Human T-cell lymphotropic virus III\!* glycoprotein (gp120) bound to CD4 determinants on normal lymphocytes and expressed by infected cells serves as target for immune attack

(antibody-dependent complement-mediated cytolysis/antibody-dependent cellular cytotoxicity/acquired immunodeficiency syndrome/fluorescence-activated cell sorting)

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ABSTRACT The lymphocyte differentiation antigen CD4 serves as a receptor for human retroviruses associated with acquired immunodeficiency syndrome (AIDS) through its interaction with the major envelope virion glycoprotein, gp120, which is also expressed on the surface of infected cells. In these experiments, purified gp120 was shown to bind to normal human T-lymphocyte populations. The gp120-CD4 complex served as a target antigen for antibody-dependent complement-mediated cytolysis by a goat serum raised against native gp120. However, patient sera that bound to gp120-adsorbed cells failed to direct their destruction in the presence of complement. In contrast, these sera were potent mediators of antibody-dependent cellular cytotoxicity. These studies demonstrate that gp120 situated on the cell surface can serve as an effective target for immune destruction by patient antibodies and effector lymphocytes. The possible contribution of this type of immunity to control of disease progression, on the one hand, and to lymphocyte destruction and immunopathology observed in AIDS, on the other, is discussed.

The most profound hematologic feature associated with acquired immunodeficiency syndrome (AIDS) is the functional impairment and quantitative depletion of the subset of lymphocytes that express the CD4 surface antigen (1-6). Infection of CD4 cells in vitro results in the rapid spread of virus throughout the culture and subsequent cell death due to the as yet undefined process of virus-induced cytopathology (7-12). This rapid in vitro lympholysis is not, however, representative of the clinical disease state. Evidence suggests that fulminant viremia accompanied by large scale infection of CD4-positive lymphocytes does not occur at any stage of disease. Harper et al. (13) reported that by in situ hybridization analysis the number of infected cells expressing viral RNA in either peripheral blood or lymph nodes remained well below 0.01% in both early and late stages of AIDS. While it is likely that more cells are infected than are actively expressing viral information, the degree of lymphocyte depletion does not appear to be solely due to infectious virus spread but may involve other processes not directly linked to virus infection.

The current body of evidence suggests that the T-cell lymphotropic properties of the causative agent virus are due, in large part, to the interaction between the major virion envelope glycoprotein (gp120) and specific epitopes of the CD4 antigen complex. Monoclonal antibodies directed against particular determinants of gp120 have been shown to block both infection (14, 15) and multinucleated giant cell formation (16), in addition to precipitating gp120-CD4 complexes from infected cells (17). Recent studies revealed that purified gp120 can effectively block cell fusion, most likely by competing for available CD4 sites on noninfected cells (18). In addition, purified gp120 readily associates with the CD4 molecules and forms a stable complex on the cell surface (19). This latter finding suggested the possibility that free gp120 bound to noninfected CD4-expressing lymphocytes could serve as a potential target for immune attack resulting in subsequent lymphyolysis.

MATERIALS AND METHODS

Cells. Normal CD4-positive lymphocyte populations (CD4 cells) were obtained by stimulating normal donor peripheral blood mononuclear cells with tetanus toxoid for 5 days, sorting for CD4 expression, and expanding in the presence of recombinant interleukin 2. CEM cell lines (CEM) or human T-cell lymphotropic virus III\!* (HTLV-III\!*) chronically infected CEM (CEM/III\!*) have been described (19).

gp120 and Goat gp120 Antisera. The gp120 was isolated from HTLV-III\!* infected H9 cells as described (18). Briefly, cells were lysed with 0.5% Triton X-100, and the soluble fraction was initially passed over an affinity resin containing human high-titered antibodies followed by lectin lectin chromatography. Details of the generation of goat antiserum to gp120 have been recently reported (20). Monoclonal antibody to HTLV-III\!* gp120 was the generous gift of Robert Ting (Biotech).

