JOURNAL OF VIROLOGY, Nov. 1988, p. 4195–4200
0022-538X/88/114195-06$02.00/0

Vol. 62, No. 11

Neutralizing Antibodies to an Immunodominant Envelope Sequence Do Not Prevent gp120 Binding to CD4

MICHAEL A. SKINNER,[1]* ALPHONSE J. LANGLOIS,[1] CHARLENE B. McDANAL,[1] J. STEVEN McDOUGAL,[2] DANI P. BOLOGNESI,[1,3] AND THOMAS J. MATTHEWS[1]

Departments of Surgery[1] and Microbiology and Immunology,[3] Duke University Medical Center, Box 2926, Durham, North Carolina 27710, and Immunology Branch, Division of Host Factors, Centers for Disease Control, Atlanta, Georgia 30333[2]

Received 7 June 1988/Accepted 8 August 1988

Animals immunized with the human immunodeficiency virus type 1 gp160 glycoprotein or certain recombinant envelope components develop potent virus-neutralizing activity. This activity is principally due to antibodies directed toward a hypervariable region of gp120 between cysteine residues 302 and 337 and is virus isolate specific. These antisera, as well as two neutralizing monoclonal antibodies directed against the same hypervariable sequence, do not appreciably block gp120 from binding CD4. In contrast, serum samples from infected humans possess high titers of antibodies that block gp120-CD4 binding; these titers approximately correlate with the serum neutralization titers. Our results suggest that there are at least two targets on the envelope glycoprotein for virus neutralization. The target responsible for the broader neutralizing activity of human serum may be a conserved region of gp120 involved in CD4 binding. The antibodies directed at the hypervariable region of the envelope inhibit a different step in virus infection which is subsequent to receptor binding. The extent to which these two different epitopes of gp120 may be involved in protection against human immunodeficiency virus infection is discussed.

As in other retrovirus systems, it is possible that a major target of neutralizing antibodies against human immunodeficiency virus (HIV) is contained within the virus envelope (5). In vitro experiments have demonstrated that serum samples from infected humans contain neutralizing antibodies which are directed against the HIV gp120 envelope glycoprotein (8, 13, 24, 25, 33). Animals immunized with recombinant HIV envelope precursor gp160 (28), envelope glycoprotein gp120 (11, 26, 34), or particular peptide sequences contained within the envelope (2, 7, 8, 21, 23, 27) have also been shown to induce neutralizing antibodies. With some of these immunogens, the neutralizing activities have been particularly strong (21, 27, 28).

The mechanism by which antibodies against the envelope of HIV block infection is unknown. While there are several steps in the virus life cycle which may be hindered (5), the most straightforward method of neutralization is to prevent virus binding to susceptible cells. Another likely mechanism might be associated with the inhibition of membrane fusion events which occur subsequent to receptor binding (31). While the details of the latter process are far from being understood, it is likely that interactions between gp120 and gp41 play an important role (6, 10).

In this study, we have investigated the biologic activity of experimental antisera and monoclonal antibodies and contrasted those activities with those of serum samples from infected humans. The results suggest several distinct mechanisms through which HIV infection may be inhibited. The implications of our findings for the effort to develop an effective vaccine are discussed.

MATERIALS AND METHODS

Proteins and antibodies. The use of the recombinant proteins gp160 (28) and PB1 (23) and the synthetic peptide RP135 (27) as immunogens in goats has been described. The human T-cell lymphotropic virus III_B (HTLV-III_B) gp120 envelope glycoprotein was purified from infected cells as previously reported (14). The 0.5β antibody was a generous gift of Shuzo Matsushita (15). The 9284 antibody was developed by R. Ting (29a) and is available from E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. The OKT4A antibody was from Ortho Diagnostics, Inc., Raritan, N.J.

