Antigen-Presenting Cell Modulation Induces a Memory Response to p24 in Peripheral Blood Leukocytes from Human Immunodeficiency Virus-Infected Individuals

Michael A. Kolber* and Maria O. Saenz
Division of Infectious Diseases, Department of Medicine, University of Miami School of Medicine, Miami, Florida

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The accurate determination of human immunodeficiency virus type 1 (HIV-1)-specific proliferative responses is critically important when evaluating immune recovery after highly active antiretroviral therapy. Using a new assay to enhance proliferative responses to recall and HIV antigen, we addressed the questions of whether viral load affects cellular immunity and whether long-term viral load suppression results in loss of antigen-specific responder cells. This assay is based on the fact that lipopolysaccharide (LPS) can augment proliferative responses to antigen after monocyte adherence to a tissue culture plate. Twenty-six HIV-1-infected individuals donated peripheral blood leukocytes (PBL). Proliferation assays against p24, using LPS and cell adherence, were performed on all samples. Medical record abstraction provided information on CD4 cell nadir and time of viral load suppression. PBL from HIV-1-infected individuals with a viral load of <200 copies/ml had a significant proliferative response and a stimulation index of >5 to p24 (12 of 15) compared to those with a viral burden (2 of 11), using the LPS-adherence assay. Proliferative responses to p24 could be found in PBL from virally suppressed donors independent of the CD4 cell nadirs and in the majority of the donors who were virally suppressed for >10 months (7 of 10). The data presented here demonstrate that LPS and monocyte adherence provide a sensitive and specific way to boost proliferative responses to recall and HIV antigens.

Materials and Methods

Materials. Casta antigen (from Candida albicans) was obtained from Greer Laboratories, Inc. (Lenoir, N.C.). The HIV-1 recombinant viral peptide Gag p24 (IIIB) was obtained from Immunodiagnostics, Inc. (Woburn, Mass.). Hepatitis C virus NS3 antigen (42 kDa) and hepatitis C virus core antigen (22 kDa) were obtained from ViroStat (Portland, Mass.). Purified hepatitis B virus surface antigen-Presenting Cell Modulation Induces a Memory Response to p24 in Peripheral Blood Leukocytes from Human Immunodeficiency Virus-Infected Individuals

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antigen was obtained from The Binding Site, Inc. (San Diego, Calif.). LPS was obtained from Sigma Chemical Co. (St. Louis, Mo.). [3H]thymidine was obtained from New England Nuclear (Boston, Mass.).

Complete medium consisted of RPMI with antibiotics, 1-glutamine, minimal essential medium nonessential amino acids, minimal essential medium sodium pyruvate, and 10 mM HEPES plus 10% human serum.

Cells. PBL were purified from whole blood by using LSM (ICN Biomedicals, Inc., Aurora, Ohio) separation. Whole blood was obtained from donors (uninfected and HIV-1 infected) after informed consent (approved by the institutional human subjects review board of the University of Miami School of Medicine) and used on the day of harvesting unless noted. HIV-1-infected donor cells were recruited from the Adult HIV Outpatient Immunology Clinics on the Jackson Memorial Hospital/University of Miami Medical School campus. Autologous serum was also obtained from normal donor whole blood and heat inactivated. Autologous serum was used in the complete medium during culture for experiments with PBL from uninfected donors. For experiments performed with PBL from HIV-1-infected donors, AB+ heat-inactivated serum was used in the complete medium. All HIV-1 donors were asymptomatic at the time of phlebotomy.

