Detection of Binding Antibodies to Native and Recombinant Human Immunodeficiency Virus Type 1 Envelope Glycoproteins Following Recombinant gp160 Immunization Measured by Flow Cytometry and Enzyme Immunoassays

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The ability of antibody induced by vaccination with recombinant gp160 (rgp160) to bind to native and recombinant human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins was measured. Thirty-three HIV-1-seronegative healthy adult volunteers were injected four times with 40 or 80 µg of an HIV-1_{LAV} envelope glycoprotein candidate vaccine per dose. The vaccine consisted of rgp160 produced in insect tissue culture cells infected with a recombinant baculovirus which contains the gp160 gene from the HIV-1_{LAV} strain. By using a flow cytometric indirect immunofluorescence assay (FIFA) to detect vaccine-induced antibody to native envelope glycoprotein expressed by target cells infected with HIV-1_{IIIB}, sera from 9 of the 33 vaccinees were positive. These included sera from eight vaccinees which stained HIV-1_{IIIB}-infected cells and sera from two vaccinees which stained target cells infected with HIV-1_{MN}, a heterologous virus strain. None of the sera stained cells infected with the HIV-1_{RF} strain. Envelope glycoprotein-binding antibody was more frequently detectable in an enzyme-linked immunosorbent assay (ELISA) by using rgp160 compared with that which was detectable in the FIFA with uninfected target cells which were pulsed with rgp160 antigen. Positive correlations were observed between the rgp160 FIFA and a whole-virus-lysate enzyme immunoassay, between the rgp160 FIFA and the rgp160 ELISA, and between the rgp160 ELISA and the whole-virus-lysate enzyme immunoassay. The ability of sera from some volunteers who received rgp160 vaccine to bind to HIV-1-infected cells suggests that further studies with this vaccine should be done.

Analysis of the humoral responses to human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein may include assays that measure the antibody which binds to antigen presented in various ways. We were interested in measuring antibodies which recognize the native envelope glycoprotein expressed on infected cell surfaces, since these antibodies may be more likely to be functional *in vivo* than those which bind to denatured protein only. The antibody response to an HIV-1_{LAV}-based recombinant gp160 (rgp160) vaccine (VaxSyn; MicroGeneSys, Inc., Meriden, Conn.) was evaluated in normal healthy adult volunteers by three assays: a flow cytometric indirect immunofluorescence assay (FIFA), an rgp160 enzyme-linked immunosorbent assay (ELISA), and a whole-virus-lysate enzyme immunoassay

(EIA). These assays compared the ability of a candidate rgp160 vaccine to induce antibody which binds to the gp160 present on cells infected with HIV-1, the vaccine rgp160 antigen, and the gp160 present in whole virus lysate.

The FIFA method with HIV-1_{IIIB}-infected H9 cells has been found to be highly sensitive and specific for the detection of anti-HIV-1 antibody in sera from HIV-1-infected individuals (18). The FIFA method was used in the current study to measure antibody in sera from uninfected volunteers injected with an investigational vaccine, rgp160. H9 cells infected with additional strains of HIV-1 and uninfected H9 cells pulsed with rgp160 were also used as targets in the current study.

MATERIALS AND METHODS

rgp160 vaccine. The HIV-1 vaccine candidate (rgp160; VaxSyn; MicroGeneSys) is the envelope glycoprotein of HIV-1 (HIV-1_{LAV} strain). The gp160 gene was derived from an infectious molecular clone of HIV-1 (1, 23). The gp160 was produced in a continuous insect tissue culture cell line by infection with a recombinant baculovirus expression vector which contained the gp160 gene (2). The vaccine was formulated with purified gp160 and aluminum phosphate gel (alum adjuvant).

Subjects. Subjects in the study were healthy adult volunteers between the ages of 18 and 55 years without evidence of serious medical illnesses as determined by history and

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physical examination. Subjects were seronegative for HIV-1 as determined by EIA and Western blot (immunoblot) assays (see below) prior to immunization. They participated in a phase I trial of the immunogenicity and safety of the rgp160 vaccine. The details of the clinical design and results of the clinical study have been reported elsewhere (4). The institutional review boards of the participating institutions approved the study, and all participants gave written informed consent.