Adsorption of gp120 to Noninfected Cells. Four million noninfected CD4 cells or CEM cells were placed in duplicate tubes containing 1 ml of medium (RPMI medium/10% fetal calf serum). Approximately 2.5 nM of purified gp120 was added in 10 nL to one tube. Cells were incubated at 37°C for 90 min with occasional resuspension. After washing in 200 vol of medium, cells were resuspended in phosphate-buffered saline containing 2% fetal calf serum and 0.1% sodium azide (PBS/FCS/NaNO\!*) to undergo fluorescence-activated cell sorting analysis.

Abbreviations: AIDS, acquired immunodeficiency syndrome; ACC, antibody-dependent complement-mediated cytotoxicity; ADCC, antibody-dependent cellular cytotoxicity; HIV, human immunodeficiency virus; HTLV, human T-cell lymphotropic virus.
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The Executive Committee of the International Committee on Taxonomy of Viruses (ICTV) has endorsed the name human immunodeficiency virus to be abbreviated HIV recently proposed by a majority of the members of a study group of ICTV as appropriate for the retrovirus isolates implicated as the causative agents of acquired immunodeficiency syndrome ([1986] Science 232, 1486 and (1986) Nature [London] 321, 644). In this paper, the term HTLV is retained for specific isolates.

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Flow Cytometry. Live cells were stained for immunofluorescence analysis by incubating $10^6$ cells in 100 μl of PBS/FCS/Na$_3$ containing a 1:20 dilution of OKT4 monoclonal antibody, OKT4A monoclonal antibody, monoclonal anti-gp120 antibody, or human serum for 30 min on ice, followed by washing (40 vol) and resuspension in 100 μl of a 1:20 dilution of affinity-purified fluorescein isothiocyanate (FITC)-conjugated F(ab')$_2$ goat anti-mouse IgG (heavy- and light-chain specific), or FITC-conjugated F(ab')$_2$ goat anti-human IgG (heavy- and light-chain specific). Cells were incubated 30 min on ice, washed repeatedly, and resuspended in PBS/FCS/Na$_3$ containing 2% formalin. Cells were then analyzed on an EPICS 753 cytofluorograph (Fig. 1) or an Ortho Cytofluorograf H50-4 (Fig. 2) and linear green fluorescence (x axis) was plotted versus relative cell number (y axis).

Antibody-Dependent Complement-Mediated Cytolysis (ACC). Four million target cells were labeled with $^{51}$Cr (250 μCi; 1 Ci = 37 GBq) for 90 min in 1 ml of medium. For gp120-adsorbed targets, 2.5 μg of purified glycoprotein was added together with the $^{51}$Cr at the beginning of the labeling period. Cells were washed in ~200 vol of medium prior to their use as targets in each assay. Recombinant interleukin 2 (5 units/ml) (DuPont) was used in all assays involving CD4 cells. All experiments were performed in triplicate. Fifty microliters of heat-inactivated serum dilutions were combined with 50 μl of target cells (2 x $10^5$ cells) in round-bottom 96-well plates. Fifty microliters of diluted Low Tox H rabbit complement was added and plates were sealed, shaken, and then incubated at 37°C for 90 min. After centrifugation at 100 x g for 10 min, the radioactivity in 50 μl of cell-free supernatants was determined. Maximum release cpm was obtained by detergent (0.5% Triton X-100) lysis of input targets, and spontaneous release cpm was determined by wells receiving only target cells and rabbit complement. Percentage specific lysis was calculated by the formula: $\frac{\text{experimental cpm minus spontaneous release cpm}}{\text{maximum release cpm minus spontaneous release cpm}} x 100$.

Antibody-Dependent Cellular Cytotoxicity (ADCC). Fifty microliters of target cells (10^4 cells) was placed in flat-bottom 96-well plates followed by 50 μl of various dilutions of heat-inactivated serum. After incubation for 20 min at 37°C, 50 μl of peripheral blood mononuclear cells (3 x $10^5$ cells) from a normal donor was added and the mixture was incubated 4 hr. Radioactivity in the cell-free supernatants and maximum release cpm were obtained as for the ACC assay. Wells containing target cells and effector cells alone were used for spontaneous release. Percentage specific lysis was calculated as for ACC.