Antigen proliferation experiments. PBL were plated in quadruplicate for each experimental point in wells (10^6 cells/well) of a 96-well U-bottom microtiter tissue culture plate for a period of time designated by the experimental conditions. For the experiments in which the effect of monocyte adherence was studied, the tissue culture plate was incubated in a 5% CO2 incubator at 37°C for 1 day prior to addition of p24 and/or Casta antigen as dictated by the experiment. When indicated, LPS was added at the designated concentrations to the experimental wells for a period of 1 h after the addition of antigen. After addition of reagents, the experimental plate was incubated for 6 days in a 5% CO2 incubator at 37°C. After the 6-day incubation 1 μCi of [3H]thymidine was added per well and the plate was incubated overnight. Wells were subsequently harvested, and DNA-incorporated radioactivity was counted. Determinations of specific counts were obtained by subtracting nonspecific counts (no antigen) from the absolute counts. Added reagents were present for the full period of incubation. Proliferation is given in counts per minute.

Nonadherent cell proliferation study. Freshly isolated PBL were incubated immediately with LPS so that PBL did not adhere before exposure to LPS. Cesta antigen was added as indicated for the experimental conditions, and radiolabeled thymidine was added and cells were harvested as described above.

Transfer experiments. In transfer experiments, PBL from normal uninfected donors were plated and permitted to adhere for 2 h. After this time, the nonadherent cells were removed and transferred to other wells. After an overnight incubation, LPS (0.01 μg/ml) was added to half of the wells (quadruplicates) containing the adherent cells, to half of the wells containing the nonadherent cells only, and to half of the wells containing the complete PBL. After incubation for 1 h at 37°C in a 5% CO2 incubator, the wells were washed twice with complete medium and the nonadherent cells were added back to the adherent cells. In this case the adherent cells preincubated with LPS received the nonadherent cells that were not incubated with LPS, whereas the nonadherent cells preincubated with LPS were added to the adherent cells that were not incubated with the LPS. After the cells were combined, Cesta antigen (1.0 μg/ml) was added to quadruplicate wells for each group and complete medium was added to the remaining four wells for each group. Proliferation was allowed to proceed, and after 6 days [3H]thymidine was added. The plate was harvested the next day to assess the incorporated radioactivity.

Statistics. The unpaired t test was used to determine significance for Fig. 1 and 2. The Mann-Whitney rank test was used to determine the significance of differences between specific p24 proliferative responses in the group with an undetectable viral load and the nonsuppressed groups (17).

RESULTS

HIV-1 PBL donor characteristics. Table 1 shows the patient demographic and immunologic characteristics at the time of harvesting PBL for the experiments described below. The median age for those with an undetectable viral burden was 47 years, and that for those with a detectable viral burden was 42 years. Nineteen of the 26 HIV-1-seropositive donors were male. There were two long-term nonprogressors, one donor who was naive to therapy, and one donor on dual nucleoside reverse transcriptase inhibitor therapy; the remainder were on HAART. Patient 2 had a history of Hodgkin’s disease and was 1 year out from chemotherapy and in remission. No patient was symptomatic with their HIV-1 disease or had active infections at the time of sampling. The mean CD4 counts were 641 cells/mm3 (standard deviation = 312 cells/mm3) for those with an undetectable viral burden and 460 cells/mm3 (standard deviation = 227 cells/mm3) for those with a detectable viral burden (P > 0.05).

Monocyte adherence augments antigen-driven proliferation by LPS. LPS has been demonstrated in a number of studies to enhance proliferation to antigen at low concentrations (18, 38) and to differentially affect cytokine production on nonadherent and adherent monocytes (24). Since we had shown (23) that adherent monocytes play an essential role in whether gp41 inhibits or stimulates antigen-driven proliferation, we evaluated whether monocyte adherence affected antigen-driven proliferation in the presence of LPS. To study this question, PBL were obtained from HIV-seronegative individuals, and prolif-
enrichment to Casta antigen when LPS was added before the monocytes were allowed to adhere or 1 day (18 h) after plating was evaluated. Results from a representative experiment (of four) are shown in Fig. 1A. In Fig. 1A it is seen that when LPS is added before the monocytes are allowed to adhere there is a diminution in the absolute proliferative response to Casta antigen \( (P < 0.025) \), whereas after 1 day of adherence there is an enhancement in the proliferative response to Casta antigen \( (P < 0.05) \). For the adherence experiments, our previous data from dose-response studies (not shown) demonstrated that 0.01 \( \mu \text{g} \) of LPS per ml was optimal for antigen-driven proliferative enhancement.