The participants whose sera were evaluated in this study received either 40 or 80 μ g of rgp160 per dose intramuscularly on days 0, 30, 180, and 545. Sera from 24 volunteers who received all four vaccine injections and from 9 volunteers who received only the first three vaccine injections were available for testing.

FIFA. The following three types of target cells were used: (i) cultures of uninfected H9 cells and CEM-NK^R cells were negative control targets; (ii) cultures of H9 cells chronically infected with HIV-1_{IIB}, HIV-1_{RF}, or HIV-1_{MN} expressed native envelope glycoprotein and other HIV-1 antigens; and (iii) cultures of H9 and CEM-NK^R cells were pulsed with rgp160 (baculovirus expressed recombinant, nonreplicating gp160, which was the immunogen). The H9 cell line was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, from Robert Gallo (10–12) and is a single cell clone derived from HUT 78 cells, which are a human cutaneous T-cell lymphoma cell line. CEM-NK^R is a human T-lymphoblastoid cell line and was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, from Peter Cresswell (7). The HIV-1_{IIB}, HIV-1_{RF}, and HIV-1_{MN}-infected H9 cell lines (human T-cell lymphotropic virus type III [HTLV-III] strain B/H9, H9/HTLV-III_{RF} NIH 1983, and H9/HTLV-III_{MN} NIH 1984, respectively) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, from Robert Gallo (5, 11–13, 17, 20). Uninfected H9 cells were added to cultures of H9 cells chronically infected with HIV-1 7 to 10 days before the assays were done to ensure good viral antigen expression on target cells.

The immunofluorescence assay was patterned after one developed by Sligh et al. (18) to detect antibody against HIV-1_{IIB}-infected cells present in sera from HIV-1-infected individuals. Target cells chronically infected with HIV-1 were simply washed and were then incubated with test serum without prior incubation with rgp160. Uninfected target cells were initially washed and were then labeled with envelope glycoprotein by incubation with 2.5 μ g of rgp160 per 10⁶ cells at 37°C for 90 min. In preliminary experiments, target cells incubated with as little as 1 μ g of baculovirus-derived gp120-fluorescein isothiocyanate (American Biotechnologies, Inc., Cambridge, Mass.) expressed >90% positive fluorescence. After the 90-min incubation with rgp160 (the immunogen), target cells were washed and resuspended in 150 μ l of RPMI 1640 medium; 25 μ l of a 1:10 dilution of the human test serum was then added. After a 20-min incubation with the serum at 4°C, the cells were washed, and fluorescein isothiocyanate-conjugated F(ab')₂ goat anti-human immunoglobulin G (IgG; Kallestad Diagnostics, Austin, Tex.) was added with bovine serum albumin for 30 min at 4°C to reduce nonspecific binding. After washing, the cells were fixed overnight in 1 to 2% (vol/vol) formaldehyde. After a final wash in phosphate-buffered saline (PBS), the logarithm of the green fluorescence intensity was analyzed on a FACScan

flow cytometer (Becton Dickinson Immunocytometry Systems, Brea, Mass.). The data were collected first into a forward-scatter versus right-scatter cytogram which displayed cell size and degree of granularity. This was gated to eliminate any particles which were not of sufficient size to be an intact cell. Cells passing through this gate were then displayed on a histogram of the logarithm of the green fluorescence intensity versus relative cell number. For each run of HIV-1-infected or rgp160-pulsed H9 cells, cells stained with control sera from known HIV-1-seropositive (infected, not vaccinated) and HIV-1-seronegative individuals were used to set an appropriate gate with which to determine the fraction of cells stained with test vaccinee sera that were shifted into the positive fluorescence intensity region as defined by the gate (see Fig. 1A and B). The fraction of cells with intensity shift above the gate (termed percent positive fluorescence) was then used as a measure of antibody content. Sera which stained envelope glycoprotein-expressing cells all resulted in a similar pattern of a shift in a single peak of cells depicted on the flow cytometric histograms (see Fig. 1A and B). This pattern of the logarithm of the fluorescence intensity shift indicated that the target cells were a homogeneous population with respect to envelope glycoprotein expression. While determination of the shift in mean channel fluorescence intensity would be an alternative method of analysis, there is precedent for our method (16, 18). In our study, the two methods of data analysis should provide similar results because of the shape of the cell histograms, because all of the vaccinee test sera stained envelope glycoprotein-expressing targets such that in no case was the fluorescence intensity of more than 90% of the cells above the gate level and because we did not observe shifts in the mean channel fluorescence intensity without a shift in the fraction of cells with fluorescence intensity above the set gate level. The location of the gate in terms of the logarithm of fluorescence intensity varied slightly from assay to assay because of variables such as the relative intensity of the fluorochrome and other instrument settings on a particular day. This did not affect the reliability of the assay, because the gate setting was based on the negative and positive control readings on any particular assay day.