RESULTS

Binding of Various Antibodies to gp120 Associated with the Cell Surface. The availability of human lymphocytes exhibiting a single viral antigen at the cell surface would greatly facilitate the determination of the role of viral products as a target for immune attack. Initial studies were thus undertaken to determine the binding characteristics of gp120 to normal CD4-positive lymphocyte populations (CD4 cells). These CD4 cells were incubated with purified gp120 isolated from HTLV-IIIB-infected cells and subsequently assessed for ligand binding by cytofluorographic analysis. Using a monoclonal antibody against gp120, goat anti-gp120 serum, and patient serum, the binding of free glycoprotein to the surface of CD4-expressing lymphocytes was readily apparent with specific staining of >90% of cells (Fig. 1 E–J). The gp120 bound to and blocked the OKT4A-defined epitope of CD4 (Fig. 1 C and D) in a manner similar to that reported by McDougal et al. (21) using intact virions. gp120 concentrations of 20 nM routinely blocked the OKT4A staining of 4 x $10^6$ normal CD4 cells. In contrast, expression of the OKT4-defined epitope (Fig. 1 A and B) was unaffected by gp120 binding, indicating the specificity of binding to a subregion of the CD4 molecule.

Since the orientation and expression of gp120 bound to noninfected lymphocytes may be different than that expressed on either virions or virus-infected cells, the ability of the anti-gp120 antisera to bind to infected cells and gp120-adsorbed cells was also examined. The lack of chronically infected CD4-positive lymphocyte cell populations necessitated the use of CEM and chronically infected CEM (CEM/IIIB) cell lines for this analysis (19). Cytofluorography revealed that all of the antibodies tested bound the virus-infected cells (Fig. 2 B, E, and H) in a manner similar to the gp120-adsorbed CD4 cells (Fig. 2 C, F, and I). However, mean fluorescence was higher in gp120-adsorbed CD4 cells, suggesting that antigen density was greater. The high-titered goat anti-gp120 antisera bound in a pattern similar to the monoclonal anti-gp120.

ACC. The extensive reactivity of these antibodies with the gp120 expressed by infected cells or adsorbed onto cells led to an investigation of their ability to mediate cellular destruction. Initially, ACC was evaluated. As shown in Table 1, ACC goat anti-gp120 antiserum specifically lysed the gp120-adsorbed cells, with maximal lysis of 49.5% at a 1:20 dilution. Furthermore, goat anti-gp120 antiserum also lysed infected cells (not shown). In contrast, randomly chosen patient sera failed to produce significant lysis over control values, even though these sera readily bound to the surface of the gp120-adsorbed target cells (Fig. 1 J). Repeat experiments using different complement sources (i.e., guinea pig, baby rabbit, and human) failed to reveal direct cytolytic activity with patient sera (data not shown), in agreement with studies using virus-infected target cells (22).

ADCC. Our previous work in murine retrovirus tumor systems had highlighted ADCC as an effective means of target-cell destruction, both in vitro and in vivo, which could occur even with antibodies that were relatively ineffective in ACC (23–27). Therefore, the ability of patient sera to participate in ADCC was investigated. The results (Table 1, ADCC) demonstrate that all patient sera tested were capable of directing ADCC activity against the gp120-adsorbed target cells. The adsorption of gp120 onto CD4-positive cells consistently increases their lytic susceptibility 8- to 10-fold in the presence of patient antibodies and normal effector cells. Antibody titrations consistently revealed that maximal ADCC was apparent only at high dilutions of patient sera (1:10^2 to 1:10^6). The inability of the goat serum to mediate ADCC may reflect the inefficient binding of goat IgG by human Fc receptor-bearing cells.

