Sequential Immunizations with rgp120s from Independent Isolates of Human Immunodeficiency Virus Type 1 Induce the Preferential Expansion of Broadly Crossreactive B Cells

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Summary
The gp120 envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) is a dominant target against which the host's humoral immune response is directed. Unfortunately, gp120 proteins from different isolates of HIV are antigenically distinct, complicating the use of the envelope glycoprotein in vaccines designed to prevent acquired immunodeficiency syndrome. Using an enzyme-linked immunosorbent spot assay (ELISA), BALB/c mice immunized and boosted with recombinant purified gp120 were studied at the single cell level for their humoral immune response to HIV-1 envelope proteins. Approximately 90% of responding B cells produced antibodies reactive with the immunizing form of gp120 but not with gp120s from other strains of HIV. A novel sandwich ELISA was then used to analyze the frequency with which individual in vivo activated B cells produced antibodies that crossreacted with heterologous gp120s. Repeated immunizations with a single gp120 or with a mixture of different gp120s resulted in the activation of primarily mono-specific (noncrossreactive) B cells. In contrast, the sequential immunization of mice with recombinant purified envelope proteins from different strains of HIV (IIIB, SF2, and Zr6) induced the selective expansion of B cells producing highly crossreactive antibodies.

Infection with HIV-1 leads to a profound and progressive impairment of T cell immunity and the development of AIDS (1, 2). Considerable evidence suggests that the major envelope glycoprotein of HIV-1 (gp120) mediates viral attachment to human T cells via the lymphocyte's CD4 receptor (3–5). Gp120 also acts as a dominant target against which the host directs its humoral and cell-mediated immune responses (6). Thus, recombinant gp120 proteins are being studied as potential immunogens in vaccines designed to prevent AIDS (6–8).

An obstacle to the development of such a vaccine is the pleiotropic nature of the gp120 molecule. Multiple variants of HIV-1 expressing antigenically distinct envelope glycoproteins have been described (9–12). Molecular analyses of these divergent viruses show differences in up to 50% of the amino acids expressed in the hypervariable regions of their gp120s (13–17). These findings are consistent with the rapid mutation rate described for HIV-1 and suggest that strong immunologically based selective pressures may be responsible for the sequential outgrowth of distinct virus strains in vivo (15).

An effective vaccine based upon gp120 must be capable of eliciting an immune response against a wide range of HIV-1 envelope glycoproteins. Yet previous studies have shown that immunizations with envelope protein derived from a single isolate of HIV-1 generally did not induce a broadly crossreactive antibody response (18–20). In the present work, a chamber ELISA was developed to investigate the crossreactivity of individual B cells activated in mice immunized with gp120s from different isolates of HIV. Results indicate that 85–95% of the B cells activated after immunization with a single isolate of gp120 reacted only with that isolate. In contrast, sequential immunization with divergent isolates of gp120 led to the preferential expansion of B cells producing highly crossreactive antibodies. These findings suggest that a protocol based upon sequential immunization might achieve the objective of eliciting a broadly protective anti-HIV antibody response.

Materials and Methods

Spleen Cell Preparation. Female BALB/c mice were obtained from The Jackson Laboratory, Bar Harbor, ME. These mice were immunized intraperitoneally with 10 μg of recombinant env 2-3 from...
the IIIB, SF2, and/or Zr6 strains of HIV-1 (Chiron Corporation, Emeryville, CA) emulsified in CFA (21). The env 2-3 preparations were full-length recombinant purified gp120 proteins (amino acids 28–509) and were produced in yeast strain 2150 under a GAP promoter. These proteins were nonglycosylated and ~90% pure. Preliminary experiments demonstrated that 10 μg/animal induced a primary gp120-specific humoral response in 100% of immunized mice. Some animals were boosted at 6–8-wk intervals with env 2-3 in IFA. All animals were bled by retro-orbital puncture 4 wk after primary immunization or 2 wk after boosting. A single cell suspension was immediately made from the spleens of these animals in medium consisting of RPMI 1640 supplemented with 2% FCS (22). Serum was stored at –20°C until assayed for the presence of neutralizing and binding antibodies as described (23).