Virus neutralization assay. The level of infectious virus was quantitated by the reverse transcriptase assay (22, 24). Briefly, serum dilutions were mixed with virus and incubated for 30 min at 37°C. The virus-serum mixture was then used to infect H9 cells. Reverse transcriptase activity was assayed after 7 to 10 days in culture, and the neutralizing titer was defined as the inverse of the dilution that reduced supernatant enzyme activity by 50% compared with untreated controls.

Cell fusion assay. Approximately 7.5×10^4 MOLT-4 cells were incubated with 5×10^3 CEM cells chronically infected with HTLV-III_B in 96-well plates (one-half area cluster plates; Costar, Cambridge, Mass.) for 24 h at 37°C in a final volume of 100 μl of growth medium. The number of giant cells per well was determined by microscopic examination at a magnification of ×40. Cell fusion was scored by occurrence of giant cells with a diameter in excess of fivefold that of MOLT-4 or chronically infected CEM cells. The syncytium inhibition titer was defined as the inverse of the lowest serum concentration which completely blocked giant-cell formation.

Cells. Uninfected CEM, MOLT-4, and H9 cells and CEM cells chronically infected with HTLV-III_B have been described (14, 19). Cells were maintained in RPMI 1640 medium (GIBCO Laboratories, Inc., Miami, Fla.) with 20% fetal calf serum.

Assay for binding of purified gp120 to CD4 and measurement of affinity constant. Purified gp120 from HTLV-III_B (14) was radiolabeled with ^{125}I by using Bolton and Hunter reagent (Amersham Corp., Arlington Heights, Ill.) to a

* Corresponding author.

specific activity of approximately 1 μCi/μg. Approximately 2.5×10^5 cpm of this solution was incubated for 30 min with 1×10^6 CD4-positive MOLT-4 cells with serial dilutions of serum or antibody in a total volume of 100 μl. The cells were washed by centrifugation through a mixture of 60% dibutyl phthalate and 40% bis(2-ethylhexyl) phthalate. The amount of gp120 bound to the cells was determined by counting the cell pellet in a gamma counter. Each point was performed in duplicate, and the OKT4A antibody was used as a positive control for complete blockade of gp120 binding to CD4. The 50% inhibitory titer was defined as the inverse of the serum dilution which reduced the bound counts per minute to one-half the counts per minute bound in the presence of normal serum. The affinity constant of gp120 binding to CD4 was determined by performing the assay in the presence of increasing levels of unlabeled gp120. The numbers of total input, bound, and free counts per minute at each point were used to numerically derive the affinity coefficient and perform a Scatchard analysis. This was done with the aid of the EBDA computer program (18). The result shown was the mean of two experiments. In all cases, the calculated standard error was less than 20%.

Assay for binding of whole HTLV-III$_B$ to CD4-positive cells. The procedure for the HTLV-III$_B$ binding assay has been previously described (17). Briefly, concentrated virus was incubated with CEM cells, washed, and suspended in a solution of fluorescein isothiocyanate-labeled anti-HIV serum. After another wash, the cells were fixed in paraformaldehyde and analyzed by a fluorescence-activated cell sorter.

RESULTS

Failure of potent HIV type 1-neutralizing antisera to prevent gp120 binding to CD4. Previous studies have shown that goats immunized with recombinant HTLV-III$_B$ gp160 envelope precursor or synthetic envelope fragments develop potent isolate-specific antivirus antibodies, as measured by virus infectivity and cell fusion assays (23, 28). Precise mapping of the responsible epitopes revealed that the major target of the envelope antisera fell within a 24-amino-acid sequence of the virus envelope glycoprotein between gp120 residues 307 and 330. This sequence was ultimately reproduced by the synthetic peptide designated RP135 (27).

The segment of the HTLV-III$_B$ envelope glycoprotein corresponding to the RP135 peptide is also the target epitope of two monoclonal antibodies, designated 9284 and 0.5β, which possess virus-neutralizing activity. The biologic activities of these antibodies (virus neutralization and inhibition of syncytium formation), as in the sera from animals immunized with the recombinant and synthetic proteins, is restricted to the III$_B$ isolate of the virus (15, 29a). Thus, independent experiments with monoclonal antibodies and sera from immunized animals have demonstrated that this hypervariable region of the gp120 envelope glycoprotein contains the immunodominant epitopes for the development of isolate-specific in vitro anti-HIV activity.

