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Antibody Cross-Competition Analysis of the Human Immunodeficiency Virus Type 1 gp120 Exterior Envelope Glycoprotein

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Forty-six monoclonal antibodies (MAbs) able to bind to the native, monomeric gp120 glycoprotein of the human immunodeficiency virus type 1 (HIV-1) LAI (HXBc2) strain were used to generate a competition matrix. The data suggest the existence of two faces of the gp120 glycoprotein. The binding sites for the viral receptor, CD4, and neutralizing MAbs appear to cluster on one face, which is presumably exposed on the assembled, oligomeric envelope glycoprotein complex. A second gp120 face, which is presumably inaccessible on the envelope glycoprotein complex, contains a number of epitopes for nonneutralizing antibodies. This analysis should be useful for understanding both the interaction of antibodies with the HIV-1 gp120 glycoprotein and neutralization of HIV-1.

The surface glycoprotein gp120 of human immunodeficiency virus type 1 (HIV-1) is the major target for virus-neutralizing antibodies (46). The mature gp120 glycoprotein comprises approximately 480 amino acids, about 30 of which are asparagine residues with N-linked carbohydrate chains that, in toto, account for nearly 50% of the molecular weight of the protein (21). O-linked carbohydrates are also present on several, undefined serine or threonine residues (2, 12). Analyses of gp120 sequences from different HIV-1 strains allowed the division of the protein into five conserved (C1 to C5) and five variable (V1 to V5) domains (24, 45). Biochemical techniques delineated nine pairs of disulfide bonds that help define the gp120 primary structure (21); the first four variable segments (V1 to V4) form simple or complex, disulfide-stabilized loops (21). Some secondary structural information on gp120 is also available; infrared spectroscopic analysis and computer modelling techniques have allowed potential alpha-helical segments to be identified (8, 32), and a folding model for gp120 has been proposed (11). However, no direct information with respect to the tertiary or quaternary structure of the HIV-1 envelope glycoproteins is available, even at low resolution. We have undertaken a program of mutagenesis and immunochemical analysis to obtain information on envelope structure and function, focussing initially on gp120.

Our first studies of the envelope glycoproteins were based on mutational analysis of protein function (19). Using this approach, we defined regions of the gp120 and gp41 molecules critical for HIV-1 fusion and infectivity. In particular, we identified the regions of the gp120 molecule important for CD4 binding and gp41 association (13, 35) and showed that both of these binding functions relied on correct three-dimensional folding of the gp120 molecule; the CD4-binding site and the gp41-associative region are both discontinuous, complex structures. Amino acids whose alteration disrupts gp41 binding are mostly confined to the N-terminal stretch of the C1 domain and the C-terminal segment of C5 (13). In contrast, substitutions of particular amino acids located in C2, C3, C4, and C5 decrease CD4 binding (7, 35).

To gain insights into gp120 topology, we used a large panel of peptide-reactive monoclonal antibodies (MAbs) whose linear epitopes had been defined with some precision. We gauged the relative exposure of different segments of monomeric BH10 gp120 by measuring whether MAbs directed against these segments reacted preferentially with native or denatured forms of gp120 (28). The variable loops were found to be mostly exposed on the surface of monomeric gp120, whereas only limited segments of the C1, C4, and C5 conserved regions were exposed. Furthermore, conserved regions accessible on the gp120 monomer were usually occluded on the oligomeric gp120-gp41 complex, probably because of their participation in intersubunit interactions (13, 28, 32).

Discontinuous epitopes for antibodies to gp120 have been characterized by the same techniques used to study the CD4-binding site (CD4BS). More than 20 different CD4BS-related epitopes have been found to share sensitivity to particular amino acid changes in C3 that were previously shown to influence the gp120-CD4 interaction (16, 37, 50, 53). Many of the MAbs directed against the CD4BS-related epitopes were also sensitive to certain amino acid substitutions throughout the other conserved domains of gp120. While no two MAbs to CD4BS-related epitopes reacted identically with the test panel of gp120 mutants, common subsets of gp120 amino acids were found to contribute to this family of conserved epitopes (50). Subtle variations in the dependence of different MAbs on individual gp120 residues can also be observed (33, 50). Of note was the finding that deletion of the V1, V2, and V3 variable loop structures generally increased the binding of CD4BS-related MAbs to gp120, demonstrating that these epitopes were predominantly confined to the conserved regions and implying that the variable loops in some way occluded these conserved domains (59, 60). This is also consistent with our recent finding that neutralizing antibody epitopes induced by CD4 binding (52) are also independent of the V1 to V3 loops and are better exposed on proteins from which these loops have been deleted (59).

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Specific interactions that take place between gp120 domains have also been defined. For example, we found that C4-directed MAbs can cross-block V3-directed MAbs and that amino acid changes in V3 affected the binding of C4-directed MAbs and vice versa. These results allowed us to infer a close interaction between sections of the V3 and C4 regions of gp120 (31, 61). Similar studies have helped us to position the V2 loop in relation to conserved (C1) elements in its stem structure (29), and others have shown functional interactions between the V2 and C4 domains (9) and between the V2 and V3 domains (18, 44). Reports from Willey et al. showed that a glycosylation site-deleting substitution in the C2 domain (267 N/Q) that reduced HIV-1 infectivity could be functionally compensated for by a reversion substitution in the C1 domain (128 S/N) (57, 58). We explored the topology of the mutant and revertant gp120s by using soluble CD4 and a panel of MAbs, finding that the C2 substitution disrupted the CD4BS, which was restored to its wild-type function by the C1 compensatory substitution (32). Furthermore, the C2 change caused the abnormal exposure, on the surface of gp120, of a large segment of the C1 domain from approximately residues 80 to 120. This segment was also restored to its wild-type configuration by the reversion substitution at residue 128. The disruptive effect of the C2 substitution also extended into a portion of the C5 domain, which earlier studies had indicated was probably linked to the C1 domain to form the gp41-associative region. Computer modelling implied that the C1 segment from residues 80 to 120 that was perturbed by the 267 N/Q substitution formed an extended alpha-helix. Helical structure was also suggested for the region surrounding asparagine 267. Thus, we consider that the 267 N/Q change might disrupt the packing of two alpha-helical stretches of the conserved, hydrophobic core of gp120, thrusting one of the helices to the surface of the molecule and disrupting the geometry of the CD4BS. In some unknown way, the reversion substitution at residue 128 restores the association between the C1 and C2 helices and repairs the disruption of gp120 conformation (32).

The above information has helped us better to understand gp120 structure and function, but our knowledge is far from complete. We have now performed a set of cross-competition analyses using 46 MAbs to linear and discontinuous gp120 epitopes. Using mutagenic and/or peptide-binding analyses, many of these epitopes have been characterized at least partially. The present analysis expands the limited set of MAb cross-competition data obtained in our previous studies (29–32, 59). By determining systematically which MAbs inhibit or enhance the binding of other MAbs, we have been able to increase our understanding of the antibody-accessible surface of the native, monomeric gp120 glycoprotein.

MATERIALS AND METHODS

MAbs and gp120. The recombinant gp120 molecule used in these analyses was a Chinese hamster ovary (CHO)-expressed protein derived from the BH10 clone of HIV-1 LAI by Celltech Ltd. (Slough, United Kingdom) and obtained from the reagent repository of the United Kingdom Medical Research Council AIDS Directed Programme (27).

