Comparison of Human Immunodeficiency Virus Antigens as Stimulants for Lymphocyte Proliferation Assays

John L. Schmitz,1* Thomas N. Denny,2 Ambrosia Garcia,2 Janet L. Lathey,3† and the Adult and Pediatric AIDS Clinical Trials Group Immunology Laboratory Subcommittees

Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina1; University of Medicine and Dentistry, Newark, New Jersey2; and Department of Pediatrics, University of California at San Diego, La Jolla, California3

Received 25 June 2001/Returned for modification 23 August 2001/Accepted 17 December 2001

CD4 proliferative responses to the human immunodeficiency virus (HIV) type 1 (HIV-1) p24 (gag) antigen inversely correlate with the plasma viral load in HIV-infected subjects who control viral replication without antiretroviral therapy. Use of a single HIV-1 protein to assess CD4 proliferative responses may not reflect the global response to this pathogen. We compared the abilities of HIV p24 and gp120 antigens from two different vendors, an inactivated whole HIV-1 MN virion preparation and an HIV-1E culture supernatant antigen, to elicit proliferative responses in HIV-seropositive and HIV-seronegative donors. Peripheral blood mononuclear cells from 12 HIV-seropositive donors (each with HIV-1 loads <4,000 copies/ml of plasma, >350 CD4 T lymphocytes/mm3, and no antiretroviral therapy) and 15 HIV-seronegative donors were assessed with multiple concentrations of each stimulant by standard lymphocyte proliferation assays. Wide variations in response rates were found, with zero, three, five, and eight individuals demonstrating stimulation indices of >3 for the HIV culture antigen supernatant, gp120, p24, and inactivated whole-virus preparations, respectively. These results suggest that the use of the inactivated whole virus resulted in a more sensitive assay for detection of CD4 T-lymphocyte function in HIV-infected subjects.

Human immunodeficiency virus (HIV)-specific immune responses are important in the control of HIV replication. CD8-positive cytotoxic T-lymphocyte (CTL) activity is associated with control of viral replication during acute infection (2, 4, 6). In addition, the frequency of HIV-specific CD8 T lymphocytes was demonstrated to inversely correlate with plasma viral load (7). CTLs may mediate control of viral replication via cytolytic mechanisms and/or the production of soluble factors that interfere with viral replication. Regardless of the mechanism, CD4 T-cell help appears to be critical to the effectiveness of the CD8 T-cell response. Strong HIV-specific CD4 T-lymphocyte responses were detected in HIV type 1 (HIV-1)-infected humans who controlled viral replication in the absence of antiretroviral therapy (10). In nonhuman primates, vaccination against HIV that resulted in the control of viral replication was associated with detectable CD4 T-cell responses (1). Further evidence suggesting a link between the HIV-specific CD4 and CD8 T-lymphocyte responses was presented by Kalams et al. (3), who demonstrated that gag-specific CD4 proliferative responses correlated with the gag-specific CTL precursor frequency. The CD4 proliferative response negatively correlated with the viral loads. Because of the importance of CD4 T-cell help in control of viral replication, methods and reagents that allow reliable and reproducible detection of this function are needed.

The assay most frequently used to assess CD4 T-cell function is the lymphocyte proliferation assay (LPA). A variety of HIV stimulants can be used in this assay. It is not clear which is the most appropriate or clinically relevant HIV-specific antigen, although recombinant HIV antigens have frequently been used. HIV p24 is commonly used because of its association with control of viral replication. However, the response elicited by a single protein may not reflect the global response to a pathogen. A whole-virus preparation may more accurately assess the global immune response to a pathogen, although there is the potential for assessment of a less specific response to a putative “protective” epitope(s).

