Enzyme-Linked Immunoassay for Human Immunodeficiency Virus Type 1 Envelope Glycoprotein 120

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Received 2 May 1990/Accepted 24 September 1990

An enzyme-linked immunosorbent assay (ELISA) that can measure picogram quantities of human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein 120 (gp120) in cell culture medium or body fluids has been developed. Recombinant, soluble CD4 immobilized in microtiter trays was used to capture gp120, which was then detected with polyclonal sheep antibody to gp120 followed by biotinylated rabbit anti-sheep immunoglobulin G and an avidin-alkaline phosphatase indicator system. With a reference recombinant gp120, the assay showed a linear relationship between optical density and concentrations ranging from 60 to 6,000 pg/100- μ l well; precision of the assay varied with the concentrations and ranged from $\pm 40\%$ with amounts smaller than 200 pg to $\pm 10\%$ with amounts larger than 200 pg. In a group of coded samples containing 60 pg (approximately 10^7 molecules) of reference gp120, the assay correctly identified the samples as containing gp120 99% of the time, with no false-positive results recorded for blank samples. Recombinant gp120 prepared in another cell culture system demonstrated a binding coefficient 13-fold lower than that of reference gp120. Mixing standard amounts of reference gp120 with increasing concentrations of human sera reduced assay sensitivity, although the linear relationship between gp120 concentration and optical density remained. With this assay we were able to detect gp120 in HIV-1 suspensions prepared from cultured lymphoblastoid cells and in the sera of HIV-1-infected patients. This ELISA for gp120 should be useful for studying the biological role of gp120 in HIV infection.

Systems for the detection of viral proteins in cell culture and body fluids have proven to be useful for diagnosis of viral infections and for understanding the pathogenesis of viral infections in patients, animal models, and cell culture. Tests for detecting the reverse transcriptase and the p24^{gag} protein of human immunodeficiency virus (HIV) are currently available in research laboratories and some clinical laboratories. However, HIV type 1 (HIV-1) envelope glycoprotein, the gp160-gp120-gp41 complex, is a virion component which is crucial to the pathogenesis of infection. Binding of gp120 to CD4 is pivotal for initiating infection as well as facilitating fusion between infected and uninfected cells (6, 10, 12, 13, 19); antibodies to gp120 and CD4 itself will block infection in many systems (7, 17). Some studies have suggested that gp120 itself is immunosuppressive (3, 16) and that CD4-bearing cells which have bound gp120 may serve as targets for immune attack (14, 25). A simple, reproducible, and sensitive quantitative assay for gp120 would be a valuable tool for studying the pathogenesis of HIV infection and might also be useful clinically for diagnosing and staging HIV infection. In this paper, we report the development of a sensitive, specific, precise, and quantitative enzyme-linked immunosorbent assay (ELISA) for gp120 employing recombinant, soluble CD4 (rsCD4) as a solid-phase capture system.

MATERIALS AND METHODS

rsCD4 cloned from human lymphocytes (SKF 106528) was a generous gift from Ray Sweet of SmithKline Beecham Pharmaceuticals, King of Prussia, Pa. (7). rsCD4 was pro-

vided at a concentration of 1 mg/ml in 50 mM sodium phosphate buffer, pH 7. Recombinant, full-length, glycosylated HIV-1 IIIB envelope gp120 (SKF 16872-031489) was a generous gift from Jeffrey Culp of SmithKline Beecham Pharmaceuticals. This material was secreted from a stably expressing *Drosophila* cell line (4) and had the same binding coefficient for rsCD4 as native HIV-1 gp120 (4). The amino terminus of this protein was determined to be GARSD, where D is at the predicted position +32 in the BH10 sequence (22); the additional four amino acids derive from the leader used in the expression system. The carboxy terminus is predicted to be R +481 (22), which is the gp120-gp41 boundary. Samples were supplied at a concentration of 1.18 mg/ml in phosphate-buffered saline (PBS), pH 7. A second recombinant, full-length, glycosylated HIV-1 gp120 prepared by baculovirus transfection of ovarian cells of the *Autographia californica* moth was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and was designated NIH:HIV gp120. This product was supplied by American Biotechnologies, Inc., Cambridge, Mass. Polyclonal sheep antisera raised against a single synthetic epitope of HIV-1 gp120 (catalog no. 6205; Enzymes International, Inc., Fallbrook, Calif.) were prepared by immunizing sheep with a single peptide corresponding to the epitope of HIV-1 envelope gp120 and purified by affinity chromatography with the same antigen conjugated to Sepharose. Antibody was reconstituted in 0.01 M PBS (pH 7.4) to a concentration of 1 mg/ml. For testing, the antibody was diluted 1:1,000 in diluting buffer (see "Assay procedure"). A polyclonal goat antiserum to polyacrylamide gel electrophoresis-purified human T-cell lymphotropic virus type IIIB (HTLV-IIIB) envelope gp120