Raw data derived from the flow cytometric histograms were used to determine the net percent positive fluorescence for each serum specimen and HIV-1 envelope glycoprotein-expressing target cell population, which was defined by the following equation: net percent positive fluorescence = [(percent positive fluorescence by using the test serum against HIV-1 antigen-expressing target cells) – (percent positive fluorescence by using the test serum against control target cells)] – [(percent positive fluorescence by using the negative control serum against HIV-1 antigen-expressing target cells) – (percent positive fluorescence by using the negative control serum against control target cells)]. A specimen was considered positive if the net percent positive fluorescence was ≥ 4.0 , which was approximately 10 standard deviations (SDs) above the mean net percent positive fluorescence of prevaccination sera tested. Positive control sera from HIV-1-infected patients and hyperimmune IgG (HIVIG; Abbott Laboratories, Inc., Abbott Park, Ill.) derived from pooled plasma of HIV-1-seropositive asymptomatic donors (3) had net percent positive fluorescence of >50. Testing of each serum sample with target cells that did not express HIV-1 antigen provided an internal control for each sample so that we could correct for antibody binding to target cells which was not specific for viral antigen.

Whole-virus-lysate EIA and Western blot. Sera from vol-

TABLE 1. Comparison of FIFA, whole-virus-lysate EIA, Western blot, and rgp160 ELISA^a

Day of study	FIFA (net % positive fluorescence) ^b		Whole-virus-lysate EIA (OD/cutoff ratio)	rgp160 ELISA reciprocal antibody titer	Anti-HIV-1 _{IIIB} reactivity by Western blot, antibody against:		
	rgp160-pulsed H9 cell targets	rgp160-pulsed CEM- NK ^R cell targets			gp160	gp120	gp41
0	0.0	1.3	0.29	<25	—	—	—
28	0.3	0.0	0.26	<25	—	—	—
30							
44	1.1	0.0	0.57	100	±	±	—
60	1.6	3.9	0.38	100	—	±	—
178	1.0	3.7	0.26	25	—	±	—
180							
194	13.9	10.3	2.39	1,600	—	+	—
365	0.0	0.0	0.44	25	±	±	—
540	0.0	0.0	0.33	25	±	—	—
545							
559	35.0	29.3	5.04	1,600	+	+	+
635	3.0	0.0	1.03	400	+	+	+

^a Sera from one volunteer who received four rgp160 vaccine doses (80 µg each on days 0, 30, 180, and 545) were tested.

^b Sera were diluted 1:10 prior to the addition of 10⁶ rgp160-pulsed target cells in suspension; the final serum dilution was approximately 1:70. Net percent positive fluorescence of ≥4.0 was considered positive.

unteers were tested for antibody to HIV-1_{IIIB} antigens by EIA by using commercially available kits (beads coated with detergent-disrupted HIV-1_{IIIB}; Abbott Laboratories, Inc., North Chicago, Ill.) and by Western blot analysis (DuPont Co., Wilmington, Del.) according to the manufacturer's guidelines. A positive result by EIA was defined as an optical density (OD)/cutoff ratio of ≥1. Western blot anti-HIV-1 antibody band intensity was considered ± if the intensity was less than that of the anti-p24 antigen band on the weakly positive control strip. The intensity of a 1+ band was at least equal to that of the anti-p24 band on the weakly positive control strip but was less than that of the anti-p24 band on the strongly positive control strip. The intensity of a 2+ band was at least equal to that of the anti-p24 band on the strongly positive control strip.