The MAbs used in the present study were all obtained from their producers or from the reagent repositories of the Medical Research Council or the National Institute of Allergy and Infectious Diseases, or they were purchased from commercial suppliers. Primary citations for these MAbs and/or references to our previous characterizations of them are as follows: M91, M85, and M90 (F. di Marzo Veronese [56]); CRA-1, CRA-3, and CRA-4 (M. Page [29, 32]); 1C1 (Repligen Inc.) (32); 522-149 (G. Robey, Abbott Inc.); 133/290, 133/192, and 135/9 (M. Niedrig [32, 34]); 4A7C6 (R. Tedder [32]); 4D4#85 and 5G11 (S. Nigida and L. Arthur, National Cancer Institute, Frederick, Md.); C11, 212A, A32, 2/11c, 48d, 17b, 15e, 21h, and F91 (J. Robinson [15, 29, 38, 48, 50, 52, 59–61]); #45, #49, and #55 (C.-Y. Kang [16]); G3-519, G45-60, G3-508, G3-536, G3-299, and G3-42, (M. Fung [31, 49]); G3-1472, G3-136, G3-4, and BAT085 (M. Fung [10, 14, 29, 48]); 110.5 (E. Kinney-Thomas [17, 20, 31]); 110.I and 110.J (F. Traincard [31]); 9284 (DuPont Inc. [43, 61]); IgG1b12 (D. Burton [5, 37]); CD4-IgG (Genentech Inc. [6]); 684-238 and SC258 (G. Robey [29]); 11/4c (C. Shotton [23, 42]); and 2G12 (H. Katinger [3, 55]). The epitopes recognized by many of these MAbs are summarized in reference 28, as well as in the reports cited above.

Cross-competition analyses. Antigen capture enzyme-linked immunosorbent assay (ELISA) was used for these studies. Briefly, BH10 gp120 was captured onto plastic (Immulon 2 microplates; Dynatech Ltd) via adsorbed sheep antibody D7324 to the gp120 C terminus (Aalto BioReagents, Dublin, Ireland) as described previously (25–27). Competitor MAbs were added to the gp120 for 15 min in a volume of 50 μl of Tris-buffered saline containing 2% nonfat milk powder and 20% sheep serum (TMSS buffer). The MAb concentration used was predetermined to be saturating (whenever this concentration could be achieved) or was the highest concentration commensurate with preserving an adequate MAb supply for completion of the analysis. Pure MAbs were generally used at 10 to 30 μg/ml, ascites fluids at 1:100 to 1:300 dilutions, and hybridoma supernatants at 1:10 to 1:30 dilutions. The detection MAbs were biotinylated as described previously (39, 40), and 50 μl of an appropriate dilution of a biotin-labelled MAb in TMSS buffer was added to the gp120-MAb mixture described above. After incubation for 1 h, unbound reagents were washed away and bound biotin-labelled MAb was detected by using alkaline phosphatase-labelled strepavidin (Dako Diagnostics, Ely, United Kingdom) and the AMPAK system (Dako Diagnostics). The amount of biotin-labelled MAb used was determined from a prior titration of the reagent against BH10 gp120 in the absence of competitor, and a stock dilution was chosen to give a net optical density at 492 nm (OD_{492}) of 0.30 to 1.30 in the linear part of the titration curve; this allowed both enhancing and inhibitory effects of the unlabelled MAb to be observed and quantitated. A few MAbs were labelled poorly with biotin, and the net OD_{492} values in assays using them were less than the above range but still adequate for determining the effects of the unlabelled MAb on the binding of the labelled MAb.

Each test MAb was tested in triplicate, and the control tests for 100% biotin-MAb binding (no competitor) were carried out at least six times. Each test plate also contained six background wells (no gp120 and no competitor); the mean OD_{492} values derived from these wells were subtracted from all other mean test OD_{492} values. After background correction, the ratio of the mean OD_{492} in the presence of each test MAb to the 100% control OD_{492} was determined as a percentage. Each biotin-labelled and unlabelled MAb combination was tested at least three times, so each binding ratio determined was the product of at least nine ELISA wells for each competing MAb. The average binding ratio for all the experiments was then calculated for each combination of biotin-labelled and unlabelled MAb. Compared with the extent of biotin-labelled MAb binding in the absence of any unlabelled MAb (100%), we deemed effects of an unlabelled MAb to be significant if the mean binding ratio fell outside the range of 75 to 125%.

Construction of a competition map of gp120. The order of the MAbs on the completed 46 by 41 competition grid was adjusted so as to group in an optimal fashion the intense bidirectional inhibitory effects along the diagonal axis. This optimal competition matrix was used to construct a map depicting the relationship among epitopes, on the basis of the following assumptions.

(i) Reciprocal inhibition was interpreted to mean that the epitopes of the two MAbs overlap, with the extent of the overlap proportional to the degree of inhibition observed.

(ii) Unidirectional positive or negative effects were interpreted to indicate proximity, but not overlap, of the epitopes.

In the construction of the gp120 competition map, an attempt was made to explain the general relationships among competition groups rather than the subtle effects pertinent to only one MAb within a competition group. The diagrams of the gp120 surface were based solely on the MAb competition data, without reference to epitope characterization or other information about gp120 structure derived from mutagenic or reversion analyses.

Initially, the antibody epitopes were placed onto a spherical structure and aligned until all of the relationships deduced from the competition matrix, using the above assumptions, were explained. The data associated with certain competition groups (V2, 2G12, C5 linear, and some C1 epitopes) could not be explained by positioning the epitope on the surface of a sphere. These data were accommodated by proposing that these epitopes reside on surface projections from the spherical gp120 core. Data on the binding effects of MAbs directed against the cognate epitopes allowed placement of the surface projections at the appropriate position over the core structures. Finally, the shape of the spherical competition map was adjusted slightly to aid the viewer in identifying the gp120 regions presumably exposed on the assembled envelope glycoprotein complex (depicted as a convex surface) and the regions less exposed on the viral spike (depicted as a concave surface).

RESULTS AND DISCUSSION

Selection of reagents for cross-competition analyses. We selected CHO-cell-expressed, recombinant gp120 from the BH10 clone of HIV-1 LAI for our initial cross-competition analysis. This protein was chosen because of the very extensive

range of rodent antibodies that have been raised against LAI gp120 over the past 8 years; although many of these are cross-reactive with the gp120 glycoprotein of other strains, some are not. Furthermore, many human MAbs have been isolated by screening them against LAI gp120 and are therefore reactive with it. In addition, we have generated a large panel of LAI gp120 mutants, containing single amino acid changes as well as large deletions, and are continuing to make more mutants. Thus, of the 481 amino acids that constitute the mature HXBc2 (LAI) gp120 glycoprotein, more than 300 have been individually altered or are contained within large deletions, with a retention of gross overall gp120 conformation. These mutants have been used to map the epitopes of the antibodies included in this study, thus providing an important context in which to understand the results of the competition analysis.