We compared the lymphocyte proliferative response (LPR) to recombinant HIV proteins and to a whole inactivated virus preparation to determine the frequency of response in a cohort of HIV-infected donors who demonstrated control of viral replication in the absence of antiretroviral therapy. Our goal was to identify a stimulant that elicited strong proliferative responses with a low background. We limited our evaluation to stimulants that were readily available from sources that would serve as a continuous, stable supplier of stimulants for use in multicenter clinical trials. The results of this evaluation demonstrated that a whole-virus preparation serves as a robust stimulus with which to assess LPR in this population.

MATERIALS AND METHODS

Study participants. Twelve HIV-1-seropositive and 15 HIV-1-seronegative donors were recruited for this study. The seropositive donors were required to have a plasma HIV-1 RNA level <5,000 copies/ml while receiving no antiretroviral therapy and have greater than 350 CD4-positive T lymphocytes/mm3. Table 1 summarizes the viral load and CD4 T-cell levels of the HIV-1-seropositive donors. All donors gave informed consent for the donation of peripheral blood samples, according to the guidelines of the institutional review board of the New Jersey Medical School of the University of Medicine and Dentistry of New Jersey, Newark.

Preparation of PBMCs. Peripheral blood mononuclear cells (PBMCs) were isolated from acid-citrate-dextrose (ACD)-anticoagulated blood collected from donors by standard procedures. Briefly, blood was diluted (1:2) with RPMI 1640 and layered onto Ficoll-Hypaque. After centrifugation at 400 × g for 30 min at room temperature, the PBMC interface was aspirated and washed two times in...
RPMI 1640. The PBMCs were then resuspended at a concentration of 10^6 viable PBMCs/ml in culture medium (RPMI 1640, 1 mM L-glutamine, 1% penicillin-streptomycin) and used on the day of preparation. Fresh PBMCs were used for all assays.

**LPA.** The following stimulants were used and were obtained from the indicated sources: pokeweed mitogen (PWM), from Sigma Chemical Co. (St. Louis, Mo.), and recombinant HIV-1 p24 and gp120, from Protein Sciences, Inc. (Meridian, Conn.), and Austral Biologicals (San Ramon, Calif.). The Austral Biologicals gp120 antigen consisted of amino acids 31 to 509 of the CSF2 isolate. The Austral Biologicals p24 antigen consisted of amino acids 139 to 369 of the SF-2 isolate. The Protein Sciences gp124 glycoprotein was a full-length glycosylated protein from the HIV-1 MN strain. The Protein Sciences p24 antigen was a full-length protein of the NY-5 strain with additional amino acids from the C terminus of p17 and the N terminus of p15. Protein Sciences provided a protein that was used as a background control. Inactivated HIV-1 (MN) particles with conformationally and functionally intact envelope glycoproteins were prepared as described previously (11) and were obtained from the AIDS Vaccine Program, National Cancer Institute, Frederick, Md. Briefly, HIV-1 MN was propagated in H9 cells, and a stock culture was treated with 2.2'-dithiopyridine for 1 h at 37°C. The inactivating reagent was removed by ultrafiltration. An uninfected control culture was prepared in the same fashion and was used for background determinations to calculate stimulation indices (SIs). The HIV-1E culture supernatant antigen and control supernatants were obtained from Rachel Schrier (University of California at San Diego). The HIV-1E culture supernatant antigen was prepared, in brief, by coculture of PBMCs from an HIV-infected donor (U.S. origin) with phytohemagglutinin-treated PBMCs from an HIV-1-seronegative donor. Cultures were maintained by periodic addition of interleukin-2 and fresh PBMCs from seronegative donors. When the p24 level in the culture supernatant was at least 400 ng/ml, the cells were removed by centrifugation. The supernatant was aliquoted and centrifuged at 23,000 × g. The pellet was then resuspended in RPMI 1640 plus 10% human serum and heat inactivated for 1 h at 56°C. An uninfected culture supernatant was prepared in a similar fashion. A 1:10 final dilution was used in the LPAs, according to the recommendation of the supplier. Culture medium was used as an unstimulated background control for PWM and the Austral Biologicals stimulants. Stimulants were used at the indicated concentrations and dilutions. Plates were prepared by aliquoting 100 µl of stimulant or control diluted in culture medium containing 20% human AB serum into quadruplicate wells of a microtiter plate, placing the plate in a Ziplock bag, and storing the bag at −70°C until use.