To further demonstrate the importance of the monocyte in LPS-driven proliferation, we performed a transfer experiment. In this experiment adherent cells (>85% monocytes by flow) were treated with or without LPS after an overnight incubation and washed, and nonadherent cells treated with or without LPS added back as described in Materials and Methods. The results of a representative experiment (of 3) are shown in Fig. 1B. There was a large enhancement in proliferation to Casta antigen when the adherent monocytes were treated with LPS compared to when the nonadherent cells were treated with LPS. It is also clear in Fig. 1B that the proliferative response to Casta antigen for adherent monocytes plus LPS-treated nonadherent cells is the same as that for the PBL in this experiment.

Taken as a whole, Fig. 1 demonstrates that monocyte adherence is an important component in whether LPS induces a proliferative or inhibitive response. This effect of monocyte adherence on LPS augmentation of a proliferative response to antigen had previously not been noted. In all of the remaining experiments described below, the PBL were permitted to adhere overnight.

LPS and adherence enhance proliferative responses to p24 in PBL from HIV-1-infected individuals. Next we wanted to evaluate whether individuals with HIV-1 infection would respond to the HIV-1 core antigen p24 when PBL from the donors were allowed to plate overnight prior to treatment with LPS and antigen. Figure 2 shows the response to antigen for PBL from HIV-1-infected individuals with a nondetectable viral load (Fig. 2A), HIV-1-infected individuals with a measurable viral load (>200 copies (c)/ml) (Fig. 2B), and non-HIV-1-infected individuals (Fig. 2C). Figure 2A demonstrates that PBL from subjects with a nondetectable viral load proliferate to p24 well after adherence and LPS treatment. The p24 (1.0 \( \mu \text{g/ml} \)) proliferative response without LPS treatment is generally low.

Stimulation indices (SI) are routinely used to evaluate proliferative responses to p24 of PBL from HIV-1-infected individuals \( (\text{SI} = \text{experimental counts per minute/control counts per minute}) \). Although the cutoff used for this parameter is relatively arbitrary, it does help with the determination of an assay’s sensitivity. If we use the criteria (3) of an SI of >5 and a difference of 5,000 cpm between experimental and control conditions, then PBL from 12 of the 15 subjects responded to p24 after LPS and adherence (Table 2). These responses were all significant \( (P < 0.05) \). Compared to those subjects that had a detectable viral load (Fig. 2B) only 2 of 11 subjects responded to p24 or to p24 after adherence and LPS treatment (subjects 16 and 25). When the numbers of p24 responders (after adherence and LPS treatment) with a nondetectable viral load (Fig. 2A) and with a viral load (Fig. 2B) are compared, there are significantly more \( (P < 0.05) \) responders in the group with a nondetectable load. The subjects in Fig. 2B are arranged in ascending order of their viral burden. Figure 2C shows the response of PBL from uninfected donors. The response to p24 alone or to p24 after adherence and LPS treatment is low. No subjects in the control group had an SI of >5 and a change in counts per minute of >5,000.

Figure 2 shows that numerous subjects had a high LPS response. The fact that some individuals have PBL that pro-
literate to LPS has been well described (see, e.g., reference 26). Whether an individual responded to LPS or not did affect whether the individual responded to p24 or to p24 after LPS and adherence. Evidence that the augmentation in response to p24 with adherence and LPS treatment is specific is shown in Fig. 3. Figure 3A shows a titration of the PBL response (of subject 26) to Casta antigen with and without LPS treatment. Even though there is an augmentation of the Casta antigen response at low concentrations with adherence and LPS, there is a significant \( P < 0.05 \) and monotonic decrease in proliferation with decreasing amounts of antigen. In Fig. 3B the PBL response (of subject 27) to a number of different antigens is shown. The proliferative responses to Casta antigen and p24 antigen are both boosted after adherence and LPS. The SI to hepatitis C virus NS3 antigen, hepatitis C virus core antigen, and hepatitis B virus surface antigen are not greater than 5, whereas the SI to p24 is 10.3 after adherence and LPS. It is notable that this patient was hepatitis C virus antibody positive and hepatitis B virus negative, indicating that the assay was not sensitive enough to reveal a response to the hepatitis C virus antigens that were used. However, Fig. 3 demonstrates that the response to antigen is titratable and that the response to p24 does not represent a general nonspecific response.