The choice of MAbs for inclusion in the competition analysis presented several challenges. A comprehensive panel of MAbs was needed to allow a coherent understanding of the relationships among gp120 epitopes to be achieved. Antibodies that neutralize HIV-1 and therefore bind native envelope glycoproteins were obvious choices. MAbs generated in HIV-1-infected humans, even if not neutralizing, were included, since many of these would be generated by native gp120 in either cell-associated or soluble forms. Much more difficult was the decision to include or exclude candidates from the scores of rodent MAbs generated to various gp120 molecules, many in denatured states. Those rodent MAbs that were shown to require native gp120 conformation for recognition were included in the analysis. We then examined more than 70 MAbs that recognized linear epitopes and, therefore, were able to bind denatured gp120. Only a subset of these antibodies, directed against the C1, V2, V3, C4, and C5 regions of gp120, was able to bind efficiently to the native gp120 glycoprotein (28). Representatives of each of these groups of MAbs were included in the competition analysis.

It was also important that the chosen MAbs exhibited roughly similar affinities for the monomeric gp120. Otherwise, one-way competition effects between MAbs binding to overlapping epitopes could result and lead to misinterpretation. Therefore, the affinity for gp120 of each antibody included in the analysis was determined, and a saturating concentration of each unlabelled MAb was used in the competition analysis whenever possible. Examples of MAb titration curves that show inhibitory or enhancing effects of an unlabelled MAb on the binding of a labelled MAb are provided in several of our previous studies (29, 31, 32, 52, 59). By using multiple MAbs to related epitopes whenever these were available, potentially spurious results derived with a single MAb could be noted and then confirmed or excluded.

The competition assay we used is a well-characterized ELISA in which gp120 is first captured onto a solid phase via a sheep antibody to its extreme C terminus. We have used this assay extensively for the past several years (25–32, 52, 59). It should, however, be noted that three antibodies are often bound simultaneously to gp120 during these experiments. In comparative studies of this ELISA technique we have only rarely noted effects that might be attributable to the presence of the C-terminal antibody and these tend to be minor in degree (59). By definition, all those test antibodies that bind to gp120 in the cross-competition experiments do so in the presence of the C-terminal antibody, which forms a discrete competition group.

Cross-competition analysis of MAb reactivity with gp120. In total, 46 MAbs were selected for the analysis, 41 of which were biotin-labelled. The cross-competition matrix therefore comprised 1,886 individual MAb combinations. The mean values derived from multiple experiments are presented in Fig. 1, in which the competitor MAbs are listed at the left and the biotin-labelled MAbs are listed on the top. The extent of competition or enhancement is depicted by the color and intensity of shading: orange and red indicate weak and strong inhibition, respectively; green indicates enhancement; and yellow denotes a neutral effect (i.e., the binding of the biotinylated MAb is unaffected by the presence of the test MAb). The values recorded within each box represent the percent binding of the labelled MAb relative to the control (100%). Values $\leq$75% and $\geq$125% are deemed significantly different from the control, and the boxes are shaded accordingly.

The order of the MAbs in each column and row has been adjusted to group in an optimal fashion the intense bidirectional inhibitory effects along the diagonal axis of the matrix (Fig. 1). Not surprisingly, there were mutually inhibitory interactions between MAbs that react with a common epitope cluster; V3 MAbs consistently cross-blocked other V3 MAbs, for example. The optimized order of MAbs provides clues to the physical proximity of the epitopes on gp120 since, in this order, moderate and strong inhibitory effects are positioned close to the diagonal axis. It is the off-diagonal patterns of inhibition or enhancement that provide the most useful information about the relationships between epitopes that do not fall within a competition group.

On the basis of these data, the MAbs can be divided into 16 competition groups. Some of these groups were expected, since the MAbs within the group were known to bind to related epitopes. The competition groups include MAbs directed against linear C5 epitopes, several groups of MAbs to linear or discontinuous epitopes with C1 components, MAbs recognizing linear C4 or discontinuous C4-V3 determinants, MAbs against V3 or V2 epitopes, MAbs against the CD4BS-related or CD4-induced (CD4i) epitopes, the CD4-IgG molecule, and MAb 2G12.

Using the assumptions detailed in the Materials and Methods, this information was used to create a diagram that depicts the relative orientation of antibody epitopes on the gp120 surface (Fig. 2). It is useful to consider some general properties of this map before discussion of the data related to each of the competition groups. Reference to the competition map should aid in placing these data in context. Conversely, the basis for the location of particular epitopes on the map should become apparent from consideration of the data.

General features of the gp120 competition map. The three-dimensional gp120 competition map consists of a core from which additional epitopes project. The core of the competition map is shown from three perspectives in the upper three illustrations in Fig. 2. The lower illustrations in Fig. 2 depict the complete competition map, including epitopes modelled as projecting structures.

The gp120 competition map can be viewed as having two faces, one containing the neutralization epitopes and the other recognized by antibodies without significant neutralizing activity. The gp120 face with the neutralization epitopes also contains the binding site for the CD4 receptor. Projecting from this face of gp120 are the V2 epitopes and the 2G12 epitope. The HIV-1-neutralizing ability of MAbs binding to epitopes located on this face of gp120 suggests that this aspect of gp120 is accessible on the assembled envelope glycoprotein complex of HIV-1 LAI.

The second face of gp120 is recognized by MAbs with little or no neutralizing activity. Many of the epitopes located on this face of the gp120 core are discontinuous and are disrupted by amino acid changes in the C1 and/or C5 region. Consistent with this association are the presence of projecting linear C1