At the time of use, the appropriate numbers of plates were removed from the freezer and placed in an incubator (37°C, 5% CO_2) to thaw. One hundred microliters of the cell suspension (10^6 cells) was added to each well of the plate containing a stimulant or control, and the plate was then incubated at 37°C in 5% CO_2 for 6 days. On day 6, the plates were pulsed with tritiated thymidine (1 µCi/well) and incubated for 6 h. The cells were then harvested onto filters that were then placed into scintillation cocktail and subjected to liquid scintillation counting.

**Data analysis.** Median counts per minute were determined for quadruplicate wells for each stimulant and control. The SI was calculated as the median counts per minute obtained with the stimulant divided by the median counts per minute obtained with the control stimulant. The change in the counts per minute was calculated as the median counts per minute obtained with the stimulant minus the median counts per minute obtained with the control stimulant. The background control wells with complete medium were used for the calculations described above for PWM and the Austral Biologicals stimulants. The control stimulants provided with the Protein Sciences p24 and gp120 antigens, the inactivated whole-virus preparation, and the HIV-1E culture supernatant were used as background stimulation controls for the respective HIV antigens.

**RESULTS**

**LPRs to HIV proteins and inactivated HIV.** The LPRs to the various HIV stimulants were assessed in 12 HIV-seropositive donors who were not receiving antiretroviral therapy and who had viral loads <4,000 copies/ml. The frequency of responses generating SIs greater than 3 and greater than 5 were determined for each concentration of stimulant (Fig. 1). Substantial differences in the abilities of the various stimulants to elicit LPRs were detected. Only 1 of 12 HIV-1-seropositive donors responded to the Protein Sciences gp120 antigen with an SI greater than 3. Three donors responded to the Austral Biologicals gp120 antigen at a concentration of 1 µg/ml, with one of the three also responding to the three higher concentrations. Three to five donors responded to the Protein Sciences p24 and Austral Biologicals p24 antigens, depending upon the concentration. The number of responders doubled when the whole inactivated HIV stimulant was used, with 7 to 8 (dependent upon concentration) of 12 responding with SIs greater than 3. There were no responses to the HIV-1E culture supernatant antigen. When an SI of 5 was used as the cutoff for a positive response, there was a loss of reactivity in one or two subjects within each stimulant group (Fig. 1).

The change in the counts per minute was also calculated for each donor-stimulant combination that achieved an SI greater than 3 (Table 2). The changes in the counts per minute ranged from 1,579 to 36,403, depending upon the stimulant and the concentration. The gp120 antigens had the lowest responses. The responses of the p24 antigens and the whole inactivated antigen were similar, with the 1:400 dilution of the MN strain yielding the highest counts per minute.

**LPRs in HIV-seronegative donors.** One seronegative donor demonstrated an SI greater than 3 for the Protein Sciences p24 antigen, with a substantial change in the counts per minute. This was the result of a spuriously high counts per minute value in one of the four replicate wells in this assay. Two seronegative donors had significant responses to the HIV-1E stimulant. The response to the whole viral immunogen in seronegative donors was dependent upon the concentration used, with four donors responding to a concentration of 14.9 µg/ml and one donor responding to a concentration of 7.4 µg/ml.