It is not known how the antibodies directed against RP135 interfere with virus infection processes. To investigate this, we initially examined the possibility that they act at the initial step in virus infection, which is thought to be gp120 attachment to CD4 (4, 9, 16). Purified gp120 from infected cells (14) was radiolabeled with iodine-125, and the characteristics of binding of the glycoprotein to CD4-expressing cells were examined. A Scatchard plot of the results is presented in Fig. 1. The dissociation constant was found to be 3.6×10^{-9} M, which is similar to the value reported for the recombinant gp120 produced in Chinese hamster ovary cells (12). The specificity of the gp120 binding is demonstrated by the blockade of binding exhibited by the OKT4A antibody (Fig. 2).

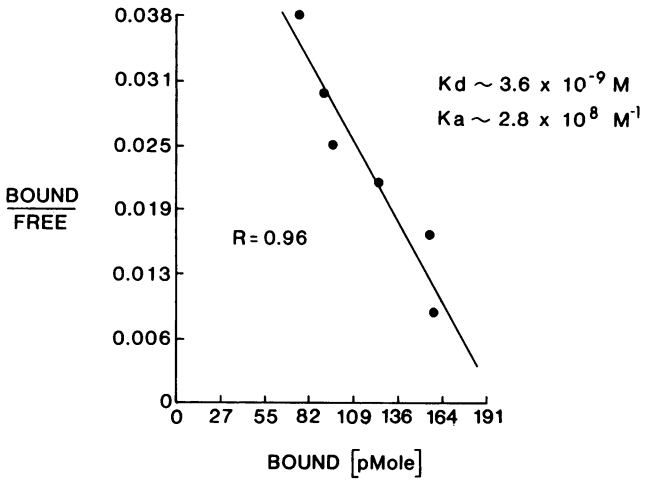

FIG. 1. Scatchard plot of purified ^{125}I-labeled gp120 binding to CD4-bearing MOLT cells.

When the gp120 binding assay was performed in the presence of the neutralizing monoclonal antibodies 0.5β and 9284, there was no inhibition of binding to the CD4+ cells (Fig. 2), even at antibody concentrations 50-fold greater than those required to block infection (29a). Similarly, antiserum to the PB1 peptide showed no inhibitory effect at a 1-to-20 dilution (Fig. 3). The neutralization titer of this serum for HTLV-III$_B$ was about 1,000. While antisera to the baculovirus-expressed gp160 did reproducibly show some blocking activity at high serum concentrations (Fig. 3), the effect was apparently related to antibodies directed at regions of the envelope other than the hypervariable sequence contained within PB1. From these data, we conclude that the potent isolate-restricted neutralizing antibodies which recognize the portion of the virus envelope represented by the RP135 peptide do not prevent the virus from binding to its CD4 receptor.

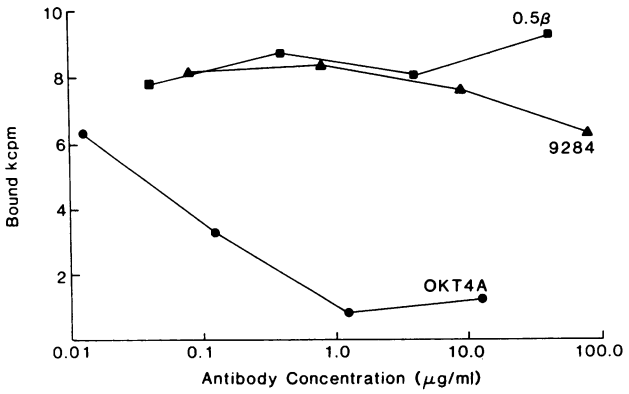

FIG. 2. Binding of purified gp120 envelope glycoprotein to CD4-positive cells in the presence of neutralizing monoclonal antibodies. Radioiodinated gp120 was allowed to bind to the cells in the presence of increasing amounts of 9284, 0.5β, and OKT4A monoclonal antibodies.