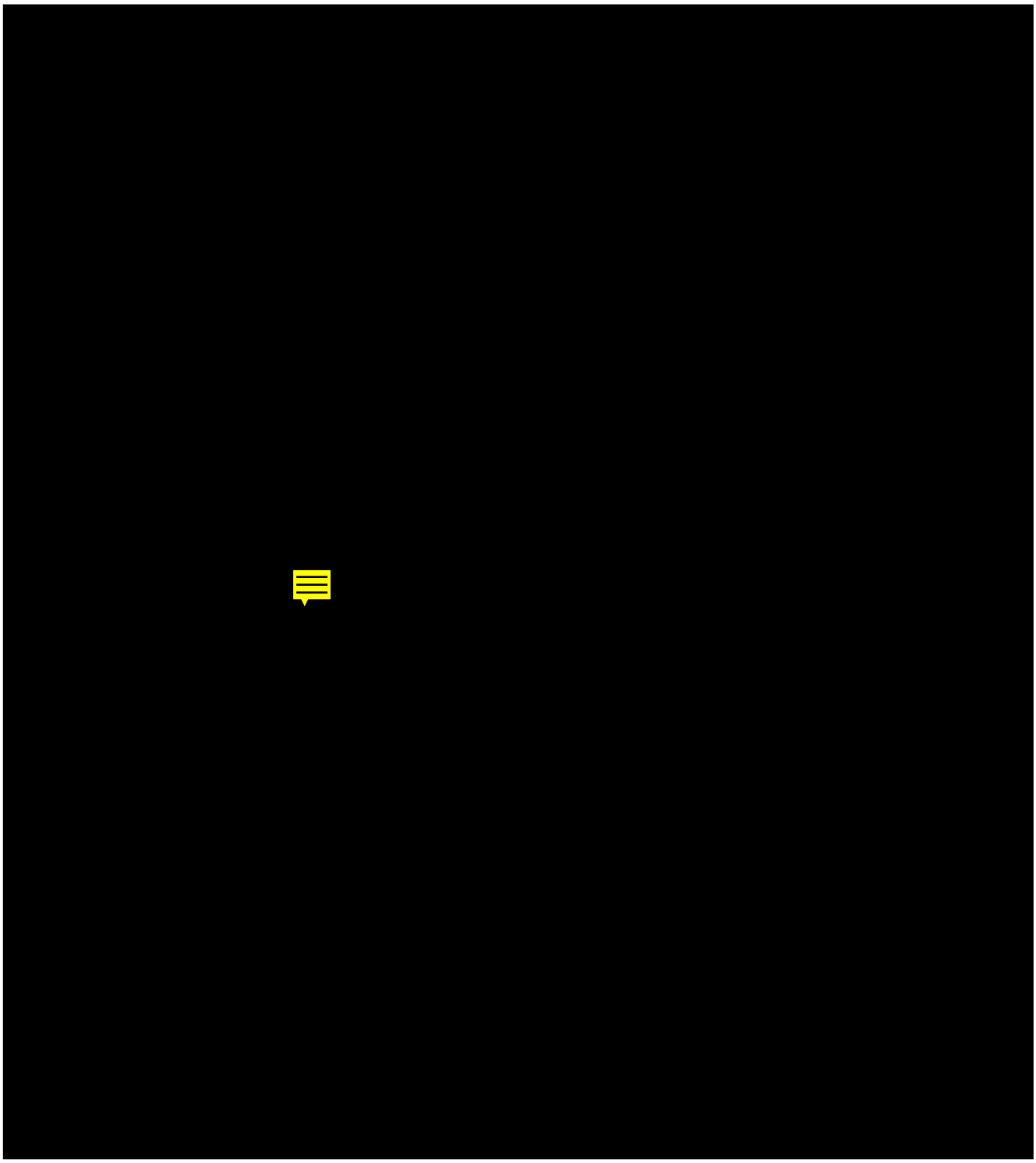

FIG. 1. Competition matrix of MAbs binding to monomeric HIV-1 gp120 glycoprotein. The effect of competition MAbs (listed on the left) on the binding of biotinylated MAbs (listed across the top) to HIV-1 gp120 captured on ELISA plates is shown. The gp120 regions recognized by the MAbs are noted in the first column on the left and at the top of the figure. The letters in parenthesis identify linear (L) or discontinuous (D) epitopes. The values shown represent the mean binding of the biotinylated MAb in the presence of the competitor MAb. A value of 100 indicates neither inhibition nor enhancement of binding of the biotinylated MAb as a result of the competitor MAb. The colors indicate the degree of inhibition or enhancement observed and are coded according to the key at the top.

and linear V5-C5 epitopes overlapping this face of gp120. We presume that on the assembled, oligomeric envelope glycoprotein complex, this face of gp120 is not accessible to MAbs because of its interaction with the gp41 transmembrane glycoprotein or with other gp120 subunits. This assumption is consistent with the observation that the C1 and C5 regions of gp20 are critical for the noncovalent interaction between gp120 and gp41 (13).

FIG. 2. Competition map of the surface of the HIV-1 gp120 glycoprotein. The top three drawings illustrate the core of the competition map of gp120 from three different perspectives, with the surface seen in the upper left-hand illustration presumably exposed on the assembled envelope glycoprotein oligomer. The binding sites for CD4 (in red) and groups of MAbs are depicted. Overlap of epitopes indicates reciprocal inhibition of MAb binding, whereas adjacent epitopes are used to model unidirectional inhibitory or enhancing effects. The lower two drawings include epitopes that were displayed as surface projections. In the lower right-hand figure, the V2 epitopes were removed for clarity.

These data as related to each of the competition groups are presented in turn.

(i) Epitopes located on the neutralization face of gp120. (a) CD4BS-related epitopes. A considerable number of human and rodent MAbs have been described that recognize an immunodominant structure(s) in proximity to the CD4BS on gp120. MAbs to this site(s) competitively inhibit CD4 binding to monomeric gp120 (3, 4, 6, 15, 16, 22, 31, 36–38, 50, 51, 53, 54). Analysis of the recognition patterns of gp120 mutants by the MAbs in this group shows that they generally differ in their precise dependence on particular gp120 residues, but there are common influences on their epitopes; for example, amino substitutions at Asp-368 and Glu-370 almost invariably destroy the binding sites for these MAbs (30, 37, 50, 53). We selected a panel of CD4BS MAbs exhibiting binding specificities representative of this group as a whole.

Despite the variations in the epitopes of these MAbs, they all cross-blocked each other, showing that they did indeed recognize very similar, overlapping sites and formed a single competition group. Reciprocal strong inhibition was observed between CD4BS MAbs and CD4-IgG, confirming the close relationship between the MAb epitopes and the discontinuous, CD4 receptor-binding site. Reciprocal inhibition was also seen between MAbs directed against the CD4BS epitopes and those, like 17b and 48d, directed against epitopes induced upon CD4 binding (CD4i epitopes) (52, 59). Weaker reciprocal inhibitory effects between CD4BS MAbs and the A32 MAb were seen. Several MAbs exhibited a unidirectional inhibitory effect on the binding of CD4BS MAbs; these include the M90 and #45 MAbs to C1 discontinuous epitopes and three MAbs (M91, 1C1, and CRA-1) directed against linear V5 and C5 epitopes. The C4-directed MAbs tended to compete with the CD4BS MAbs, but this was not always seen; the binding of one CD4BS MAb (#55) was generally unaffected by C4-directed MAbs, with G45-60 being a only weak competitor, and the binding of CD4BS MAb F91 was actually enhanced by four of six C4 or C4-V3 MAbs. MAb F91 was further distinguished from the other CD4BS MAbs by the unusual degree to which its gp120 recognition was increased by several V3-directed and V2-directed MAbs. However, gp120 binding by other CD4BS MAbs was sometimes increased by some V3- and V2-directed MAbs, notably by the V3 MAb 5G11 and the V2 MAb G3-136. The CD4BS MAb IgG1b12, which is unique among the CD4BS MAbs in being sensitive to V2 amino acid substitutions (37), was the most susceptible MAb of this group to inhibition by V2-directed MAbs, and its binding was not enhanced by any V2 MAb.

(b) CD4-IgG. The influences of MAbs on the CD4BS of gp120 were probed by using CD4-IgG as test ligand, because this resembles the MAbs in our panel by being dimeric (6). CD4-IgG demonstrated strong reciprocal inhibition of binding of MAbs directed against CD4BS-related epitopes and, to a lesser extent, MAbs directed against C4 or C4-V3 epitopes. Weaker reciprocal inhibitory effects were noted between CD4-IgG and the M90 and #45 MAbs. In general, those MAbs that decreased or increased the binding of the CD4BS MAbs to gp120 tended to have a similar effect on CD4-IgG binding, although there were several differences. Among these were the finding that CD4-IgG binding was more sensitive than CD4BS MAb binding to the inhibitory effects of V2-, C4-, C4-V3- and V3-directed MAbs but was insensitive to C5-directed MAbs. Note, however, that although some V3-directed MAbs could reduce CD4-IgG binding to gp120, the inhibition was fairly weak.