Effect of CD4 cell nadir and duration of viral load suppression on LPS-induced proliferative augmentation. There are numerous studies that have evaluated the effect of CD4 T-cell nadir (6, 25, 40) and the duration of undetectable viral load as determinants of cellular immune recovery after HAART (33, 37, 40). The data have been varied, with some studies able to show a response after viral load suppression (1, 2, 35, 37) and others not able to do so (4, 13, 34, 40). In Table 2 we show the available data for HIV-1-seropositive PBL donors with an un-

| Patient no. | CD4 (cells/mm³) | Time ND (mo) | SP | p24/medium | p24-LPS/PS |
|-------------|-----------------|--------------|----|------------|------------|
| 1           | 924             | 14           | 4.8| 9          |
| 2           | 1,085           | 32           | 1.2| 4          |
| 3           | 362             | 10           | 4.4| 6.3        |
| 4           | 311             | 11           | 10.3| 13.8      |
| 5           | 684             | 3            | 4.7| 51.4       |
| 6           | 222             | 32           | 4.5| 5.6        |
| 7           | 1,230           | 6            | 2.2| 11.7       |
| 8           | 379             | 247          | 2.2| 3.2        |
| 9           | 725             | 5            | 82.7| 17.9      |
| 10          | 492             | 30           | 19.6| 3.2       |
| 11          | 604             | 269          | 3.4| 18.3       |
| 12          | 243             | 154          | 13.2| 94.3      |
| 13          | 610             | 512          | 12.8| 4.8       |
| 14          | 790             | 15           | 5.1| 7.6        |
| 15          | 958             | >52          | 4.9| 10.3       |

a Time ND, time with a nondetectable viral load. IF the time ND was ≥10 mo, the value is in boldface.

b p24/medium, cpm_p24/cpm_medium (SI_p24). A boldface value indicates that the SI was >5 and the cpm was >5,000.
c —, not available.
detectable viral load for current CD4 cell count, nadir CD4 cell count, and time that a donor had an undetectable load. Although the amount of data displayed is limited, a number of points can be made. First, for the 10 patients with long-term suppression (≥10 months), 7 of the 10 generated a significant proliferative response to p24 with LPS and adherence treatment as determined by the SI and a change in counts per minute of >5,000, compared to 3 of 10 patients with a significant proliferative response to p24 alone. Second, eight patients had a documented CD4 cell nadir (Table 2). In seven of the eight cases a vigorous proliferative response to either p24 alone or p24 augmented by LPS and adherence was found. It is notable that individuals who are LPS responders have a reduction in their SI relative to those without LPS (e.g., subjects 18, 19, and 23 in Table 2). These data suggest that proliferative responses and responder cells are present even after extended periods of viral load suppression or reconstitution from any given CD4 cell nadir.