rgp160 ELISA. An ELISA with plates coated with the vaccine antigen was developed. The wells of 96-well Immulon 2 microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated with baculovirus-derived rgp160 (2 µg/ml diluted in PBS) at 4°C overnight and were then washed on the following day with water containing 0.05% Tween 20. Serial 1:4 dilutions of test sera (the lowest dilution was 1:25) were added, and the plates were incubated at 37°C for 1 h and were then washed again with 0.05% Tween 20 in water. Background was determined by the addition of the same serum dilutions to wells which had not been coated with rgp160. Rabbit anti-human IgG (gamma chain) antibody conjugated with horseradish peroxidase (Dako Immunoglobulins a/s, Denmark and Dako Corporation, Carpinteria, Calif.) was added; after 0.5 h at 37°C, the plates were washed again with 0.05% Tween 20 in water and *o*-phenylenediamine · 2HCl reagent (Abbott Laboratories, Inc., North Chicago, Ill.) was added. After incubation at room temperature for 30 min, the reaction was stopped by the addition of 2.5 M H₂SO₄. The OD at 490 nm was read. The antibody titer was that dilution of serum with an OD of >0.3 in rgp160-coated wells which was at least twice the background OD and no lower than the OD of that vaccinee's prevaccination serum diluted 1:25. The reciprocal titer of hyperimmune IgG (HIVIG) against baculovirus rgp160 was 400,000.

Statistical methods. Sera were tested as single replicates in

serologic assays, and results of repetitive testing in a subset of sera were reproducible. If the results for the positive and negative controls included in each assay were not as expected, the entire assay was repeated.

The mean net percent positive fluorescence and the mean OD/cutoff ratio are reported with the SDs of the means. Reciprocal geometric mean anti-rgp160 antibody titers are reported for the rgp160 ELISA results. The net percent positive fluorescence by rgp160 FIFA and the log (OD/cutoff ratio) and log (reciprocal antibody titer) values for each serum sample tested were compared by linear regression and by the Spearman rho correlation test. Significance testing for comparison of proportions was done by using chi-square analysis and Fisher's exact test. The Mann-Whitney test was used to compare means of unpaired datum points.

RESULTS

Comparison of the serologic assays. Sera collected at multiple time points from a subject who received 80 µg of rgp160 vaccine per dose were tested against H9 and CEM-NK^R cells coated with rgp160 (Table 1). Sera from day 194 (14 days after the third vaccine injection) and day 559 (14 days after the fourth vaccine injection) were both positive by rgp160 FIFA. The serum sample from day 559 did not stain H9 cells infected with HIV-1_{IIIB}, although it was the most positive by rgp160 FIFA of the sera from this vaccinee. Sera collected at other time points (days 44, 60, 178, 365, and 540), when a ± or 1+ envelope glycoprotein antibody band was present by Western blot analysis, were negative by rgp160 FIFA.

Anti-gp160 antibody titers by rgp160 ELISA were first detectable on day 44 in the volunteer described above (Table 1). The peak reciprocal antibody titer at day 559 (14 days after the fourth rgp160 vaccine injection) was 1,600. The whole-virus-lysate EIA became positive later than did the rgp160 ELISA, after the third and fourth vaccine injections. The highest OD/cutoff ratio occurred 14 days following the fourth vaccine injection.

Representative flow cytometric histograms show the staining of rgp160-pulsed cells and HIV-1_{IIIB}-infected cells to be a shift in a single peak, so that a greater portion of the

FIG. 1. Flow cytometric histograms showing relative cell number as a function of the logarithm of green fluorescence intensity. The vertical line is the gate, to the right of which cells were considered to be in the positive fluorescence intensity region. The narrow solid lines represent H9 cells stained with serum from vaccinee 1 obtained 14 days after the fourth 80- μ g rgp160 injection, and the bold solid lines represent H9 cells expressing HIV-1 antigens stained with the same serum sample. The dashed lines represent staining of H9 cells with the positive control anti-HIV-1 IgG preparation (HIVIG), and the dotted lines represent H9 cells expressing HIV-1 antigens stained with the same IgG (HIVIG) preparation. (A) HIV-1 antigen-expressing H9 cells were pulsed with rgp160; (B) HIV-1 antigen-expressing H9 cells were infected with HIV-1_{IIIB}. The percent positive fluorescence for each target cell population, which represents the fraction of cells with fluorescence intensity greater than that of the gate, was derived from these histograms. Net percent positive fluorescence was then calculated by further subtracting the background percent positive fluorescence of the test serum sample and a negative control serum sample (see text for equation).