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(517/D-00145) was kindly provided by Stephen W. Pyle and Larry O. Arthur, AIDS Vaccine Program, Program Resources, Inc., NCI-Frederick Cancer Research Facility, Frederick, Md., under contract N01-C0-74102 (21, 23). A second polyclonal sheep HIV-1 envelope gp120 antiserum was obtained from Michael Phelan, Food and Drug Administration, through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and was designated NIH: antiserum to HIV-1 gp120. Biotin-conjugated, affinity-purified rabbit anti-sheep immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was diluted 1:1,000 (0.7 µg/ml) with diluting buffer.

Assay procedure. Immulon-2 96-well, flat-bottom microtiter trays (Dynatech Laboratories, Inc., Chantilly, Va.) were incubated overnight at 4°C with 1 µg of rsCD4 in 100 µl of coating buffer (0.1 M sodium carbonate, pH 9.6) per microtiter well. After incubation, rsCD4 was removed and replaced with 200 µl of diluting buffer (human sera from pooled HIV-seronegative blood obtained from M. A. Bioproducts, mixed one part to nine parts of 0.1 M PBS-0.05% Tween 20-0.1% sodium azide-1% bovine serum albumin [BSA], pH 7.5) per well, and the mixture was incubated for 45 min at 25°C. Gentle agitation during each of the following incubations was used to improve mixing. Diluting buffer was removed, and replicate 100-µl samples of reference gp120, virus suspensions, or serum specimens diluted in the appropriate amount of diluting buffer were placed into each well. Samples were incubated for 25 min at room temperature. Three consecutive washes with 200 µl of wash buffer (0.1 M PBS-0.05% Tween 20-1% BSA-0.1% sodium azide, pH 7.5) followed this and each of the incubation steps that followed. Then 100 µl of primary antibody, sheep anti-gp120 diluted in diluting buffer, was added to each well, and the mixture was incubated for 25 min, followed by three consecutive washes with wash buffer. One hundred microliters of affinity-purified, biotin-conjugated, rabbit anti-sheep immunoglobulin G diluted 1:1,000 in diluting buffer was incubated at room temperature for 25 min, followed by three consecutive washes. Avidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc.) was diluted 1:2,000 in diluting buffer, 100 µl of substrate (1 mg of *p*-nitrophenylphosphate per ml in 1 M diethanolamine from Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was added to each well, and the mixture was analyzed at a wavelength of 405 nm for change in optical density between 3 and 33 min after the addition of substrate. Samples were analyzed with a Multiscan MCC/340 MK II type 347 automated ELISA reader (Flow Laboratories, Inc., McLean, Va.), which reads the entire 96-well plate in 2 s. The mean optical density value for the blanks for each run was subtracted from the sample values for data presentation analysis. Blanks and calibration standards were always run in the same assay as the unknown specimens.

RESULTS

This ELISA for gp120 demonstrated a linear relationship between the reference gp120 concentration and optical density over a 100-fold range, from 60 to 6,000 pg/100-µl assay well (Fig. 1). With more than 6 ng, substrate conversion occurred too rapidly to provide accurate optical density readings over the 30-min period selected for the assay. The lower limit of reliable detection of reference gp120 was between 30 and 60 pg. All samples tested with 60 pg/100 µl showed optical densities consistently above background,

while only 50% of the samples containing 30 pg registered optical densities above background. When run in one assay, 25 replicate samples each containing 60 pg of reference gp120 showed a mean optical density of 0.049 with a standard deviation of 0.007 (Fig. 2), while 25 blank samples registered a mean optical density of 0.027 with a standard deviation of 0.003. As these results were reproducible, 99% of samples containing ≥ 60 pg should have activity above background. Not surprisingly, the precision of the assay varied with the concentration of gp120 being measured. At concentrations below 200 pg/100-µl well, the variation in the assay increased progressively, such that at 60 pg/100-µl well, sample values had a range of 40% from the mean (Fig. 1). In contrast, at concentrations above 200 pg/100-µl well, the range was less than 10% of the sample value (Fig. 1).

Various attempts were made to improve the sensitivity and specificity of the assay. Increasing the concentration of the rsCD4 above 1 µg/100-µl well, increasing the incubation period with rsCD4 from 0.5 to 4 days, or precoating the microtiter trays with poly-L-lysine did not increase assay sensitivity. No differences in rsCD4 binding efficiency were noted among several brands of microtiter plates. Increasing the concentrations of the detector antibody resulted in increased background and did not improve assay sensitivity; however, the avidin-biotin system was clearly more sensitive than conventional single-antibody systems (data not shown).

