CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, May 2002, p. 525–529 Vol. 9, No. 3
1071-412X/02/$04.00+0 DOI: 10.1128/CDLI.9.3.525–529.2002

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

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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/mm^3, 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/mm^3. 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

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TABLE 1. Immunologic and virologic characteristics of HIV-seropositive donors

Donor no.	Viral load (no. of copies/ml)	CD4 count (no. of cells/μl)
1	<400	763
2	952	438
3	<400	839
4	935	693
5	852	441
6	<400	589
7	<400	780
8	650	589
9	1,250	800
10	2,846	359
11	1,104	869
12	3,968	689

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 gp120 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 interkeukin-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 type 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 (10^5 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 stimulant control. 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 stimulant control. 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 per 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

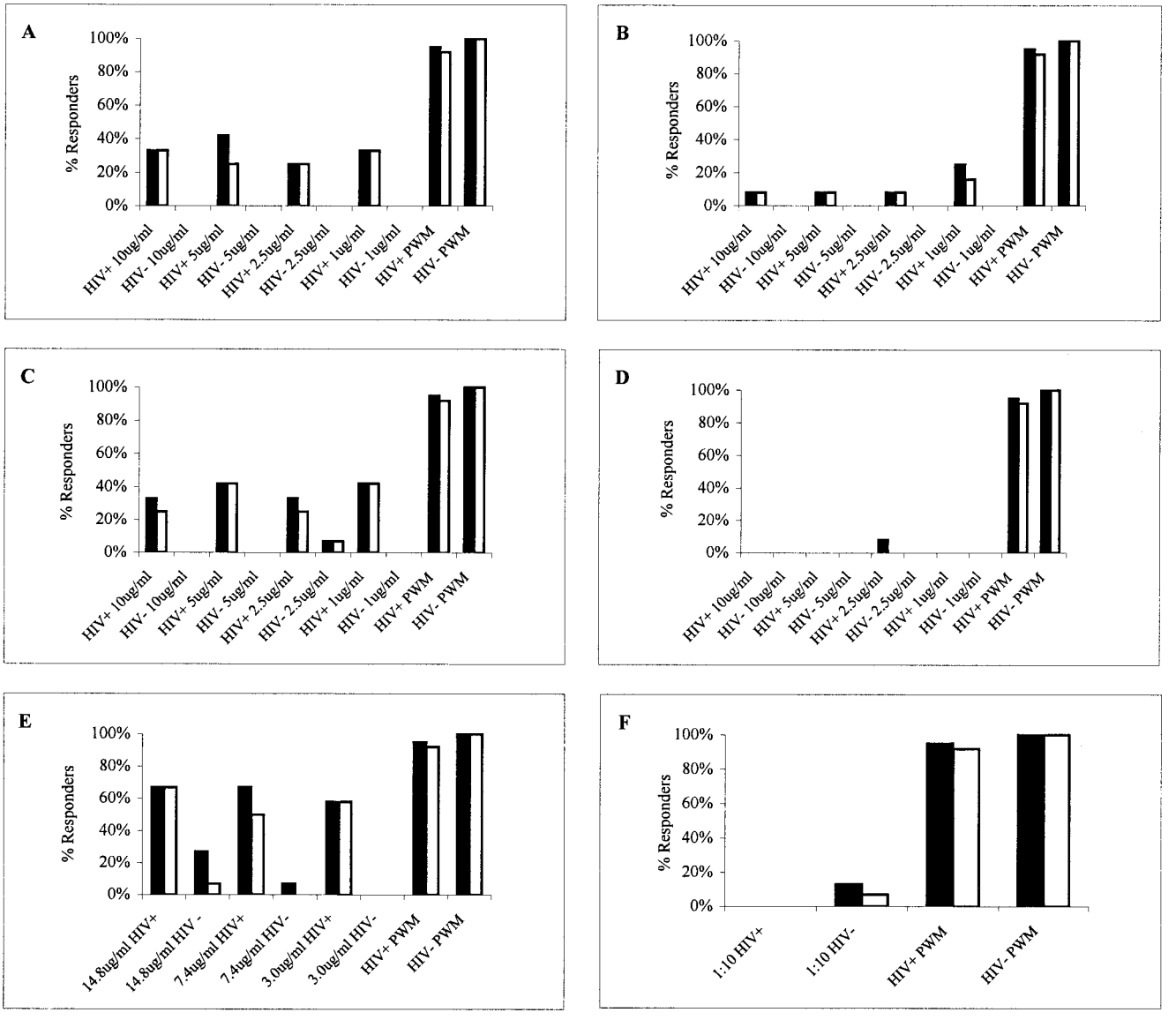

FIG. 1. Proportions of HIV-seropositive (+; $n = 12$) and HIV-seronegative (−; $n = 15$) donors with SIs >3 (solid bars) and >5 (open bars) for the Austral Biologicals p24 (A) and gp120 (B) antigens, the Protein Sciences p24 (C) and gp120 (D) antigens, the inactivated whole-virus HIV-1 MN preparation (E), and the HIV-1E culture supernatant antigen (F; final dilutions are indicated). The frequency of response to PWM along with that to each stimulant is indicated.

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

TABLE 2. Changes in counts per minute for HIV-seropositive donors

Stimulant and dilution or concn	No. of samples[a]	HIV-positive donors			HIV-negative donors			
		Change in cpm[b]	SD[c]	Range[d]	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	836–31,962	0	NA	NA	NA
HIV-1E, 1:10	0	NA[e]	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	5,370	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	225–20,941	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

[a] Number of samples with SIs>3.
[b] Mean counts per minute with stimulant minus mean counts per minute with control.
[c] SD, standard deviation.
[d] Minimum minus maximum change in counts per minute.
[e] NA, not applicable.

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 LPRs to p24 (10). These LPRs to p24 are hypothesized to provide help to CD8 T-cell effectors, which are mediators of the antiviral effect (3).

The present study was conducted because of the importance of identifying and obtaining reliable and, it is hoped, clinically relevant stimulants for use in multicenter clinical trials. In addition to comparing different vendors for the same stimulants (p24 and gp120), we also compared whole-virus and viral culture supernatant antigen preparations. The rationale for inclusion of the last two stimulants was their potential to provide a more complete assessment of the global CD4 proliferative response to HIV.

Similar to other investigators (3), in the present study gp120 stimulation led to limited proliferative responses in HIV-infected subjects. Only one subject demonstrated significant proliferation at the three highest concentrations of the Austral Biologicals gp120 antigen tested. Three and two subjects had SIs above 3 and 5, respectively, with the lowest concentration of this stimulant. This finding likely reflects individual variability in the optimal stimulant concentration for the induction of proliferation. One subject responded to the 2.5-μg/ml concentration of the Protein Sciences gp120 stimulant. Both of the gp120 stimulants were very specific, in that they did not elicit significant 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 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 regent. 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

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 poor proliferative responses. The reason(s) for this poor response is not known. We cannot rule out the use of a suboptimal concentration of stimulant in this study. We used the recommended final dilution but did not verify its suitability in our assay.

While the whole-virus preparation elicited responses in a larger number of immunologically and virologically well off donors, it remains to be determined if the response to this stimulus correlates with virologic control, as the response to 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.

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