**Viruses Neutralization Assay.** 500 tissue culture infectious doses of HIV-1 viruses were incubated for 90 min with heat-inactivated mouse serum diluted 1:4 to 1:512 in microtiter plates. 1.5 x 10^5 Molt-3 cells, which had been pretreated in media containing 2 μg/ml of polybrene, were then added. Plates were centrifuged at 700 g for 45 min and placed in 37°C humidified 5% CO2 for 5 d. 50% inhibition of giant cell formation was taken as the endpoint for virus inhibition. Well-to-well variation was <15% (23).

**ELISAs.** Flat-bottomed Immulon microtiter plates (Dynatech Labs, Alexandria, VA) were coated with optimal concentrations of env 2-3 from IIIB, SF2, Zr6, or feline leukemia virus (FELV) (Chiron Corporation), or ssDNA, TNP-KLH, OVA, or rabbit anti–mouse IgG (Cappel Laboratories, Cochranville, PA) and then blocked with 1% BSA in PBS as previously described (21, 24, 25).

**Spot ELISAs.** Serial dilutions of single cell suspensions made from the spleens of immunized mice, starting with 10^6 cells/well, were incubated for 6 h on antigen-coated plates in 5% CO2 in an air incubator at 37°C. The cells were removed by washing the plate with PBS/0.05% Tween 20, and the wells were overlaid with phosphatase-conjugated anti–mouse IgG antibody for 2 h. The antibodies produced by individual B cells that bound to the plate were visualized by addition of a 5-bromo-3-chloro 4-indolyolphosphate solution (Sigma Chemical Co., St. Louis, MO) in a low-melt agarose kept at 50°C. Phosphatase acts on this substrate to produce a blue spot that cannot diffuse through the agarose once it solidifies at room temperature (26). That dilution of cells producing ~30 spots/well was used to quantify the total number of antibody-specific B cells per sample. The presence of BSA in the FCS used to conduct these assays inhibited the binding of BSA-specific antibodies to BSA-block plates, giving the assay a zero background. The sensitivity and specificity of this assay was documented by antigen inhibition tests and in studies involving antigen-specific hybridoma cell lines (26–28; and data not shown).

**Sandwich ELISA Technique.** Plastic microscope slides (Thomas Scientific, Philadelphia, PA) were coated with antigen and blocked with 1% BSA by the procedures described above for coating microtiter plates. Sandwich ELISA chambers were constructed by taping two antigen-coated slides together using strips of Scotch Brand double-sided tape (3M Co., St. Paul, MN). Four equally spaced strips of tape were used to create three independent compartments per chamber (see Fig. 1). Approximately 50 μl of a single cell suspension containing 10^5, 10^6, or 10^7 cells/ml was pipetted into these compartments and the chamber placed in humidified 5% CO2 in an air incubator at 37°C for 10 h. The chamber was then split apart and processed as described in the spot ELISA described above.

After processing, the number of spots per compartment on each slide was counted. The two slides were then juxtaposed (through use of alignment markings placed on each slide before incubation), and overlapping spots (representing antibodies that reacted with antigen on both slides) were quantitated. The percent of crossreactive B cells for each set of antigens was determined independently in at least three mice per experiment. All experiments were performed at least twice.

**Results**

**Serum Antibody Levels after gp120 Immunization.** BALB/c mice were immunized with gp120 (env 2-3 preparation) from the IIIB, SF2, or Zr6 strains of HIV-1. The serum antibody response of these mice was analyzed after 4 wk (preliminary studies showed that maximal IgG anti-gp120 production was present at this time). Whereas 100% of these mice developed significantly elevated serum IgG antibody titers to the immunizing isolate of gp120, only 33–83% of these animals produced antibodies that bound to gp120s expressed by other HIV isolates (Table 1). In addition, the concentration of antibody reactive with the immunizing gp120 was 15–30-fold higher than that against envelope proteins from other HIV strains. Secondary immunization increased the total anti-gp120 response but not the relative proportion of antibodies reactive with heterologous gp120 (Table 1). It also induced a low-titered (1:8 to 1:32) neutralizing antibody response that was type specific (i.e., neutralization was specific for the HIV-1 strain from which the gp120 was derived).

**Number and Specificity of B Cells Producing Antibodies against gp120.** The concentration of anti-gp120 antibodies in serum reflects the time-averaged balance between their rate of production and degradation. To directly measure the ongoing response to gp120, a spot ELISA was used to monitor the number and specificity of B cells actively secreting Ig in vivo.