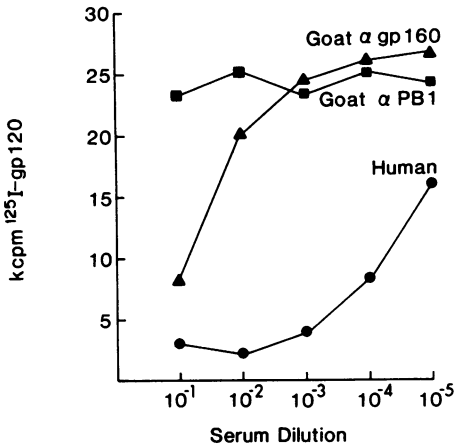

FIG. 3. Blockade of gp120-CD4 binding by HIV-infected human serum and immunized goat serum. The amount of radiolabeled gp120 bound to CD4 was measured with serial dilutions of serum as described in Materials and Methods.

In contrast to the heterologous antibodies, serum taken from humans infected with HIV exhibited high titers of blocking antibodies which prevent purified gp120 from binding to CD4. This is demonstrated in Fig. 3, in which the patient serum is compared with PB1 and gp160 antisera. To ensure that those data were not an artifact resulting from the use of the purified glycoprotein, the sera were also tested for the ability to prevent whole-virus binding to CD4-bearing cells (17). As previously found (17), serum from HIV-infected humans prevented the virus particle from binding to CD4, in agreement with the results obtained using soluble gp120 (Table 1).

Association of human neutralizing activity and gp120-CD4-blocking antibodies. To examine the antivirus activity of human serum, a number of serum samples from HIV-infected patients were screened for inhibition of syncytium formation. In general, only a minority of these serum samples have been found to exhibit inhibitory activity even at a final dilution of 1 to 10 in the assay. One of the most active human serum samples we have identified is shown in Fig. 4A titrated in parallel with a goat anti-gp160 serum for the inhibition of syncytium formation. The human serum induced complete fusion inhibition at a 1-to-20 dilution, while partial protection was seen at a 1-to-40 dilution. Figure 4B shows the results when the same serum was tested at the 1-to-40 dilution with increasing levels of the HTLV-III$_B$ recombinant PB1 peptide. The peptide did not abrogate the fusion inhibition of the human serum, and this result contrasted sharply with the ability of the peptide to inhibit the antifusion activity of gp160 antiserum. Similar results have been obtained with the limited number of other human serum samples that we have found to prevent HTLV-III$_B$-induced syncytium formation. Thus, the syncytium-inhibiting activity of human serum differs from those of the experimental sera raised against the envelope glycoprotein of HTLV-III$_B$ in that the human serum activity is not attributable to antibodies against the peptide sequence contained in the RP135 region.

TABLE 1. Serum blockade of purified-gp120 and whole-virus binding to CD4

Serum	Blocking titer for:	
	Purified gp120	Whole-virus HTLV-III$_B$
Normal human	<10	<100
Infected human	2,000	800
Normal goat	<10	<100
Goat anti-RP135	<10	<100
Goat anti-PB1	<10	<100
Goat anti-gp160	37	<100

