant gp120s. Thus, whether the titer of broadly neutralizing antibodies is sufficient to prevent heterologous virus infection is still

Table 2. Effect of Amino Acid Substitutions on Binding of Mabs to Recombinant HxB2 gp120

Mab	Mutant with Substantially Impaired Binding
(A) "CD4 Binding Site" Mabs	
MAG 55	102 E/L, 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, 475 M/S, 477 D/V
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, 314 G/W, 368 D/R, 368 D/T, 370 E/R, 370 E/Q, 384 Y/E, 421 K/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 D/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
(B) 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
(C) V3 - Mabs	
MAG 49	120/121 VK/LE, 313 P/S, 314 G/W, 308-310 ....., Δ V3, Δ V1/2/3, 477 D/V
MAG 53	120/121 VK/LE, 313 P/S, 314 G/W, 308-310 ....., Δ V3, Δ V1/2/3
MAG 56	Not Tested
MAG 109	Not Tested

N.B. Mutants underlined show only partial reduction in Mab binding.

in question . In contrast, several studies analyzing anti-gp120 antibodies in HIV infected assymptomatic individuals have clearly indicated the existence of high titers of broadly neutralizing anti-gp120 antibodies in the sera . Therefore, an important question should be answered - what causes the difference 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 dificiency in eliciting broadly neutralizing antibodies by immunization with recombinant gp120 antibodies may be due to the unstable conformation of recombinant gp120 when mixed with an adjuvant or due to the structural unstability when delivered into the immune system. In any case, we reasoned that CD4 could complex with rgp120 and stabilize the gp120 conformational structure. The complexed CD4-gp120 may preserve overall conformational structure until delivered to the immune system. This complex however may be dissociated in a certain degree when it interact with cells in 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 result strongly support these assumptions. First, thirteen of the seventeen Mabs elicited by the complex were directed against conformation-sensitive epitopes, indicating that the complex as a immunogen sussesfully preserve the gp120 conformational strcture. Secondly, nine of the seventeen Mabs recognized epitopes near the CD4 binding site. This suggest that free gp120 dissociated from the complex could expose the CD4 binding site epitopes. Comparing the immune responses induced by the complex with the immune responses followed by HIV-1 infection, there are similarity and difference. A similar phenomenon was that both cases induced substantial amount of CD4 binding site specific, broadly neutralizing antibodies. This is encouriging since we beleive this kind of antibodies are most important in protecting HIV infection. However, a difference was observed in the V3 specific antibodies. Most V3 specific antibodies elicited by natural infection exhibit highly potent ,however, type-specific neutralizing activities. In contrast, the Mabs elicited by the complex exhibit more cross reactive, however , less potent neutralizing activity. We simply interpretate that bound CD4 molecule on

Table 3. Neutralizing Activity of gp120 Specific Murine Mabs

Mab	Virus Neutralization in		
	HIV_{MN}	HIV_{IIIB}	HIV_{RF}
A) "CD4 Binding Site" Mabs			
MAG 55	+	+++	+
MAG 72	+	+++	++
MAG 86	+++	+++	++
MAG 96	-	++	-
MAG 116	+	+++	++
MAG 3B	-	-	-
MAG 12B	-	+	-
MAG 29B	-	++	-
MAG 6B	-	-	-
(B) NH_2 - Terminal Mabs			
MAG 45	-	-	-
MAG 95	-	-	-
MAG 97	-	-	-
MAG 104	-	-	-
(C) V3 - Mabs			
MAG 49	++	+	+
MAG 53	+	-	-
MAG 56	+++	-	-
MAG 109	+++	+	-

*+++, 10 ug/ml >50% inhibiting concentrations (IC_{50}); ++, 10ug/ml <IC_{50}<50ug/ml; +, 50ug/ml<IC_{50}<200ug/ml; -, no detectable neutralizing activity below 200ug/ml.

gp120 may influence the V3 region structure and preferentially expose certain epitopes over naturally existing epitopes on polymeric form of gp120. Since there are no descriptions on the existence of antibodies directed to conformational C1 epitope in the sera of HIV-1 infected individuals, the induction of MAG 45, 95, 97, 104 by the complex may be related to monomeric form of the rgp120. The CD4-gp120 immunization could induce not only anti-gp120 antibodies but also induce anti-CD4 antibodies in mice as described by others. However, we beleive CD4 molecules in the complex may be not problematic for the development of human vaccine since the molecules should be tolerogenic in human. Therefore, we suggest that immunization of CD4-gp120 complex may be worth evaluating in monkeys and human.