To test the ability of the assay to detect gp120 in patient sera, a reconstruction experiment was performed. Reference gp120 at defined concentrations between 30 and 5,000 pg/100 µl was mixed with increasing concentrations of pooled normal human sera in diluting buffer, and the resulting mixtures were then routinely assayed. The sensitivity of the assay was reduced by human serum (Fig. 3). Undiluted sera from five patients (without HIV infection) showed the same results, reducing the apparent gp120 concentration by about 75%. The use of normal serum did not increase background optical density readings (data not shown). However, completely omitting human serum from the diluting buffer greatly reduced the reproducibility of the assay at lower concentrations because of high background optical density readings. Reproducibility was maximized by the use of 10% normal human sera in diluting buffer for all blocking, sample incubation, and antibody incubation steps in the assay.

In marked contrast to normal serum, however, the sera of patients with HIV infection blocked detection of gp120, even when 100 ng/ml was added (Fig. 3). Sera from HIV-infected patients (all of which contained antibodies to the virus by immunoblot) prevented detection of added reference gp120 until diluted between 100- and 100,000-fold in diluting buffer. However, preincubation of the rsCD4 capture system with the same patient sera prior to the addition of reference gp120 did not affect assay sensitivity (data not shown).

To confirm the specificity of this assay for gp120, two other anti-gp120 antibodies were substituted for the sheep anti-gp120 (standard anti-gp120): a polyclonal sheep antibody elicited to the whole gp120 molecule (sheep anti-gp120) and a polyclonal goat anti-gp120 (goat anti-gp120). Standard anti-gp120 and sheep anti-gp120 each produced superimposable standard curves for reference gp120 concentrations between 60 and 500 pg/100-µl well volume (Fig. 4). With more than 500 pg of reference gp120, assay values with sheep anti-gp120 were beyond the interpretable optical density range. Goat anti-gp120 also demonstrated a linear relationship between optical density and reference gp120 concentration but with less sensitivity than standard anti-gp120

FIG. 1. Relationship between reference gp120 concentration and optical density change. The inset provides a detail of this relationship at lower concentrations of reference gp120. Individual assay values are shown (●); values are the means and standard deviations (error bars).

(Fig. 4). While all three antisera to gp120 worked in this assay, the standard anti-gp120 provided the widest range of gp120 concentrations and the greatest sensitivity.

We have used this system to compare binding and detection of another recombinant, full-length, glycosylated gp120 molecule obtained from the AIDS Research and Reference and Reagent Program (moth gp120; see Materials and Methods for details). Moth gp120 demonstrated a binding coefficient 13-fold lower than that of reference gp120 (Fig. 5). This was not due to altered binding of the gp120 standard detector antibody, as the goat and sheep antisera gave equivalent results (data not shown).

Serum samples from 41 patients with HIV infection (sero-positive risk group member with AIDS or AIDS-related complex), all of whom were HIV p24 antigenemic with antigen concentrations up to 2,200 pg/ml (quantitation with Abbott HIV antigen ELISA and the AIDS Clinical Trial Group Virology Reference Laboratory Standards), were tested for the presence of circulating gp120. Only 11 of these samples had detectable gp120 in concentrations ranging from 250 to 2,000 pg/ml; no correlation was demonstrated between gp120 and p24 antigen concentrations (data not shown). In the 11 specimens positive for gp120, a confirmatory ELISA was performed in which the aforementioned sheep anti-gp120 was substituted for standard anti-gp120; all

11 remained positive, and the measured concentrations were within assay precision. We have also tested HIV suspensions derived from lymphoid cell lines and macrophages and have detected gp120 in them as well.

DISCUSSION

We have developed a simple, reproducible, sensitive, and specific assay for an important component of the HIV virion, the gp120 surface glycoprotein. The assay demonstrated a number of desirable features. The reagents used for the assay were largely defined proteins available in moderately large quantities. The assay could detect gp120 at ≥ 0.5 ng/ml, in the same range of sensitivity reported for other immunoassays, including the HIV p24 antigen assay. The specificity was also very high and was confirmed with other indicator antibodies. The performance of the assay system itself was typical of solid-phase microimmunoassays in that it was economical of reagents, rapid, semiautomated, and reproducible. The relationship between the concentration of reference gp120 and optical density was linear over a 100-fold range, from 60 to 6,000 pg/100- μ l well volume, and 60-pg amounts of gp120 could be detected reliably and without false-positive reactions. Despite many adjustments in assay

FIG. 2. Reproducibility of the gp120 ELISA at low gp120 concentrations. Twenty-five replicate samples each of diluting buffer (blank) and 60 pg of reference gp120 per 100- μ l microtiter well are compared. Individual assay values (●) are shown; optical density (◇) and standard deviations (error bars) are indicated next to each set of datum points. The separation between blank and 60-pg optical density readings (---) is shown.

procedure and materials, detection of gp120 at a concentration of less than 50 pg/100- μ l well was not reliable.