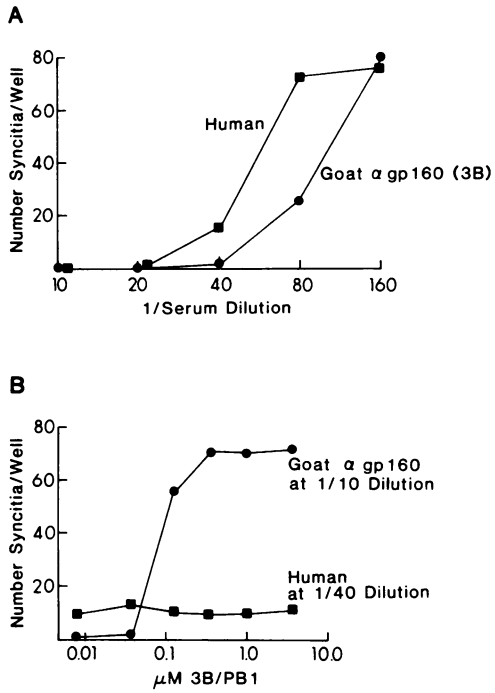

FIG. 4. Syncytium inhibition by sera from an infected human and a goat immunized with gp160 from HTLV-III$_B$. The assay was performed as stated in Materials and Methods. (A) Titration curve for these sera. (B) The assay was performed in the presence of the PB1 peptide from the III$_B$ virus isolate. Here, the concentrations of the human and goat antisera were held constant at a level sufficient for inhibition of infected-cell fusion.

It is therefore possible that the blockade of gp120-CD4 binding is the mechanism by which the human serum prevents viral infection when measured by in vitro neutralization assays. To investigate this, we compared the titers for virus binding and neutralization in 20 human serum samples. The results were plotted as shown in Fig. 5, and the data were subjected to a least-squares analysis. A correlation coefficient of 0.92 was found between the two assays. Although not conclusive, these findings are consistent with the notion that the broadly acting antivirus activity present in HIV-infected humans may be related to antibodies which prevent the virus from binding to its receptor.

DISCUSSION

Animals immunized with full-length HTLV-III$_B$ envelope gp160 (28) and with the recombinant sequence PB1 (23) produce high-titer isolate-restricted neutralizing and cell-fusion-blocking antibodies. More recent studies have shown that the principal blocking antibodies in these sera were directed at an immunodominant portion of the envelope which was reproduced with a synthetic peptide 24 residues

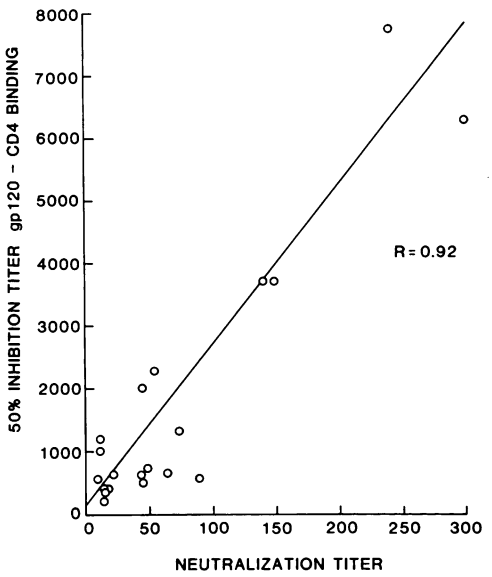

FIG. 5. Plot of virus neutralization versus blockade of gp120-CD4 binding. The neutralization and binding inhibition data from 20 HIV-infected humans were subjected to linear regression analysis, and the best-fit line was constructed as shown. R, Correlation coefficient.

long (27). Taking independent approaches, several other researchers have also identified synthetic peptides representative of this segment of gp120 which induce neutralizing antibodies (6a, 8, 21; W. Kenealy, personal communication). Similarly, two neutralizing monoclonal antibodies have also been found to bind the same region of the envelope glycoprotein (15, 29a). Thus, the portion of gp120 lying between residues 307 and 330 is apparently a major epitope for the development of high-titer, isolate-specific neutralizing antibodies against HIV type 1. Recently, four additional monoclonal antibodies against the envelope glycoprotein which possess isolate-restricted neutralizing properties have been described (32), although mapping studies to determine the precise binding epitope need to be performed.