No MAb increased CD4-IgG binding to gp120; this was unusual in that this lack of binding enhancement by any other MAb was a property shared by only two other MAbs in our test panel, the C5 MAbs CRA-1 and 1C1. Of particular note is the finding that whereas CD4-IgG binding increased the gp120 reactivity of MAbs A32, 48d, and 17b, prior binding of these three MAbs to gp120 inhibited CD4-IgG binding. Thus, the conformational changes in gp120 induced by CD4-IgG that better expose the epitopes for A32, 48d, and 17b (52, 59) are not reciprocated by the MAbs themselves.

(c) CD4-induced epitopes. Two human MAbs, 48d and 17b, have been described that recognize gp120 epitopes that become exposed better upon CD4 binding (52, 59). Consistent with earlier results, CD4-IgG caused enhancement of both 48d and 17b binding to gp120. The gp120 reactivity of both these MAbs was also increased by MAbs A32 and 2/11c, suggesting that these MAbs cause a conformational change in gp120 that exposes the 48d and 17b epitopes. The only other MAbs to have this effect were the V3 MAb 5G11, which increased 17b binding only, and five V2 MAbs, which increased 48d binding only. These differential effects on the 48d and 17b epitopes are consistent with others noted above; the epitopes for these two MAbs overlap, as 48d and 17b strongly cross-block one another but are not identical. Deletions within the V3 loop, for example, affect the integrity of the 48d epitope more than that of the 17b epitope (52, 59).

The CD4i epitopes are the gp120 discontinuous structures most sensitive to disruption by detergent or by amino acid changes (52, 59). It was not surprising, therefore, to observe that the binding of many other MAbs to gp120 exerted negative effects on the binding of either one or both of the 17b and 48d MAbs. In contrast to the enhancing effect of CD4-IgG on the binding of 48d and 17b, all the MAbs to the CD4BS-related epitopes strongly inhibited both 48d and 17b binding. This reinforces the notion that MAbs to the CD4BS-related epitopes do not react with the CD4-binding site itself but with structures that are in close proximity to it. MAbs to the C4 domain were also consistently inhibitory to the binding of 48d

and 17b, while a subset of MAbs to the C1 and C5 domains were weakly inhibitory, especially to 48d binding. One MAb, #45, to a discontinuous C1 epitope was a particularly effective competitor of 48d binding and also inhibited 17b reactivity, whereas M90, to a very similar discontinuous C1 epitope, was without effect on either—another example of the subtlety of some of the interactions we have noted. MAbs to the V3 loop were generally strong inhibitors of 48d binding but weak inhibitors of 17b binding, and we noted that one V3 MAb, 5G11, actually increased 17b reactivity with gp120. Furthermore, whereas five V2-directed MAbs increased 48d reactivity, three (CRA-3, CRA-4, and SC258) were competitors and CRA-3 also reduced 17b binding. Again, we presume that the precise geometry with which different MAbs react with similar binding sites influences their effect on other gp120 structures.

(d) C4 domain and C4-V3 discontinuous epitopes. The C4 domain contains elements of the CD4BS (35); several MAbs to C4 sequences have been described and are able to inhibit gp120 binding to CD4 or CD4-IgG (49). In addition, we have shown a close association between segments of the C4 and V3 domains of gp120 and two MAbs (G3-42 and G3-299) may actually recognize a discontinuous epitope spanning the C4 and V3 regions (31, 61). The results of the cross-competition analysis are consistent with these ideas; C4-dependent MAbs were inhibited by other C4-dependent MAbs, by CD4-IgG, and by V3 MAbs. Weaker inhibition by MAbs to the discontinuous CD4BS epitopes, by MAbs to the CD4-induced epitopes, and by the A32 and 2/11c MAbs was seen. Of note, however, was the enhancement of the binding of C4-dependent MAbs by MAbs to several other regions of gp120. These include various MAbs to epitopes in the C1 and C5 domains and a subset of MAbs reactive with conformationally sensitive V2 epitopes. Furthermore, gp120 binding of the V3-sensitive, C4-dependent MAb G3-299, but not of the other two C4-dependent MAbs, was enhanced by two (#55 and F91) of the six MAbs to the CD4BS epitopes. However, the other four MAbs to the CD4BS-epitope cluster were inhibitors of G3-299 binding to its C4-V3 epitope. These opposing effects of MAbs within the same epitope cluster implies that there must be subtle variations in the epitopes of the CD4BS-related MAbs. These variations presumably influence the geometries with which the MAbs bind in relation to the V3 and C4 domains of gp120, to cause either enhancement or inhibition of G3-299 binding. Other examples of this exquisite variation in the structure of epitope clusters are outlined below.

(e) V3 loop. Elements of the V3 region of gp120 are located in close proximity to the C4 domain (31, 61), and there is also a description of interactions between the V3 and the V2 loops (18). The cross-competition data are consistent with these findings. Thus, C4-directed MAbs consistently inhibited the binding of most biotin-labelled V3 MAbs, the strongest inhibition being of MAbs 110.I and 110.J to epitopes on the carboxy-terminal side of the V3 loop. MAb 48d to the CD4-induced epitope, but not MAb 17b to a very similar overlapping site, also weakly inhibited V3 MAb binding to gp120. The subtle distinction between the epitopes for MAbs 48d and 17b was further exemplified by the enhancement of MAb #49 binding by 17b but not by 48d. MAbs to several other sites significantly enhanced V3 MAb binding; most prominent among these were MAbs to the CD4BS epitopes and to the V2 loop. However, not every CD4BS- or V2-directed MAb increased the binding of every V3 MAb, indicating that the precise geometry of the interactions between the different antibodies was important. In contrast to the CD4BS MAbs, CD4-IgG did not enhance the binding of V3-directed MAbs but was either neutral or weakly inhibitory in its actions. Other enhancing effects were noted; the binding of three V3 MAbs, 5G11, 9284 and #49, was increased by some MAbs to epitopes in C1 and C5, most notably by MAb 135/9 to residues 110 to 120 in the predicted C1 alpha-helix.

(f) V2 loop structures. Several MAb epitopes in and around the V2 loop of gp120 have been described, some continuous in nature and others not (10, 14, 23, 29, 48). We selected for analysis eight MAbs covering the range of V2 epitopes. In general, the V2 MAbs were cross-inhibitory for each other, albeit to various extents. However, it was notable that the binding of MAb 11/4c to its continuous epitope in the amino-terminal flank of V2 was enhanced by several MAbs to discontinuous V2 epitopes and that, reciprocally, MAb 11/4c enhanced the binding of several of these MAbs. In contrast, 11/4c and BAT085, which recognize adjacent continuous epitopes in V2 (10, 23), were mutually cross-competitive. One nonreciprocal pattern of inhibition within V2 was the finding that 11/4c strongly inhibited CRA-3 binding to its discontinuous epitope, which involves the stem structure at the base of the V1-V2 loops (29), but CRA-3 was without effect on 11/4c binding.