**DISCUSSION**

The goal of this evaluation was to compare the abilities of two different recombinant HIV proteins from two vendors, a whole viral preparation and an HIV culture supernatant antigen, to induce LPRs in HIV-infected donors. Donors were selected to provide a cohort of subjects with a high likelihood of demonstrating CD4 LPRs to HIV antigens. Donors were HIV seropositive and had viral loads less than 4,000 copies/ml while receiving no antiretroviral therapy. HIV stimuli could be arranged in three groups on the basis of the frequencies of responses in this cohort of HIV-infected subjects. The gp120 protein from both vendors and the HIV-1E supernatant were very poor stimulators of an LPR. HIV p24 antigens from both
vendors were moderately successful in inducing LPRs. Finally, the inactivated, whole viral preparation was superior in inducing LPRs in this cohort of donors.

Numerous assays have been developed to assess CD4 T-cell responses to infectious agents. Determination of intracellular cytokine expression (8) and enzyme-linked immunospot assays (5) are used as tools to study the CD4 response to HIV infection. Class II major histocompatibility complex tetramer assays (B. Y. Diab, S. Younes, G. Breton, A. McNeil, N. Bernard, K. MacDonald, M. Conners, and R. P. Sekaly, Abstr. 8th Conf. Retrovir. Opportunistic Infect., abstr. 155, 2001) are also being developed to assess this response. Although these are powerful assays and have distinct advantages associated with their use, LPA with p24 stimulation is the only available assay that demonstrates a significant correlation with viral load (10) or decline in CD4-cell numbers (9) in HIV-infected subjects.

LPAs have been the standard assays used to assess the CD4 response to antigenic stimuli. Several types of stimulants can be used in this assay, including mitogens, alloantigens, recall antigens, and neoantigens. All of these stimuli provide unique information on the quality and strength of the CD4 T-lymphocyte response. Recall antigens, however, are frequently used to assess the state of cellular immunity. Various formulations of recall antigens may be used, including whole-pathogen preparations and purified or recombinant proteins from the pathogens.

Most studies of HIV-specific CD4 T-cell responses to date have used recombinant HIV proteins, most notably, the p24
antigen. Important information has been provided by use of this antigen. Specifically, HIV-infected donors with long-term nonprogressive disease or acute-phase seroconverters who receive antiretroviral therapy are prone to demonstrate strong CD4 proliferative responses in HIV-seronegative donors. Proliferative responses to p24 were more prevalent than responses to envelope proteins. Up to 42% of donors responded to the Austral Biologicals p24 antigen with SIs greater than 3, while up to 33% responded with SIs greater than 3, while up to 33% responded with SIs greater than 5. Similarly, up to 42% of donors responded to the Protein Sciences p24 antigen with SIs greater than 3, while up to 42% responded with SIs greater than 5. One HIV-seronegative donor responded to the Protein Sciences p24 antigen at a concentration of 2.5 μg/ml with an SI of 6.6 and a change in the counts per minute of greater than 6,000. Even so, good specificity was obtained with this reagent. Compared to the Austral Biologicals p24 antigen, the changes in the counts per minute generated with the Protein Sciences reagent were nearly doubled when the reagents were used at equivalent concentrations. The low frequency of response to gp120 may be a reflection of the greater variability in the env proteins compared to that in gag proteins (3).

The other reagents tested were an inactivated whole HIV-1 MN preparation and an HIV-1E culture supernatant. We expected to detect robust responses to these antigens due to the