relative cell number exhibited logarithmic green fluorescence intensity above the gate compared with that of the fraction of H9 cells that did not express envelope glycoprotein stained with the same serum with fluorescence intensity above that gate (Fig. 1A and B). The gate used to define the level of the logarithm of green fluorescence above which stained cells were considered to be positive was determined by using known negative and positive control standard sera (see Materials and Methods). This pattern was seen with the positive control anti-HIV-1 IgG (HIVIG), positive control sera from HIV-1-infected patients, and positive sera from vaccinees.

Sera from 24 volunteers (14 received 40 μ g of rgp160 per dose; 10 received 80 μ g of rgp160 per dose) obtained 14 days after the fourth rgp160 vaccine dose were tested by all three methods. All sera were positive by the rgp160 ELISA (Table 2). Sixteen of the 24 serum samples were positive by the FIFA by using rgp160-pulsed H9 cells, and 4 serum samples were positive by using H9 cells infected with HIV-1_{IIIB}.

Fifty-three available serum samples obtained after the third and fourth rgp160 vaccine injections (27 from 16 recipients of 40 μ g of rgp160 per dose; 26 from 17 recipients of 80 μ g of rgp160 per dose) were most likely to be positive by rgp160 ELISA. The 51 (96%) of 53 serum samples positive by rgp160 ELISA (reciprocal geometric mean titer of those 51 serum samples, 1,259) was a significantly greater proportion than the 36 (68%) of 53 serum samples that were positive by rgp160 FIFA (mean $\pm$ SD net percent positive fluorescence of those 36 serum samples, 23.4 ± 16.0) and the 24 (45%) of 53 serum samples positive by whole-virus-lysate EIA (mean $\pm$ SD OD/cutoff ratio of those 24 serum samples, 2.77 ± 1.65) ($P < 0.001$). The proportion of serum samples positive by rgp160 FIFA (36 of 53) was also significantly greater than that positive by whole-virus-lysate EIA (24 of 53) ($P < 0.05$). The two serum samples that were negative by rgp160 ELISA were collected from two volunteers 14 days after the third 80- μ g vaccination and had net percent positive fluorescence of 9.2 and 0 by rgp160 FIFA.

While rgp160 ELISA, rgp160 FIFA, and whole-virus-lysate EIA correlated with each other in the 33 volunteers and the 53 serum samples tested (Spearman rho test, $P < 0.001$), the closest linear relationships were seen between log (OD/cutoff ratio) and net percent positive fluorescence against rgp160-pulsed H9 cells ($r = 0.723$; $P < 0.001$) (Fig. 2) and between log (OD/cutoff ratio) and log (reciprocal antibody titer to rgp160) ($r = 0.716$; $P < 0.001$) (Fig. 3). Log (reciprocal antibody titer to rgp160) and net percent positive fluorescence against rgp160-pulsed H9 cells were correlated less closely ($r = 0.526$; $P < 0.001$). Sera from the 80- μ g rgp160 vaccine dose recipients tended to have higher OD/cutoff ratios, reciprocal antibody titers, and net percent positive fluorescence compared with those of sera from the 40- μ g dose recipients (Fig. 2 and 3).