As seen in Table 2, normal BALB/c mice had no splenic B cells producing IgG antibodies reactive with gp120. In contrast, 4–9% of all IgG-secreting cells from gp120-immunized mice secreted antibodies reactive with the immunogen. These cells did not react with the unrelated antigens OVA, TNP-KLH, or DNA, or with gp120 from FELV (an env 2-3 preparation produced by the same process used to make gp120 from HIV-1). Consistent with the above serum studies, only a small fraction of B cells from immunized mice produced antibodies capable of binding to heterologous forms of envelope protein (Table 2).

Mice were then primed and boosted twice with the same gp120. 11–19% of the IgG-secreting splenic B cells from multiply immunized mice produced antibodies reactive with the immunogen (Table 3). While some broadening of the
Specificity of Serum Antibodies from rgp120-immunized Mice

Table 1.

| Immunizations | Percent of mice Ab positive* | Reciprocal Ab concentration |
|---------------|-----------------------------|-----------------------------|
|               | IIIB | SF2 | Z6 | IIIB | SF2 | Z6 |
| Primary       |      |     |    |      |     |    |
| IIIB          | 100  | 83  | 33 | 1,280| 38 | 20 |
| SF2           | 50   | 100 | 33 | 36   | 980| 46 |
| Z6            | 33   | 33  | 100| 22   | 24 | 880|
| Secondary     |      |     |    |      |     |    |
| IIIB          | 100  | 83  | 50 | 16,400| 160| 126|
| SF2           | 67   | 100 | 50 | 300  | 6,800| 210|
| Z6            | 67   | 50  | 100| 156  | 144| 28,000|

Mice were immunized with 10 μg of env 2-3 in CFA and bled 4 wk later. Ab concentration was determined in comparison to a standard curve generated using a high titered antiserum of known specificity. A minimum of six mice/group were independently examined.

* A mouse was considered antibody positive if its serum yielded an anti-gp120 concentration that exceeded the mean of unimmunized mice by >2 SD.

Reactivity pattern was noted, on average, <10% of these gp120-specific B cells produced antibodies reactive with heterologous gp120s (Table 3).

Crossreactivity of Anti-gp120-producing B Cells. An assay was developed to identify and quantitate in vivo activated B cells secreting crossreactive anti-gp120 antibodies. Freshly isolated splenic lymphocytes from immunized mice were sandwiched between plastic slides that had been coated with env 2-3 from the IIIB, SF2, or Z6 strains of HIV-1 (see Fig. 1). In vivo activated B cells continued to secrete Ig when incubated for 10 h in these sandwich ELISA chambers and produced antibodies that bound to the antigens coating one or both sides of the chamber. In preliminary experiments, both sides of the chamber were coated with the same gp120. Splenic B cells from mice immunized with that antigen (but not naive animals) generated ELISA spots in precisely the same position on both sides of the chamber (visualized by superimposing but slightly off-setting the slides, as illustrated in Fig. 1). When OVA rather than gp120 was used to coat one side of the chamber, there was no concordance between the location of the ELISA spots produced. Similarly, no crossreactivity was detected when chambers were constructed using slides coated with env 2-3 from FELV as the antigen on one side and env 2-3 from HIVIIIB on the other. These findings indicate that: (a) B cells producing crossreactive antibodies produced ELISA spots in the same position on both sides of an ELISA chamber, whereas mono-specific B cells produced spots on only one side of the chamber (Fig. 1); (b) spot formation was antigen specific (a finding confirmed by

Table 2.

Reactivity of B Cells from rgp120-immunized Mice

| Immunogen | IIIB | SF2 | Z6 | FELV | Other* |
|-----------|-----|-----|----|------|-------|
| IIIB      | 6.5 | 0.56| 0.28| 0    | 0     |
| SF2       | 0.39| 9.8 | 0.83| 0    | 0     |
| Z6        | 0.65| 0.26| 4.4 | 0    | 0     |
| FELV      | 0   | 0   | 4.7 | 0    | 0     |
| PBS       | 0   | 0   | 0.12| 0    | 0     |

Six mice/group were immunized intraperitoneally with 10 μg of env 2-3 or PBS in CFA. 4 wk later, spot ELISA was used to determine the number of B cells producing IgG antibodies reactive with each gp120. The percent of cells secreting antibodies of each specificity was calculated as a function of the absolute number of IgG-producing cells per spleen by the formula: 100 × (no. of IgG anti-gp120-secreting cells/total no. of IgG-secreting cells). It should be noted that B cells from mice immunized with gp120 also bound to gp160 (Microgenics, West Haven, CT) and total viral lysates (DuPont Co., Wilmington, DE) produced from that isolate.