| Stimulant and dilution or concn | No. of samples | HIV-positive donors | HIV-negative donors |
|-------------------------------|----------------|---------------------|---------------------|
|                               |                | Change in cpm | SD | Range | No. of samples | Change in cpm | SD | Range |
| HIV-1 MN                      |                |                |    |       |                |                |    |       |
| 1:200                         | 8              | 14,624         | 13,699 | 2145–45,078 | 4              | 1,582         | 914 | 541–2,767 |
| 1:400                         | 8              | 13,761         | 13,272 | 364–36,403  | 1              | 1,043         | NA  | NA     |
| 1:1000                        | 7              | 10,027         | 9,708  | 856–31,962  | 0              | NA           | NA  | NA     |
| HIV-1E, 1:10                  | 0              | NA             | NA    | NA       | 2              | 15,881        | 6,620 | 8,278–20,368 |
| Austral Biologicals p24      |                |                |    |       |                |                |    |       |
| 10 μg/ml                      | 4              | 10,285         | 7,008  | 3,192–19,819 | 0              | NA           | NA  | NA     |
| 5 μg/ml                       | 5              | 7,495          | 5,570  | 2,270–16,679 | 0              | NA           | NA  | NA     |
| 2.5 μg/ml                     | 3              | 14,077         | 5,572  | 6,812–20,352 | 0              | NA           | NA  | NA     |
| 1 μg/ml                       | 4              | 10,404         | 4,112  | 5,033–14,651 | 0              | NA           | NA  | NA     |
| Austral Biologicals gp120    |                |                |    |       |                |                |    |       |
| 10 μg/ml                      | 1              | 1,774          | NA    | NA       | 0              | NA           | NA  | NA     |
| 5 μg/ml                       | 1              | 1,579          | NA    | NA       | 0              | NA           | NA  | NA     |
| 2.5 μg/ml                     | 1              | 2,075          | NA    | NA       | 0              | NA           | NA  | NA     |
| 1 μg/ml                       | 3              | 2,297          | 1,231  | 1,208–4,017 | 0              | NA           | NA  | NA     |
| Protein Sciences p24          |                |                |    |       |                |                |    |       |
| 10 μg/ml                      | 4              | 14,475         | 12,024 | 129–30,918  | 0              | NA           | NA  | NA     |
| 5 μg/ml                       | 5              | 14,272         | 13,382 | 225–29,941  | 0              | NA           | NA  | NA     |
| 2.5 μg/ml                     | 4              | 11,229         | 8,779  | 2,197–20,941| 1              | 6,596        | NA  | NA     |
| 1 μg/ml                       | 5              | 19,515         | 9,044  | 2,010–27,818| 0              | NA           | NA  | NA     |
| Protein Sciences gp120        |                |                |    |       |                |                |    |       |
| 10 μg/ml                      | 0              | NA             | NA    | NA       | 0              | NA           | NA  | NA     |
| 5 μg/ml                       | 0              | NA             | NA    | NA       | 0              | NA           | NA  | NA     |
| 2.5 μg/ml                     | 1              | 7,805          | NA    | NA       | 0              | NA           | NA  | NA     |
| 1 μg/ml                       | 0              | NA             | NA    | NA       | 0              | NA           | NA  | NA     |
| PWM                           | 11             | 48,114         | 32,170 | 5,942–114,887 | 15           | 44,064        | 31,252 | 8,719–10,883 |

* Number of samples with SIs>3.
* Mean counts per minute with stimulant minus mean counts per minute with control.
* SD, standard deviation.
* Minimum minus maximum change in counts per minute.
* NA, not applicable.
broad array of epitopes that would be presented compared to the array presented by a single recombinant protein. As expected, almost twice as many donors responded to the HIV-1 MN preparation than to the p24 antigen. This finding is in contrast to that of Wahren et al. (12), in which responses to p24 were more prevalent than responses to whole HIV-1. However, a different population was tested in the present study. When changes in the counts per minute were compared, this whole-virus preparation gave results similar to those obtained with the Protein Sciences p24 antigen except at a concentration of 7.4 μg/ml. The maximal counts per minute were higher for the HIV-1 MN antigen, resulting in an overall wider range of reactivities. The HIV-1E culture supernatant elicited higher for the HIV-1 MN antigen, resulting in an overall wider range of reactivities. The HIV-1E culture supernatant elicited a correlation of p24 does (with our limited numbers, there was a negative correlation of −0.23 between viral load and SI [data not shown]). The findings in this small study suggest that the whole-virus preparation is a more robust stimulus with which to elicit CD4 T-cell responses. On the basis of the data presented here, it is recommended that a concentration of the MN inactivated whole-virus antigen of 7.4 or 3.0 μg/ml be used in HIV LPAs.