Antibody to native envelope glycoprotein expressed by HIV-1-infected H9 cells. Antibody to envelope glycoprotein expressed by HIV-1_{IIIB}-infected H9 cells was detectable in 12 serum samples from 8 of the 33 volunteers (Table 3). All but 2 of these 12 positive serum samples had antibody titers of $\geq 1,600$ against rgp160 by ELISA, and 6 were positive by whole-virus-lysate EIA (Table 3). The reciprocal geometric mean antibody titer by rgp160 ELISA, the mean OD/cutoff ratio by whole-virus-lysate EIA, and the net percent positive fluorescence against rgp160-pulsed H9 cells for the sera which stained infected H9 cells were not significantly different from the corresponding mean values for sera which did not stain the infected cells (reciprocal geometric mean titers, 1199 versus 1109; mean OD/cutoff ratios, 1.31 ± 1.01 versus 1.57 ± 1.71 ; mean net percent positive fluorescence, 20.9 ± 18.0 versus 15.7 ± 17.0 , respectively). Patterns of anti-envelope glycoprotein antibody band reactivity by Western blot analysis were similar among those sera which stained H9 cells infected with HIV-1_{IIIB} compared with those which did not.

Of the 12 serum samples positive by FIFA against HIV-1_{IIIB}-infected H9 cells, one (volunteer 7; Table 3) was also positive against HIV-1_{MN}-infected H9 cells (net percent positive fluorescence, 4.1). None were positive against the HIV-1_{RF}-infected cells. Serum collected 28 days after the

TABLE 2. Comparison of FIFA, whole-virus-lysate EIA, and rgp160 ELISA 14 days after the fourth rgp160 vaccine dose in 24 volunteers

Assay method (units of measurement)	No. of positive serum samples/ total no. tested	Mean $\pm$ SD ^a	Range
rgp160 FIFA (net % positive fluorescence)	16/24 ^b	11.6 $\pm$ 12.1	0.0–35.0
HIV-1 _{IIIB} -infected cell FIFA (net % positive fluorescence)	4/24 ^c	3.6 $\pm$ 7.5	0.0–27.1
Whole-virus-lysate EIA (OD/cutoff ratio)	10/24 ^c	1.40 $\pm$ 1.65	0.18–6.55
rgp160 ELISA (reciprocal antibody titer)	24/24 ^{b,c}	1,131	100–25,600

^a Values are arithmetic means for net percent positive fluorescence (FIFA), and OD/cutoff ratio (whole-virus-lysate EIA) and geometric means for the rgp160 ELISA reciprocal antibody titer. All 24 serum samples tested were included in the calculations.

^b Proportion of sera positive by rgp160 ELISA greater than that by rgp160 FIFA, $P < 0.01$.

^c Proportion of sera positive by rgp160 ELISA greater than those by whole-virus-lysate EIA and HIV-1_{IIIB}-infected cell FIFA, $P < 0.001$.

fourth 80- μ g dose of the rgp160 vaccine from an additional vaccinee stained H9 cells infected with HIV-1_{MN} (net percent positive fluorescence, 7.2) and H9 cells pulsed with rgp160 (net percent positive fluorescence, 39.7).

The mean net percent positive fluorescence of the HIVIG-positive control anti-HIV-1-purified IgG preparation against HIV-1_{IIIB}-infected H9 cells was 74.0, against HIV-1_{RF}-infected H9 cells it was 50.4, against HIV-1_{MN}-infected H9 cells it was 69.7, and against uninfected H9 cells pulsed with rgp160 it was 82.8.

DISCUSSION

The FIFA with virus-infected target cells provided a quantitative way in which to assess the ability of the rgp160 vaccine to induce antibody which recognizes the native envelope glycoprotein on the surfaces of infected cells. The fact that sera from at least a minority of vaccinees were positive against HIV-1-infected cells at these low vaccine doses is encouraging. Also, the activities in two serum samples were cross-reactive with a heterologous strain of HIV-1. Staining of cells infected with HIV-1 was not simply a reflection of the height of anti-gp160-binding antibody titer by ELISA and EIA.