A recently described ELISA for gp120 and gp160 quantitation (18) compared five different antibodies against envelope glycoprotein in both the solid-phase capture and the detection systems. This assay, in which a particular sheep polyclonal antiserum is used for the solid phase and mouse antiserum is used as a detector, was sensitive to between 50 and 100 pg/100 μ l; however, no precision analysis was provided. Our system reliably detected 60 pg of gp120 in all samples tested and in 50% of samples containing 30 pg/100- μ l well. Samples analyzed in the assay described by Moore and Jarrett (18) did not include human sera, which

FIG. 3. Effect of human serum on detection of gp120. Increasing concentrations of reference gp120 were added to diluting buffer containing one of three concentrations of normal human sera (0, 20, and 100% NS) and were assayed. Reference gp120 was also assayed in undiluted sera from five HIV-seropositive patients (AIDS sera).

FIG. 4. Comparison of alternate detector antisera on quantitation of gp120. Two other antisera against gp120, sheep anti-gp120 (1:1,000) and goat anti-gp120 (1:1,000) were substituted for standard anti-gp120 (1:1,000) in assays of serial dilutions of reference gp120.

may limit its application to human specimens. With a solid-phase rsCD4, our ELISA provided specific, high-affinity binding of gp120 and allowed direct analysis of glycoprotein-CD4 interaction.

A 13-fold difference in binding coefficient was noted between reference gp120 and moth gp120. Interestingly, prior independent comparisons of the reference gp120 and moth gp120 with native gp120 by using the anti-gp120 solid-phase ELISA and the HIV binding competition assay found both similar to native gp120 (4, 8). The differences noted between these gp120 molecules, both recombinant, full-length, glycosylated proteins, suggested that minor variations in structure existed. In addition, the capture system utilized in this ELISA provided improved sensitivity, which allowed it to discriminate between these two gp120 molecules. Recent data from several investigators (5, 11) suggest

FIG. 5. Comparison of reference and moth gp120 binding to solid-phase rsCD4. Serial dilutions of reference gp120 were compared with those of another recombinant, full-length, glycosylated gp120 produced in an *A. californica* moth ovary cell culture system by using the described assay system.

that gp120s from different HIV strains may vary in their CD4-binding affinity and that gp120s from native isolates (recovered from patients without serial *in vitro* passage) have a lower CD4 affinity than laboratory strains such as HIV HTLV-III_B (12, 20, 22). The effect of these variations on the sensitivity of this assay system needs to be explored.

Sera from patients with HIV infection blocked detection of added reference gp120, and this inhibitory effect persisted until the sera were diluted 100- to 100,000-fold. However, the mechanism of this effect did not appear to be through binding to CD4 (17). These data suggest that these sera contain antibodies to gp120 of various titers, which prior studies have shown may block the gp120-CD4 interaction (1, 2, 15, 24), and further studies of this phenomenon are in progress. Potentially more interesting was the observation that sera from normal, HIV-uninfected subjects also reduced the assay sensitivity. Theoretically, this could result from proteolytic degradation of gp120; however, storage of reference gp120 with uninfected serum at various time periods had no influence on assay sensitivity, suggesting that proteolytic degradation had not occurred. Since CD4 is a constituent of normal cells, it is unlikely to bind serum proteins, and we were unable to block gp120 binding by prior incubation of the rsCD4 capture system with normal serum. Therefore, gp120 may bind normal serum components, such as the mannose-binding protein described by Ezekowitz and his collaborators (9), and further experiments to elucidate this phenomenon are under way.

We were able to detect native gp120 circulating in sera from some infected patients and in virus suspensions produced from cultured cells. Given the dual specificity of the assay system (rsCD4 and anti-gp120) and confirmatory testing with another anti-gp120, these reactions almost certainly indicate the presence of circulating HIV gp120. This system should therefore permit quantitative studies of gp120 to assess its role in the pathogenesis of HIV infection.

ACKNOWLEDGMENTS

Nancy McManus is thanked for skillful performance of p24 antigen assays, and Tarek Elbeik offered much helpful advice. Ray Sweet, Jeffrey Culp, and Martin Rosenberg, all of SmithKline Beecham, are thanked for generous supplies of rsCD4 and gp120.

This project was supported in part by NIH grant AI-25329 to J.M.

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