ACKNOWLEDGMENTS

This work was supported by NIAID Immunology Quality Assessment contract NO1-AI-95356 and the Adult AIDS Clinical Trials Group (grant U01 AI38858–01).

We acknowledge Jeffrey Lifson (NCI) for supplying the HIV-1 MN stimulant and Rachel Schrier (University of California, San Diego) for supplying the HIV-1E culture supernatant antigen.

REFERENCES

1. Barouch, D. H., S. Santra, J. E. Schmitz, M. J. Kuruda, T. Fu, W. Wagner, M. Bliska, A. Craut, X. X. Zheng, G. R. Krivulka, K. Beauudy, M. A. Lifton, C. E. Nickerson, W. L. Trigona, K. Punt, D. C. Freed, L. Guan, S. Dubey, D. Casimiro, A. Simon, M. Davies, M. Chastain, T. B. Strom, R. S. Gelman, D. C. Montefiori, M. G. Lewis, E. A. Emini, J. W. Shiver, and N. L. Letvin. 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science 290:886–942.

2. Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. A. Oldstone. 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J. Virol. 68:6103–6110.

3. Kalams, S. A., S. P. Buchbinder, E. S. Rosenburg, J. M. Billingsley, D. S. Colbert, N. G. Jones, A. K. Shea, A.K. Trocha, and B. D. Walker. 1999. Association between virus-specific cytotoxic T-lymphocyte and helper responses in human immunodeficiency virus type 1 infection. J. Virol. 73:6715–6720.

4. Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. 68:4650–4655.

5. Moss, R. B., E. Webb, W. K. Giernakowska, F. C. Jensen, J. R. Savary, M. R. Wallace, and D. J. Carlo. 2000. HIV-1-specific CD4 helper function in persons with chronic HIV-1 infection on antiviral drug therapy as measured by ELISPOT after treatment with an inactivated, gp120-depleted HIV-1 in incomplete Freund’s adjuvant. J. Acquir. Immune Defic. Syndr. 24:264–269.

6. Muñoz, L., J. Hughes, T. Schacker, T. Shea, L. Corey, and M. J. McElrath. 1997. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. N. Engl. J. Med. 337:1267–1274.

7. Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, A. Hurley, M. Markowitz, D. D. Ho, D.F. Nixon, and A. J. McMichael. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 280:2103–2106.

8. Picker, L. J., M. K. Singh, Z. Zdravetski, et al. 1995. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. Blood 86:1408–1419.

9. Pontesilli, O., M. Carlesimo, A. R. Varani, R. Ferrara, G. D’ORezi, and F. Aiuti. 1994. In vitro lymphocyte proliferative response to HIV-1 p24 is associated with a lack of CD4+ cell decline. AIDS Res. Hum. Retrovir. 10:113–114.

10. Rosenberg, E. S., J. M. Billingsley, A. M. Caliendo, S. L. Boswell, P. E. Sax, S. A. Kalams, and B. D. Walker. 1997. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. Science 278:1447–1450.

11. Rossi, J. L., M. T. Esser, K. Suryanarayana, D. K. Schneider, J. W. Bess, Jr., G. M. Vasquez, T. A. Wiltrout, E. Chertova, M. K. Grimes, Q. J. Sattentau, L. O. Arthur, L. E. Henderson, and J. D. Lifson. 1998. Inactivation of HIV-1 infectivity with preservation of conformational and functional integrity of virion surface proteins. J. Virol. 72:7992–8001.

12. Wahren, B., L. Morfeldt-Mansson, G. Biberfeld, L. Moberg, A. Sonnerborg, P. Ljungman, A. Werner, R. Kurht, R. Gallo, and D. Bolognesi. 1987. Characteristics of the specific cell-mediated immune response in human immunodeficiency virus infection. J. Virol. 61:2017–2023.