MAbs reactive with epitopes outside the V1-V2 structure were generally unable to inhibit the binding of any V2-directed MAb to gp120, the most consistently, albeit weakly, inhibitory MAb being IgG1b12 to the CD4BS. We noted above that IgG1b12 is sensitive to amino acid substitutions in V2 (37) and that its binding is weakly reduced by several V2-directed MAbs. The binding of the 11/4b MAb was enhanced by IgG1b12. CD4-IgG was able to inhibit the gp120 reactivity of several V2 MAbs. Inhibition of the binding of V2 MAbs by CD4-IgG binding may relate to the movement of the V2 structure that occurs upon CD4 binding (59). The V2 MAbs 684-238 and CRA-3 were also weakly blocked by some MAbs to CD4BS-related epitopes. An extensive range of MAbs to non-V2 epitopes was able to enhance V2 MAb binding to gp120. These MAbs included several to epitopes in the C1 and C5 domains, all the V3-, V3-C4-, and C4-directed MAbs, and several of the CD4BS MAbs. Not every V2 MAb was affected in the same way, but four MAbs (CRA-3, G3-136, G3-4, and SC258) to conformationally sensitive V2 epitopes were particularly sensitive to MAb-induced enhancement, whereas CRA-4 and 11/4c were relatively insensitive.

The multiple MAb groups that demonstrated effects on the binding of V2 MAbs made it difficult to model this region on a simple spherical structure. A solution that satisfied this dilemma was to represent the V2 loop as a structure projecting over the gp120 surface. The depiction of the V2 loop as an umbrella that partially masks the CD4 binding site, the CD4BS epitopes, the CD4i epitopes, and the A32 epitope is consistent with several observations. First, the V2 loop can be deleted without disrupting the conformation of most gp120 epitopes, suggesting that the V2 loop does not contribute to a core gp120 structure (59, 60). Second, removal of the V1 and V2 loops results in an increase in the binding of MAbs directed against the CD4BS and CD4i epitopes (59, 60). Third, CD4 binding to gp120 has the effect of moving the V1 and V2 loops, resulting in a demasking of the CD4i and A32 epitopes (59).

The CRA-3 epitope was modelled as a structure at the base of the projecting V2 loop. This reflects the higher degree of competition seen between the CD4i epitopes and CRA-3 than that between the CD4i epitopes and other V1 and V2 MAbs. This depiction is consistent with a greater sensitivity, compared with other V2 MAbs, of the CRA-3 MAb to amino acid changes in the conserved stem of the V1-V2 stem-loop structure (29).

(g) 2G12 epitope. Human MAb 2G12 recognizes a conformationally sensitive epitope on monomeric gp120 that is

destroyed by amino acid substitutions affecting N-linked glycosylation sites near the base of the V3 loop and in the amino-terminal flank of the V4 loop (55). We are unaware of any other MAb with the same pattern of sensitivity to amino acid substitutions. Consistent with this, the cross-competition analysis found that no MAb, other than 2G12 itself, was able to inhibit 2G12 binding to gp120. Neither was 2G12 able to inhibit the binding of any other MAb. These findings suggest that 2G12 lies within a unique competition group and is the only member of this group identified to date. Certain MAbs were, however, able to increase 2G12 binding; among these were several MAbs to epitopes in the C4, C4-V3, and V3 regions and three MAbs to CD4BS-related epitopes. This observation allowed us to model the 2G12 epitope as a surface projection near the C4 and V3 epitopes.

(ii) Epitopes located on a face of gp120 inaccessible to neutralizing antibodies. In contrast to the epitopes described above, which can be targeted by neutralizing antibodies, there are a number of epitopes exposed on the monomeric HIV-1 gp120 glycoprotein that are recognized by MAbs that exhibit little or no neutralizing activity. In almost every case examined, these MAbs bind poorly to the assembled oligomeric envelope glycoproteins, suggesting that these epitopes are occluded by interactions of gp120 with gp41 or with other gp120 subunits (32). Many of these nonneutralizing antibody epitopes are affected by changes in the C1 and C5 domains, which have been shown to be critical for the noncovalent gp120-gp41 interaction (13).

(a) A32 and 2/11c discontinuous epitopes. The consistency and potency with which the A32 and 2/11c MAbs neutralize HIV-1 are generally very low. For example, no neutralization of the HXBc2 or ADA HIV-1 isolates by the A32 MAb was observed in an *env* complementation assay, consistent with the undetectable binding of the MAb to these envelope glycoproteins expressed as oligomers on a cell surface (31, 46a, 47). The A32 and 2/11c MAbs recognize discontinuous epitopes that are sensitive to amino acid substitutions in several regions of gp120, but particularly in the C1 and C4 domains (31). Of the two MAbs, 2/11c has the lower affinity and could not be adequately labelled with biotin for binding studies. The binding of A32 was strongly inhibited by MAbs M90 and #45, and it was also significantly inhibited by five of six MAbs reactive with epitopes centered on and around the C4 domain. This pattern of inhibition is consistent with the dependence of A32 binding on amino acid residues in the C1 and C4 domains (31). However, A32 binding was unaffected by MAbs to C1 continuous epitopes, except for a weak inhibition by 4D4#85. The 522-149 MAb, which recognizes a discontinuous epitope in the C1 domain (30), also exhibited weak, reciprocal inhibition of A32 binding. As MAbs to the C5 domain also reduced A32 binding, the A32 epitope might lie in proximity to the C1-C5 structure involved in gp41 association. It was also notable that MAbs to the discontinuous CD4BS epitopes partially blocked A32 binding, as did MAb 17b (but not MAb 48d) to the CD4i epitope, two MAbs (CRA-3 and CRA-4) to the V2 domain, and two MAbs (9284 and #49) to V3 epitopes. These observations are consistent with the complex nature of the A32 epitope and the probable involvement of C4-dependent structures in it. Of particular note is the major increase in A32 reactivity caused by CD4-IgG; the A32 site is thus a CD4-induced epitope (59). However, the A32 site is clearly distinct from the CD4i epitope(s) recognized by the 48d and 17b MAbs, as the binding of the latter two MAbs was greatly enhanced by both A32 and 2/11c.

(b) M90 and #45 epitopes in C1. Two murine MAbs, M90 and #45, recognize discontinuous epitopes located primarily, if not exclusively, within the C1 domain (16, 30, 56). Strong reciprocal competition was observed between the M90 and #45 MAbs and the A32 MAb and between the M90 and #45 MAbs and the C11 and 212A MAbs. The latter MAbs recognize discontinuous epitopes dependent on C1 and C5 structures (32). Reciprocal inhibition between M90 and M85, which recognizes a linear C1 epitope (28, 56), was observed, but while binding of the #45 MAb also inhibited M85 binding, this inhibitory effect was not reciprocated. Some reciprocal inhibition between CD4-IgG and the M90 and #45 MAbs was observed. The binding of the M90 or #45 MAbs also inhibited the binding of CD4BS MAbs, but this inhibition was, in most cases, not reciprocated. Mild reciprocal inhibition was seen for the binding of CRA-3 and the M90 and #45 MAbs. The 1C1 MAb weakly inhibited the binding of both M90 and #45. The binding of MAb #45, but not M90, strongly inhibited the binding of CD4i MAbs, but there was little or no reciprocal inhibition of #45 or M90 binding by MAbs 48d or 17b. Little evidence for any interactions between the V3 loop and the M90 and #45 epitopes was observed, although the V3 MAb 5G11 did enhance the binding of both M90 and #45. This enhancement by 5G11 binding was also seen for the adjacent C11, 212A, and M85 epitopes.