Previously, sera collected after the fourth rgp160 inocula-

tion from 24 vaccinees were tested for their neutralizing activities by microtiter syncytia formation inhibition assay (4, 22) and complement-mediated antibody-dependent enhancement (4, 15). Serum samples from five vaccinees had low titers of neutralizing activity against HIV-1_{IIIB} infection in vitro. Three vaccinees who had antibodies to cells infected with HIV-1_{IIIB} or HIV-1_{MN} by FIFA were among these 5 with neutralizing antibody, and 2 with a positive infected cell FIFA were among the 19 without detectable neutralizing antibody after the fourth vaccine dose (3 of 5 versus 2 of 19 vaccinees; $P = 0.04$, Fisher's exact test). Two of the eight vaccinees with antibodies against HIV-1-infected cells in our study following the third vaccine dose were not tested for the presence of neutralizing antibody, because they did not receive a fourth vaccine dose. Six of the 24 volunteers had complement-mediated antibody-dependent enhancement after the fourth vaccination (4). Of these, two had activity against HIV-1-infected cells in the FIFA. The other three with a positive infected cell FIFA after the fourth dose were among the 18 volunteers without complement-mediated antibody-dependent enhancement (2 of 6 versus 3 of 18 vaccinees; P was not significant). Thus, there was some

FIG. 2. Net percent positive fluorescence by rgp160 FIFA plotted as a function of log (OD/cutoff ratio) by whole-virus-lysate EIA for 53 serum samples from 33 volunteers obtained after the third and fourth rgp160 vaccine doses. The best-fit line defined by these points was determined by linear regression [$r = 0.723$; $P < 0.001$; net percent positive fluorescence = $27.76 [\log (\text{OD/cutoff ratio})] + 16.95$]. Solid circles represent sera collected from 40- μ g rgp160 vaccine recipients, and open circles represent sera collected from 80- μ g rgp160 vaccine recipients.

FIG. 3. Log (reciprocal antibody titer) by rgp160 ELISA plotted as a function of log (OD/cutoff ratio) by whole-virus-lysate EIA for 53 serum samples from 33 volunteers obtained after the third and fourth rgp160 vaccine doses. The two serum samples that were negative by ELISA at a serum dilution of 1:25 were arbitrarily assigned a reciprocal antibody titer of 12.5. The best-fit line defined by the 53 points was determined by linear regression [$r = 0.716$; $P < 0.001$; $\log (\text{reciprocal antibody titer}) = 1.327 [\log (\text{OD/cutoff ratio})] + 3.06$]. Solid circles represent sera collected from 40- μ g rgp160 vaccine recipients, and open circles represent sera collected from 80- μ g rgp160 vaccine recipients.

TABLE 3. Comparison of FIFA, whole-virus-lysate EIA, Western blot, and rgp160 ELISA in eight rgp160 vaccinees with detectable antibody by FIFA against H9 target cells infected with HIV-1_{IIIB}

Vaccinee no.	Dose of rgp160 (μ g)	Vaccine injection	Day postvaccine injection	FIFA (net % positive fluorescence) ^a		Whole-virus lysate EIA (OD/cutoff ratio)	rgp160 ELISA reciprocal antibody titer	Anti-HIV-1 _{IIIB} Reactivity by Western blot, antibody against:		
				HIV-1 _{IIIB} -infected H9 cells	rgp160-pulsed H9 cells			gp160	gp120	gp41
1	80	Fourth	14	27.1	33.7	1.38	1,600	+	+	-
			28	7.5	33.6	1.70	6,400	+	+	-
2	80	Third	14	9.6	33.7	2.06	1,600	+	+	+
3 ^b	80	Third	14	9.8	9.2	0.30	<25	-	±	-
4	40	Fourth	14	24.2	18.1	1.21	1,600	+	+	-
			28	10.3	34.0	1.54	6,400	+	+	-
			90	7.8	1.1	0.87	1,600	+	+	+
5	40	Third	14	13.3	1.9	0.95	1,600	±	+	-
6	40	Fourth	14	13.2	12.2	0.75	1,600	+	±	-
7 ^b	40	Third	14	4.0	53.9	4.01	1,600	+	2+	+
8	40	Third	14	6.3	0.9	0.37	400	-	±	-
		Fourth	14	5.2	0.2	0.60	1,600	-	-	-

^a The net percent positive fluorescence against HIV-1_{IIIB}-infected H9 cells by HIVIG was 74.0%.^b The vaccinee did not receive a fourth dose of vaccine.

concordance between the presence of neutralizing antibody and antibody reactive against HIV-1-infected cells by FIFA. Sera from most vaccinees that were positive for neutralizing and enhancing antibody after the fourth vaccine inoculation were positive by rgp160 FIFA; in the sera from one vaccinee, only enhancing antibody was detected. However, a negative rgp160 FIFA was not always predictive of a lack of staining of cells infected with HIV-1.