Sequence analysis studies have shown that the immunodominant region of gp120, recognized by the neutralizing animal sera and monoclonal antibodies 0.5β and 9284, lies within a hypervariable region of the virus (1, 30, 35). This segment is thought to exist as a loop structure formed by the two disulfide-linked cysteine residues 302 and 337 (T. Gregory, Genentech, personal communication). The two cysteines are themselves highly conserved within the virus envelope glycoprotein. Thus, although the amino acid sequence within this loop is variable, the loop itself is conserved. While the function of this segment of the envelope is unknown, genetic manipulation studies have suggested that the region may be involved in the association of gp120 with the transmembrane component gp41 (10). In any case, the results reported here suggest that the site is not associated with CD4 binding domains of gp120, since antibodies to this region do not interfere with gp120-CD4 binding. It is therefore likely that such antibodies prevent virus infection at some step after virus attachment.

Serum samples taken from HIV-infected humans differ from those raised against envelope components in that human serum generally exhibits broad neutralizing activity (3, 13, 34) and contains a high titer of antibodies which block gp120 binding to CD4 (17). Presumably, the latter antibodies are directed at more conserved structures within gp120 associated with CD4 binding (10, 12). These antibodies may also be related to the broader neutralizing activity often noted in human serum. The approximate correlation of the gp120-CD4-blocking titer with neutralization titer (Fig. 5) is consistent with the possibility that these two activities are mediated by the same set of antibodies. However, the CD4 binding structure is apparently not the only viral target against which broadly neutralizing antibodies can be made. Indeed, other sites have been identified as conserved targets both on gp120 (7, 8) and gp41 (2, 8), as well as on the p17 gag protein (29).

Whatever the nature of the broader blocking activity recovered from human serum, our experiments indicate that it has been difficult to duplicate this response in animals immunized with virus envelope subunits. This raises the possibility that the envelope subunit immunogens used in the animal experiments were in large part denatured and thus did not display the appropriate epitopes which may be conformational in nature. However, it is also possible that such epitopes may naturally be poorly immunogenic and require prolonged exposure for an appropriate response. The poor immunogenicity of these epitopes is supported by studies of sequential sera from an HTLV-III_B-infected laboratory technician (W. A. Blattner, D. P. Bolognesi, M. Skinner, and T. Matthews, studies in progress) and from HTLV-III_B-infected chimpanzees (20). In these cases, an isolate-restricted neutralizing humoral response was noted shortly after seroconversion and this activity was primarily directed at the hypervariable loop (6a, 27; Blattner et al., in progress). Broadening of the humoral response to include other virus isolates was noted only later (over a year after seroconversion in the case of the laboratory technician), and the onset of broader antivirus activity was roughly associated with the gradual development of significant gp120-CD4-blocking activity.

Taken together, the observations reported here suggest that there are at least two classes of biologically active antibodies to HIV. One class is isolate restricted and is primarily directed to a hypervariable loop structure of gp120 that is not involved in CD4 binding. This site is immunodominant, and sensitive epitopes can be reproduced with synthetic peptides. The second class of neutralizing antibodies is directed at more conserved structures which may include those involved in CD4 binding.

It may be possible to induce protective immunity against the virus with a vaccine which takes advantage of either class of epitope, although there are many challenges yet to overcome. In order for the hypervariable loop to be used effectively, the problem of its extreme virus isolate specificity will need to be addressed, perhaps by using an appropriate "cocktail" of peptides from different viral isolates. Further insight into the usefulness of this epitope may be gained when its mechanism of action is more precisely determined. In order for the CD4 binding site of the envelope to be used effectively as an immune target, a method must be devised of presenting the epitope to the host in such a fashion that antibodies which will out-compete the high-affinity virus-receptor interaction are elicited.

ACKNOWLEDGMENTS

This work was supported by Frederick Cancer Research Facility (NCI) grant FOD-0758 and National Cancer Institute grant 5PO1-CA43447-03.

We gratefully acknowledge the expert assistance of Vicki Gibson in the preparation of the manuscript.

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