* In all experiments, B cells were tested for reactivity with control antigens (DNA, TNP-KLH, and OVA). Results represented the highest background reactivity detected against any of these antigens.

Table 3.

gp120 Reactivity of B Cells from Multiply Immunized Mice

| Immunogen | IIIB | SF2 | Z6 | FELV | Other |
|-----------|-----|-----|----|------|-------|
| IIIB      | 12.18| 0.91| 0.81| 0.06 | 0.08  |
| SF2       | 0.82 | 18.52| 2.38| 0.00 | 0.00  |
| Z6        | 1.67 | 0.88| 10.53| 0.18 | 0.12  |
| FELV      | 0.00 | 0.14| 11.76| 0.00 | 0.00  |

Six mice/group were immunized intraperitoneally with 10 μg of gp120 in CFA. 8 and 14 wk later, they were boosted with the same immunogen in IFA. A spot ELISA was performed as described in the legend of Table 3 2 wk after the final immunization.
testing hybridomas of known antigenic specificity and in Ag inhibition tests; data not shown); and (c) low-level contamination of the env 2-3 preparations with yeast-derived impurities (<10% by weight) was not responsible for the B cell stimulation detected in this assay, since activated B cells did not crossreact with gp120 from FELV.

When B cells from immunized mice were analyzed in sandwich ELISA chambers, only 2-15% of lymphocytes reactive with the immunizing form of gp120 crossreacted with heterologous gp120s (Table 4). Repeatedly boosting mice with a single isolate of gp120 did not increase the proportion of crossreactive B cells (although it did increase the absolute number of B cells producing antibodies against heterologous gp120s; Table 5).

Selection of Crossreactive B Cells by Sequential Immunization with Distinct gp120 Isolates. The above findings suggested that immunization protocols using envelope proteins from a single isolate of gp120 would be limited in their ability to promote the outgrowth of crossreactive B cells. In an attempt to preferentially activate crossreactive lymphocytes, mice were immunized sequentially with gp120s from different virus strains. As seen in Table 6, this strategy resulted in the preferential activation/proliferation of crossreactive lymphocytes. Mice immunized with gp120IIIB and boosted with gp120SF2 expressed a repertoire in which ~40% of Ag-specific B cells reacted with all gp120s examined, including Z6. The expansion of crossreactive B cells was further enhanced by immunizing sequentially with all three gp120s in turn (Table 7). While we could not determine the neutralizing capacity of the antibodies secreted by individual B cells, serum from sequentially immunized mice neutralized the MN strain of HIV-1 whereas serum from mice immunized with a single isolate of gp120 did not (Table 6). The preferential induction of crossreactive B cells apparently depended upon the sequential presentation of gp120 antigens, since repeated immunization with a single mixture containing all three gp120s did not lead to the selective proliferation of these cells (Table 7).

**Figure 1.** Chamber ELISA. (A) Plastic microscope slides were coated with antigen and secured together using double-sided tape to form an ELISA chamber. Freshly isolated splenic lymphocytes were added between the slides to form a monolayer. Chambers were then placed in a humidified 5% CO2 in-air incubator for 10 h. Antibodies produced by in vivo activated B cells bound to the antigen-coated slides and were visualized by a conventional colorimetric spot developing ELISA. (B) The number of spots on each slide, representing the number of B cells producing antigen-specific antibodies, were then counted. If the same antigen was used to coat both sides of an ELISA chamber, 100% of the spots present on one slide were present in the same position on the other slide. Crossreactive B cells were detected by the appearance of ELISA spots in the same position on slides that had been coated with different antigens.