Our results suggest that one or more of the following situations were operative: (i) that the envelope glycoprotein epitope specificities of antibodies induced by rgp160 vaccine at doses of 40 and 80 μ g in a majority of vaccine recipients are, at best, a subset of those induced by HIV-1 infection; (ii) that in a majority of vaccinees the titer of HIV-1-infected cell-binding antibody induced by vaccine doses of 40 or 80 μ g and the regimen used in this study was too low to be detected by FIFA with HIV-1-infected cells; or (iii) that most epitopes which react with antibody stimulated by rgp160 vaccination may be hidden or present at a low density on the infected cell surface. Reactivity against gp41 by Western blot did not identify those sera which were likely to stain cells infected with HIV-1.

Lyerly et al. (9) adsorbed gp120 isolated from HIV-1_{IIIB}-infected H9 cells onto CEM cells and found that goat antiserum raised against the same HIV-1_{IIIB} gp120 stained these cells more brightly than did HIV-1_{IIIB}-infected CEM cells. Since the goats were immunized with the gp120 derived from HIV-1_{IIIB}-infected H9 cells, this is consistent with our findings; however, the reasons remain unclear.

The correlation between rgp160 FIFA and rgp160 ELISA was surprising. Presumably, much of the rgp160 was bound to the H9 cells in one specific orientation. This baculovirus-derived rgp160 may bind to CD4⁺ cells at a different site or by a different mechanism than does rgp120, which appears to bind to CD4 molecules (8). If binding of the rgp160 to H9 cells was via the carboxy-terminal half of gp120, which is

important in CD4 binding (19), then the amino-terminal half of gp120 and the gp41 amino acid sequences were exposed for interaction with antibody. Another portion of rgp160 may have been responsible for binding, however, leading to a different orientation of the molecule on the surface of the H9 cells. The rgp160 was bound noncovalently to the plastic microtiter plates, presumably in multiple orientations, allowing additional antibody interactions with the epitopes which were hidden when rgp160 was bound to H9 cells. While not an explanation for a correlation between the two assays, the differences in rgp160 orientation is one possible reason for the greater sensitivity of the rgp160 ELISA compared with that of the rgp160 FIFA.

Despite the few positive sera by whole-virus-lysate EIA, there was a good correlation between the OD/cutoff ratios and the corresponding values derived from the rgp160 ELISA and rgp160 FIFA. The gp160 in the EIA was bound noncovalently to polystyrene beads, is not recombinant, may be denatured during lysis of the virus, but may be underrepresented compared with the representation of other viral proteins (6, 14). Thus, the correlation between the whole-virus-lysate EIA and the rgp160 ELISA was less surprising than that between the whole-virus-lysate EIA and the rgp160 FIFA.

Viscidi et al. (21), using a methodology different from ours, found that a gp160 EIA was also sensitive for the detection of antibody to HIV-1 envelope glycoprotein in sera collected from individuals in the prior study (4) who received rgp160 at low doses. They reported that antibody was directed against epitopes on both gp120 and gp41, but seroresponses to the immunodominant epitopes on gp41 were infrequent and no seroresponse to the neutralization epitope in the V3 region of gp120 was detected (21).

In conclusion, low doses of rgp160 vaccine induced antibody which binds to HIV-1 envelope glycoprotein presented in several ways. The rgp160 ELISA was most sensitive for

the detection of antibody induced by the vaccine, and the methods with rgp160 in the ELISA and FIFA and gp160 in whole-virus-lysate EIA correlated with respect to the relative amounts of antibody detected. At least some sera bound native envelope glycoprotein on cells infected with HIV-1_{IIIB} and HIV-1_{MN}. There was an association between the activity against infected cells in this study and that of HIV-1_{IIIB}-neutralizing antibody measured in a prior study (4). The FIFA technique with HIV-1-infected cells and that with rgp160-pulsed cells should be studied further to determine their usefulness in the characterization of antibody responses induced by envelope glycoprotein vaccines.

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