(c) C11 and 212A epitopes. The human MAbs C11 and 212A recognize discontinuous gp120 epitopes dependent on C1 and C5 amino acids (32). Reciprocal inhibition was observed between the C11 and 212A MAbs and the M90, #45, and M85 MAbs. Several unidirectional effects were also observed. The A32, 2/11c, and 4D4#85 MAbs moderately inhibited the binding of the C11 and the 212A MAbs. The C11 and 212A MAbs moderately inhibited the binding of CD4BS MAbs, but CD4-IgG binding was inhibited by 212A only. The 212A MAb inhibited the binding of both CD4i MAbs, but C11 binding exhibited weaker inhibition of only the 48d MAb.

(d) Continuous epitopes in the C5 domain. MAbs to the C5 domain of gp120 bind relatively poorly to nondenatured, monomeric gp120, indicating that these epitopes are not optimally accessible on the surface of the folded protein (28). However, three MAbs (M91, CRA-1, and 1C1) were selected that were able to show weak but significant binding to nondenatured gp120. Thus, at least a proportion of the binding signals derived from the use of these MAbs results from their reactivity with native gp120 molecules. In general, MAbs M91, CRA-1, and 1C1 cross-blocked one another, but it was notable that while the binding of M91 was strongly enhanced by 1C1, the reciprocal combination was inhibitory. This implies that MAb binding to the 1C1 epitope (residues 475 to 485) exposes the nearby M91 epitope (residues 461 to 470) but not vice versa.

The C5 epitopes were depicted as a surface projection overlapping the core of the gp120 competition map. This feature of the competition map helped to explain the nonreciprocal nature of the effects of C5 MAb binding on the binding of MAbs to the CD4BS, C1, CD4i, A32, and V2 epitopes (Fig. 1). The 1C1 MAb exhibited mild inhibitory effects on the binding of the 212A, M90, and #45 MAbs and positive effects on the binding of C4 MAbs and 5G11. MAbs to these and other gp120 epitopes had little or no effect on binding of the biotinylated MAbs to the C5 domain.

(e) Epitopes in the C1 domain. Seven MAbs used in the competition analysis recognized epitopes within the C1 domain of gp120. One of these epitopes, that of the 522-149 MAb, is discontinuous (30), while the others are linear (28, 32). There are probably four competition groups formed by these seven MAbs. The 522-149 and 133/290 MAbs exhibited mutual binding inhibition, as did the 4A7C6 and 133/192 MAbs. M85 binding to gp120 was also inhibited by MAb 4D4#85, but the

reciprocal combination could not be tested. These patterns of inhibition are probably attributable to epitope proximity: the continuous epitope for MAb 133/290 lies within residues 61 to 70, and the discontinuous epitope for 522-149 is probably centered on the same region, as 522-149 binding is destroyed by the 69 W/L gp120 amino acid substitution (30). MAbs 4A7C6 and 133/192 recognize residues 81 to 90 and 91 to 100, respectively, both sites lying within a predicted alpha-helix (28, 32). MAbs M85 and 4D4#85 both bind to epitopes approximately located within residues 35 to 50 at the extreme amino terminus of gp120 (28, 32, 56). A few other weak, one-way inhibitory effects were noted, involving MAbs to continuous and discontinuous C1 epitopes, and it was also apparent that M85 binding was weakly reduced by MAbs to the C4 domain and to some discontinuous epitopes.

More common than cross-competition was the enhancement of MAb reactivity with C1 epitopes induced by other C1 MAbs and by MAbs directed at the C5 epitopes. This suggests that the C1 epitopes tend to be in regions of the protein less than optimally accessible to MAbs and that MAb-induced conformational changes in gp120 increase the exposure of several sites in the C1 domain; this is consistent with results of our previous studies of epitope exposure in the C1 domain (28, 32). Furthermore, the effect of C5 MAbs on the binding of C1 MAbs is also consistent with the notion of a close structural association between the amino- and carboxy-terminal domains of gp120, probably involving the formation of the discontinuous gp41-binding site (13, 32). It was also notable that the binding of M85 to the amino terminus of gp120 was strongly enhanced by MAb 5G11, an antibody reactive with a conformationally sensitive V3 epitope (30). This MAb also increased the binding of MAbs M90 and #45 to discontinuous C1 epitopes and of C11 and 212A to discontinuous C1-C5 epitopes. These observations suggest that elements within the V3 loop can directly or indirectly affect the exposure of the C1 and/or C5 gp120 regions. The 5G11 antibody is unique among V3 antibodies in enhancing the binding of the MAbs mentioned above and of MAbs directed against the CD4BS epitopes. The binding of the 5G11 antibody may alter the conformation of the V3 loop or adjacent structures in unique ways, leading to the observed enhancing effects.

Although the M85 epitope could be readily depicted on the core competition map, placement of other C1-determined epitopes required the proposal of a projecting element (Fig. 2). Enhancing effects observed with C5-directed MAbs and MAb M85 guided the placement of this element on the gp120 competition map, although the relative lack of influence of other MAbs on C1 MAb binding made this assignment more tentative than those of other gp120 epitopes.

(iii) Conclusions. The availability of a large panel of MAbs directed against the HIV-1 gp120 glycoprotein has allowed us to complete a relatively comprehensive cross-competition matrix describing the interactions of antibodies with gp120. We have also attempted to summarize this body of data in a three-dimensional competition map of gp120, making the assumption that the epitopes of antibodies that exhibited negative or positive cooperativity in reacting with gp120 were overlapping or adjacent. The compatibility of the competition map with the data suggests that, in most cases, this assumption is valid. Nonetheless, recognition of the shortcomings of our approach will prevent overinterpretation of the competition map. The map is designed to explain the antibody competition data and should not be taken as a model of the exact spatial relationships of gp120 epitopes. While the clustering of binding sites for neutralizing and nonneutralizing antibodies on gp120 is obvious, definition of the precise boundaries and positions of epitopes on the gp120 surface will require methods possessing higher resolution. In addition, in assembling the competition map, no attempt was made to distinguish the sometimes different behaviors of individual MAbs within a competition group. These differences probably reflect subtle variations in the ways that MAbs bind to topologically related epitopes. Good examples of these variations are seen in the interactions between CD4BS MAbs and MAbs to the V2 or V3 loops; different V2 or V3 MAbs can either enhance or inhibit the binding of CD4BS MAbs to gp120. Variations in the ability of one MAb to induce the binding of another could account for the observations that only some combinations of V3 and CD4BS MAbs interact synergistically to neutralize the infectivity of T-cell line-adapted strains of HIV-1, such as IIIB (1, 4, 22, 36, 51, 54). A further example of the subtlety with which different MAbs to a single epitope cluster may induce conformational changes in the envelope glycoproteins is provided by MAb-induced enhancement of the fusogenicity of certain primary HIV-1 strains; while some CD4BS MAbs do this, others such as IgG1b12 neutralize (41, 47).

The competition map of the HIV-1 gp120 glycoprotein presented here should promote understanding of the relationships among epitopes on this important molecule and should guide future structural analysis.