**Table 4. Crossreactivity of B Cells from gp120-immunized Mice Detected by Chamber ELISA**

| Immunogen | Percent gp120-reactive cells | Percent crossreactivity between immunogen and: |
|-----------|-----------------------------|---------------------------------------------|
| IIIB      | 6.7                         | 100 8.8 2.3 0 0                           |
| SF2       | 10.3                        | 5.5 100 15.5 0 0                          |
| Z6        | 4.8                         | 12.5 8.8 100 0 0                          |

Sandwich ELISA chambers were constructed in which one side was coated with the immunizing antigen and the other with gp120 from a different isolate of HIV-1. Freshly isolated splenic lymphocytes from immunized mice were cultured in these chambers for 10 h. The number of spots per slide was counted (cell numbers yielding no greater than 125 spots/slide were used). The two sides of the chamber were then superimposed and spots present in the same location on both (representing crossreactive B cells) counted. To determine the assay background (representing the chance appearance of two spots in the same position on both sides), slides from two different chambers were superimposed. This background (average of 0.3 spots/chamber) was subtracted from all data. Results represent the mean of at least two independent experiments involving a minimum of three independently studied mice per group.

**Table 5. Crossreactivity of B Cells from Multiply Immunized Mice Detected by Chamber ELISA**

| Immunogen | Percent gp120-reactive cells | Percent crossreactivity between immunogen and: |
|-----------|-----------------------------|---------------------------------------------|
| IIIB      | 12.7                        | 100 6.8 3.9 0.4                           |
| SF2       | 19.1                        | 3.7 100 13.4 0.0                          |
| Z6        | 11.0                        | 8.2 5.4 100 0.3                           |
| FELV      | 11.8                        | 0.0 0.7 100 0.0                           |

Immunization schedule was described in the legend of Table 3, and the assay was conducted as described in the legend of Table 4.
Table 6. Effect of Sequential Immunization on B Cell Crossreactivity

| IIIB Immunized, boosted with: | Percent gp120 reactive | Neutralizing Abs* | Percent crossreactivity between boosting rgp120 and: |
|-------------------------------|------------------------|------------------|--------------------------------------------------|
| IIIB                          | 8.4                    | –                | IIIB                                             |
| SF2                           | 15.1                   | +                | SF2                                             |
| Z6                            | 10.0                   | +                | Z6                                              |

Mice were immunized intraperitoneally with 10 μg of rgp120IIIB. 8 wk later, these animals were immunized/boosted with the antigen shown in the first column. The percent of IgG anti-gp120-secreting cells was quantitated 2 wk later by spot ELISA as described in Table 2. Crossreactivity was studied simultaneously using a chamber ELISA, as described in Table 4. Data represent results of two independent experiments involving three independently studied mice per group. Background crossreactivity with rgp120 from FELV was <0.2%.

* Sera from these mice were analyzed for the presence of neutralizing antibodies. A + indicates neutralizing titers ≥ 1:16 against both the MN and IIIB strains of HIV-1 (23).

Table 7. Expansion of Crossreactive B Cells Requires Sequential Immunization with Divergent gp120s

| Immunization sequence | Percent gp102-reactive cells | Percent crossreactive with IIIB |
|-----------------------|------------------------------|--------------------------------|
| 10                    | SF-2                         | Z6                             |
| IIIB IIIB IIIB        | 12.2                         | 2.8                            |
| IIIB SF2 Z6           | 18.3                         | 31.0                           |
| IIIB Z6 SF2           | 26.6                         | 42.8                           |
| SF2 IIIB Z6           | 16.8                         | 42.6                           |
| Z6 IIIB SF2           | 21.9                         | 57.6                           |
| All All All            | 5.1                          | 3.5                            |

See legend to Table 4 for experimental details. Data in the last row represent mice immunized with a mixture containing 5 μg/isolate of each rgp120.

Discussion

The possibility that recombinant gp120 could be used in a vaccine to prevent HIV-1 infection has attracted considerable interest (6–8, 29). In the present work, the immunogenicity and crossreactivity of recombinant gp120s from various HIV strains was examined in BALB/c mice. Results indicate that ~90% of the B cells responding to envelope proteins from a single isolate of gp120 produced antibodies that reacted with that isolate but not with gp120s from other strains of HIV. Boosting with the same gp120 induced serum antibodies with increased binding to heterologous isolates. However, this was due to an increase in the number (and perhaps affinity) of anti-gp120-producing cells rather than an increase in the frequency of crossreactive B cells. By comparison, sequential immunization of mice with gp120s from different isolates of HIV-1 led to the preferential expansion of lymphocytes producing antibodies that crossreacted with a variety of gp120s but not with unrelated antigens. These findings indicate that a vaccination protocol involving sequential immunization might optimize the production of a broadly protective anti-HIV humoral response.