The CEM and chronically infected CEM cell lines were again used to determine the role of ADCC against infected cells. As shown in Fig. 3, patient sera failed to lyse noninfected CEM cells in the presence of normal donor lymphocytes. A direct comparison of ADCC against CEM/IIIB and CEM with gp120 adsorbed on the surface revealed distinctive features (Fig. 4). First, the lysis of gp120-adsorbed cells was consistently greater than that of chronically infected CEM with serum from patient 1 as well as other patient sera tested (not shown). Second, whereas maximum lysis of CEM/IIIB cells always occurred at the highest concentration of patient sera, peak lysis of gp120-adsorbed targets was consistently preceded by a "prozone" of suboptimal lysis. Most sera had a peak lysis of gp120-adsorbed cells at dilutions of 1:10^2 to 1:10^4. Differences between lysis of infected and gp120-adsorbed cells may be due to either qualitative or quantitative expression of gp120. Moreover, the exposed epitopes of gp120 on adsorbed cells may not be expressed to the same
extent on chronically infected cells. Determining the relative sensitivity of infected cells to ADCC, however, is greatly confounded by the increased susceptibility of tissue culture lines to natural killer (NK)-cell lytic activity present in human peripheral blood mononuclear cell preparations. Furthermore, other viral antigens may also be expressed on the surface of infected cells, which could serve as additional targets for immune attack.

**DISCUSSION**

Antibodies in patients directed against gp120 have already been shown to exhibit virus-neutralizing activities (20) and may, therefore, be involved in preventing further spread of the virus. The finding that patient sera can direct the cell-mediated destruction of gp120-bearing cells indicates the presence of an additional barrier to HIV infection and disease progression. Generation of antibodies mediating ADCC following viral infection could lead to the elimination of infected cells expressing viral proteins on their surface. Moreover, if disease transmission involves not only free virus but also transfer of infected cells, pre-existing antibodies mediating cell lysis may thus be critical. The amount of antibody required to mediate ADCC is small. In a recently completed survey of 75 seropositive patients, we found that peak lytic activity was most frequently observed with serum dilutions in the range 1:10^2 to 1:10^4 (data not shown). Furthermore, ADCC mediated by patient sera appears to be broadly reactive, effectively directing the lysis of cells expressing gp120 from widely divergent HIV isolates (H.K.L., unpublished observation). These findings, in addition to a recent report demonstrating the absence of complement-dependent lytic activity against infected cells by patient sera (22), indicate that ADCC may assume an increasingly important role in the immune response to HIV infection.

**Fig. 1.** Reactivity of OKT4, OKT4A, and anti-gp120 monoclonal antibodies, goat anti-gp120 antiserum, and patient serum on CD4-positive lymphocytes (CD4 cells) and CD4 cells adsorbed with gp120 (CD4 cells + gp120). Linear green fluorescence (x axis) was plotted versus relative cell number (y axis).

**Fig. 2.** Reactivity of anti-gp120 monoclonal antibody, goat anti-gp120 antiserum, and patient serum on CEM cells, HTLV-IIIa-infected CEM cells (CEM/IIIa), and CEM cells adsorbed with gp120 (CEM + gp120). Linear green fluorescence (x axis) was plotted versus relative cell number (y axis).
Table 1. Antibody-mediated cytolysis of CD4-positive lymphocytes bearing gp120

<table>
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<tr>
<th>ACC</th>
<th>CD4</th>
<th>CD4 + gp120</th>
<th>ADCC</th>
<th>CD4</th>
<th>CD4 + gp120</th>
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<tr>
<td>Normal goat</td>
<td>2.1</td>
<td>5.1</td>
<td>2.9</td>
<td>4.6</td>
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<tr>
<td>Goat anti-gp120</td>
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<td>49.5</td>
<td>2.5</td>
<td>4.2</td>
<td></td>
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<tr>
<td>Normal human</td>
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<td>3.6</td>
<td>4.1</td>
<td>4.4</td>
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<td>3.6</td>
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<tr>
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<td>0.8</td>
<td>2.9</td>
<td>18.7</td>
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<tr>
<td>Patient 3</td>
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<td>1.5</td>
<td>3.0</td>
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</tr>
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<td>Patient 5</td>
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<td>1.7</td>
<td>15.0</td>
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</tr>
</tbody>
</table>

Results are expressed as % specific lysis.

role as a possible preventive or therapeutic approach to patients with HIV infections.