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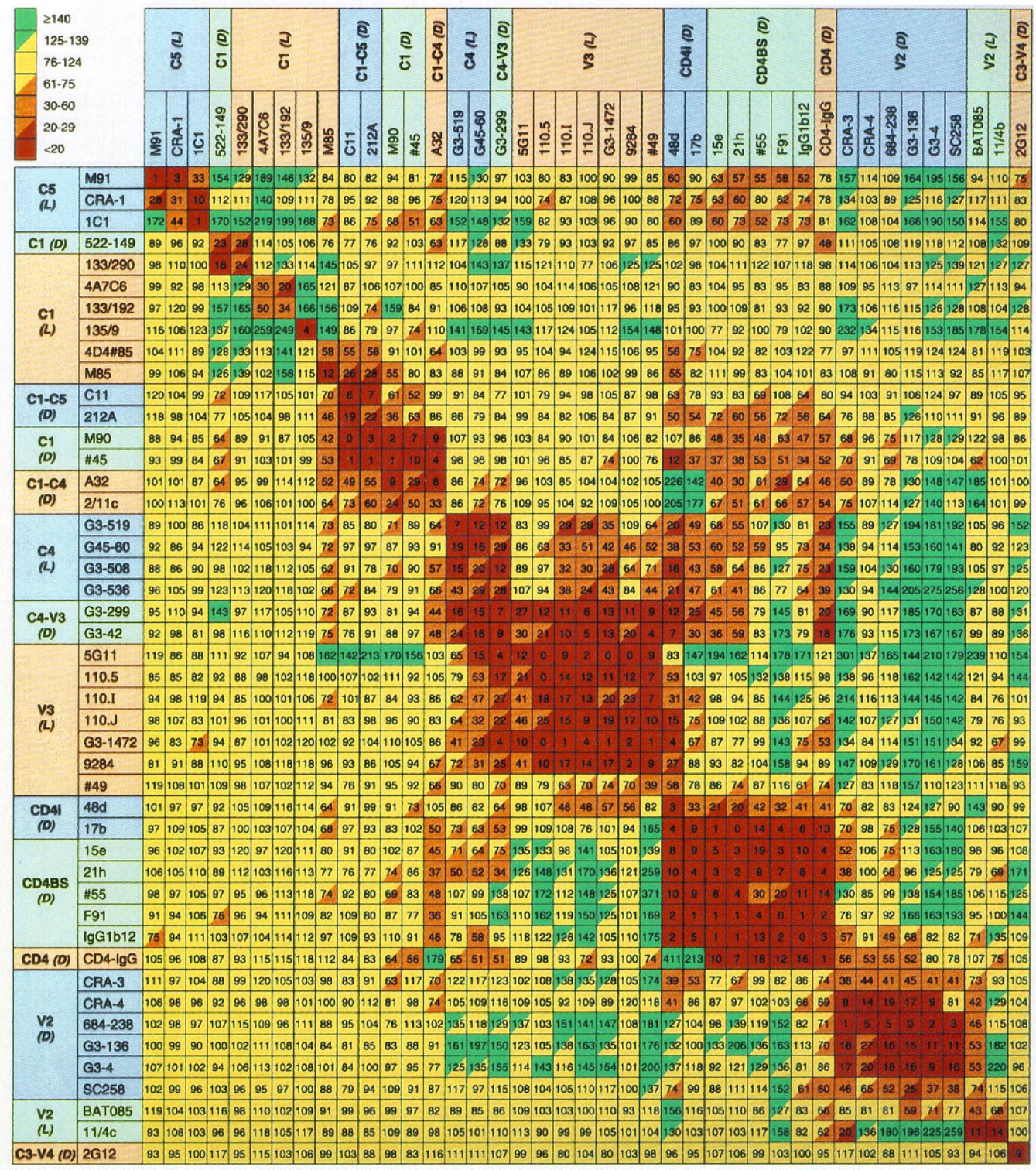
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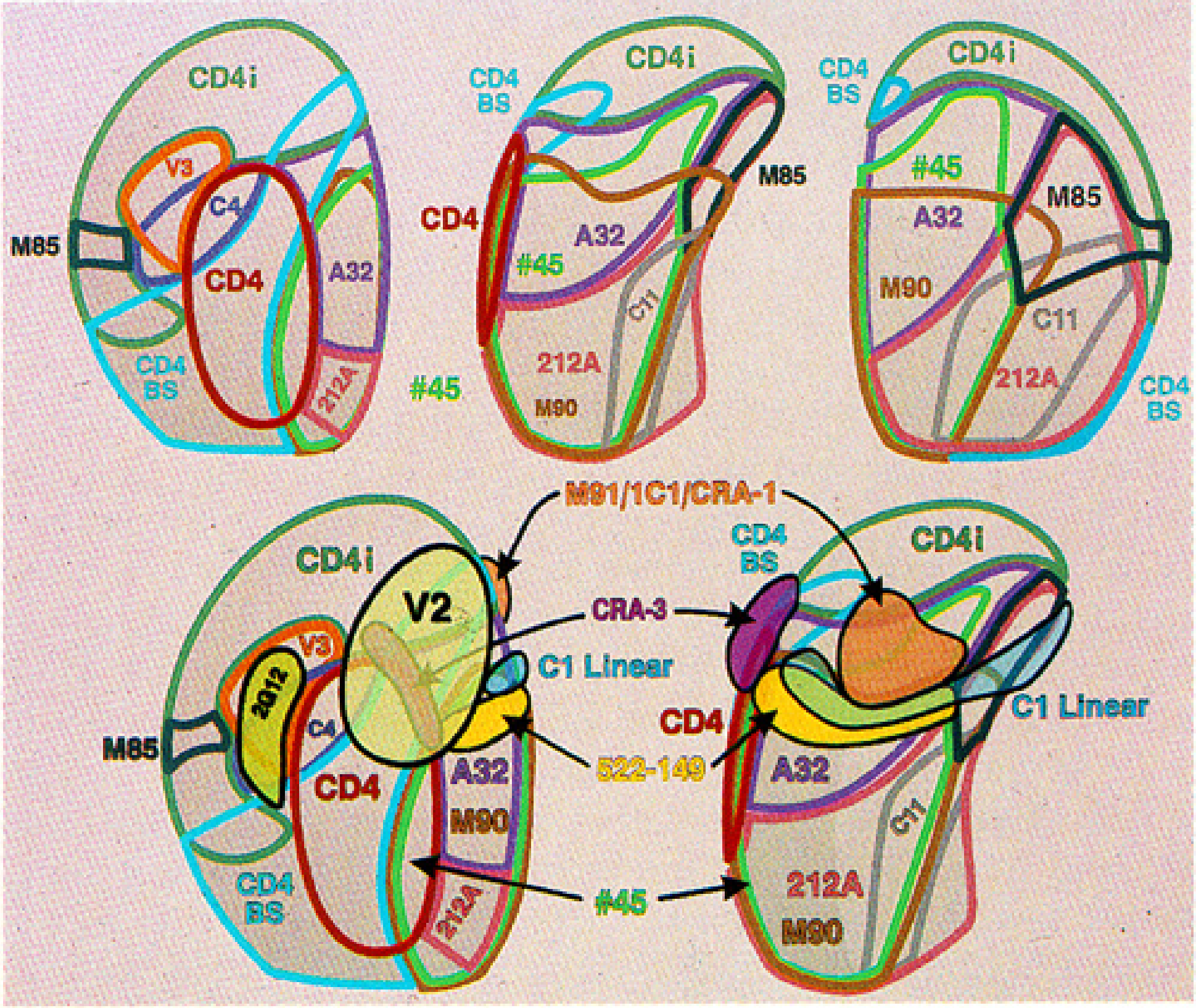
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