A number of investigators have shown that animals immunized with nonglycosylated gp120 produce strain-specific neutralizing antibodies (8, 29). For example, goats immunized with gp120IIIB produced antibodies that neutralized HIVIIIB but not HIVRF, and vice versa (30, 31). By comparison, non-neutralizing antibodies from similarly immunized animals showed a limited degree of crossreactivity, suggesting that non-neutralizing antibodies might differ in concentration, affinity, and/or epitope specificity from those with neutralizing capacity (31). In the present report, serum from sequentially immunized mice neutralized the MN virus at titers ranging from 1:16 to 1:128. In contrast, serum from mice repeatedly immunized with a single isolate (or mixture) of gp120s did not produce detectable crossneutralizing antibodies. Similarly, we found that sera from sequentially immunized mice bound to gp120s from multiple different isolates of HIV-1, whereas sera from mice immunized multiple times with a single gp120 reacted predominantly with only that isolate of envelope glycoprotein.

The crossreactivity detected in serum could have been due to the presence of: (a) multiple antibody populations, each specific for a different gp120; (b) highly crossreactive antibodies capable of binding to numerous unrelated antigens (such as have been described in various autoimmune states); or (c) antibodies specific for epitope(s) shared by different isolates of HIV-1. To differentiate among these possibilities, a novel sandwich spot ELISA was developed to detect individual in vivo activated B cells secreting crossreactive antibodies. This assay was a significant improvement over previous techniques used to study B cell crossreactivity. It did not require the mitogen stimulation or cloning of B cells and thus eliminated the possible repertoire bias introduced by such in vitro manipulations. Unlike the technique of Cunningham and Pilarski (32), the chamber ELISA yielded a permanent record of results, was simple to perform, and could be used to study
antibodies of different isotypes reactive with a wide variety of antigens. The specificity and sensitivity of this assay was similar to that of conventional spot ELISAs (shown by antigen inhibition tests and assays using hybridomas of known antigenic specificity; data not shown). Using this assay, we found that at least 90% of the responding B cells from mice immunized with one isolate of rgp120 were reactive only with that isolate. Multiple immunizations did not increase the proportion of B cells secreting crossreactive antibodies.

That only a small proportion of B cells activated after immunization with a single isolate of gp120 were crossreactive is consistent with results from other laboratories showing that conserved regions are less immunogenic than hypervariable regions on the gp120 molecule (16, 29, 33–35). Indeed, recent findings suggest that antibodies against conserved regions cannot be induced unless hypervariable regions are also present (21) — a phenomenon that may be associated with the recognition of hypervariable regions by T cells (36).

A major goal in the development of an HIV vaccine is the induction of antibodies capable of recognizing all possible strains of virus (31). Unfortunately, achieving this objective has been complicated by the high degree of polymorphism expressed by HIV-1 envelope proteins (16) and the poor immune response generated against conserved gp120 determinants (21, 29). To circumvent this problem, we sequentially immunized mice with different isolates of gp120. Results indicate that sequential immunization induced the specific expansion of B cells secreting IgG antibodies that crossreacted with a variety of rgp120 but not with the unrelated antigens DNA, TNP-KLH, OVA, or rgp120envL. Indeed, 43–67% of the B cells induced by immunizing with rgp120B konntegular followed by rgp120 HIM and then rgp12026 reacted with all three HIV rgp120 envelope proteins. In addition, sera from these mice were capable of neutralizing unrelated strains of the HIV-1 virus. Our data indicated that sequential immunization was ~15-fold better at stimulating crossreactive B cells than a protocol involving multiple immunizations with a single isolate of gp120 or a mixture of all gp120s simultaneously.

We postulate that sequential immunization elicited an anamnestic response to conserved epitopes on the HIV-1 envelope protein. An animal immunized with three different gp120s on three different occasions would mount a secondary immune response against conserved epitopes that were expressed on all of the gp120s (33, 34). In contrast, hypervariable region epitopes expressed uniquely on different gp120s would induce only primary immune responses. A similar situation might account for the broadening of the anti-HIV neutralizing response, which develops over time in humans infected with HIV-1 (37–39). When the infecting virus mutates in vivo, the immune system is confronted repeatedly with conserved epitopes but sequentially with distinct hypervariable region determinants, a situation that might select for the activation of crossreactive B cells. These findings suggest that a protocol involving sequential immunization might be of value in optimizing the production of broadly protective anti-HIV antibodies.

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