The effector cells used to measure ADCC in the present study were obtained from normal donor peripheral blood mononuclear cells. We have recently found that similar cellular elements also exist in asymptomatic HIV infected individuals (H.K.L., unpublished observation). Whether these effectors are present in all HIV-seropositive patients is under investigation, particularly in relation to disease progression. If this mechanism is operative in vivo, control of virus infection may be limited by effector cell depletion or dysfunction in spite of the presence of antiviral antibodies. Therapeutic approaches might thus include stimulation of patient effector cells or adoptive immunotherapy with lymphokine-activated or expanded cells. Patient ADCC effector cell function is highly augmentable with interleukin 2 (29).

While destruction of chronically infected cells may be a beneficial result of ADCC in infected patients, such antibodies could also direct the lysis of noninfected CD4-positive cells if they adsorbed gp120 in vivo and became susceptible to immune attack as demonstrated here. Such an event could represent one of the many possible mechanisms of lymphocyte destruction contributing to the overwhelming lymphopenia seen in AIDS. A major issue to be resolved within the context of normal lymphocyte destruction in virus-infected individuals is whether free gp120 is released from infected cells and is actually able to circulate and bind to CD4 cells. A search for gp120 bound to CD4 cells in patients has thus far been unsuccessful. However, most seropositive individuals exhibit detectable anti-gp120 antibody reactivities, suggesting that gp120 sites on such cells may be covered by a patient's own antibody. The finding here and in another study (19) that purified gp120 blocks subsequent staining with OKT4A but not OKT4 monoclonal antibodies may provide an indirect means of assessing the extent to which gp120-adsorbed cells may exist in patients. Discrepancies in OKT4A and OKT4 staining of patient lymphocytes would at least be suggestive of gp120 situated on the cell surface. Likely sites for detecting gp120-adsorbed CD4 cells might be secondary lymphoid organs such as lymph nodes and spleen. While residing therein, such cells could not only serve as a target for immune attack but also as a stimulus to other immunocytes including CD8 cytotoxic T cells. A cascade of gp120 adsorption, immune stimulation, and target cell lysis could ultimately result in CD4 depletion and CD8 cell expansion—a scenario typical in lymph nodes of AIDS patients.

A recent report by Rook and co-workers (28) suggested that HIV p24 may be a target for antibodies directing ADCC based on a correlation between the disappearance of anti-p24 antibodies and ADCC activity. Although in our present study gp120 is clearly an ADCC target, we have little direct evidence of a similar role for p24. Cytotoxicity analysis of infected cells using high titered monospecific anti-p24 sera revealed very low levels of cell surface p24. Furthermore, our preliminary results indicate that a number of sera devoid of anti-p24 activity by immunoblot can still direct ADCC (H.K.L., unpublished data). In contrast, we have been unable to demonstrate ADCC using sera devoid of gp120...
antibodies. Taken together these results suggest that the role of p24 as a target antigen for ADCC is, at best, unclear.

Quite separate from the possible clinical significance of the present findings, the adsorption of purified gp120 to normal lymphocytes provides an important investigative tool since it allows one to define the specificity of an immune response by individualizing the target antigen. Specific cellular anti-viral immunity appears to exist in infected individuals when tested against autologous CD4 cells with adsorbed gp120 (K.J.W., H.K.L., T.J.M., Ahearne, K. C. Stine, D. T. Durach & D.P.B., unpublished data). This approach lends itself to the analysis of various arms of the cellular compartment of the immune system as to how each responds to the infecting agent.

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