**DISCUSSION**

In this paper we have demonstrated a sensitive and specific assay that can be used to reveal proliferative responses to p24 in PBL from individuals infected with HIV-1. Although other studies have used LPS to enhance antigen-driven proliferation (18, 38), they have not identified monocyte adherence as important in augmenting the proliferative response. This was demonstrated in Fig. 1. In an earlier paper (23), we demonstrated that gp41 will enhance proliferation to recall antigen if the monocytes are permitted to adhere. This, together with the present study, suggests that the state of the monocyte may be a critical component in interventions that strive to boost immunologic responses to antigen or to evaluate such responses. The unique finding of this study is that the proliferative enhancement was dependent on both the LPS and monocyte adherence. The mechanism by which this augmentation in proliferation to antigen occurs is not known. There are, however, data showing that monocyte adherence modifies the production of various cytokines, such as tumor necrosis factor alpha (TNF-α) (24). Complementing that work are a number of studies. One study demonstrates that TNF-α supports gamma interferon-driven production of interleukin-12 (IL-12) (15). In a murine study, LPS given in vivo supported lymphocyte survival after injections of staphylococcal enterotoxin A (41). This was mediated in part through TNF-α (41). Furthermore, monocyte adherence and LPS will modify B7 expression of monocytes (see, e.g., reference 38), affecting proliferative responses as well. It is anticipated that enhancement of the proliferative effect to antigen is a complex combination of both cytokine and costimulatory molecules acting to drive the response.

The enhanced sensitivity of the LPS and adherence assay contributes to the field, since there has been considerable variation in the ability of different laboratories to generate a proliferative response to p24 from PBL obtained from HIV-1-infected individuals. This problem has been recognized as so significant by the Adult and Pediatric AIDS Clinical Trials Group Immunology laboratory subcommittees that an attempt was made to determine whether this variability was attributable to the p24 antigen preparation as well as concentration (39). The results of that study suggested that because of the variability of proliferative results to p24, whole virus might be more useful than the subunit preparations commonly used. More sophisticated assays such as ELISPOT or intracellular cytokine staining have been used to study antigen responses. Although they provide some advantages over the usual lymphocyte proliferation assays, they measure only one cytokine at a time. This may work well for CD8 assays, in which the cytokine is a readout for a terminal event, but for effector responses in which multiple cytokines participate in the proliferative response, correlations between cytokine and proliferation may not be found (15).

Factors that affect immunity to HIV are important in guiding therapeutic interventions. Plasma viral load, time of suppression after HAART, and CD4 cell nadir have all been
studied in regard to addressing questions such as when to begin therapy and the importance of viral load suppression. In the present study we demonstrate that although PBL proliferative p24 responses were low in patients with a nondetectable viral load, monocyte adherence and LPS treatment augment the response in the majority of the patients studied. This augmentation was not found in PBL from HIV-1-infected individuals with a viral load greater than the limits of detection (>200 c/ml). This finding is consistent with data from other studies demonstrating that PBL from HIV-1-seropositive individuals with a plasma viral burden are poorly responsive to HIV-1 antigen-driven proliferation (28, 30, 37). However, some studies have not found an effect of viral load on either proliferation (5) or gamma interferon production (33). This variability may be attributable to a difference in the sensitivity of the assays used or in the indicator variable used to evaluate the response.

The enhanced antigen responsiveness of PBL from virally suppressed individuals is consistent with results from clinical trials as well. Data from IL-2 studies suggest that individuals with a measurable viral burden elevate their CD4 counts after treatment to a lesser degree (32) than those individuals with a suppressed viral load (14). In a macaque study evaluating therapeutic vaccination after early simian immunodeficiency virus infection, the development of a CD8 cellular response and containment of viral burden were dependent on the administration of antiretroviral therapy and not vaccination (21). In a recent therapeutic vaccination trial, individuals were vaccinated with an HIV-1 immunogen and evaluated for changes in proliferative responses to p24 as a function of time. Those individuals with a viral burden of >400 c/ml had little or no proliferative response to p24, compared to a significant response in the individuals with a suppressed viral burden, 28 weeks after vaccination (29). These studies are consistent with our data suggesting that the ability to boost immune responsiveness is impaired in those individuals with a viral burden. It would be worthwhile to evaluate, in a prospective manner, whether an inability to boost responsiveness to antigen predicts an inability to respond after in vivo interventions such as IL-2 administration or vaccination.

Numerous groups have examined proliferative responses to p24 as a function of time after initiation of HAART. For patients with extended viral suppression, some groups have shown a proliferative response to p24 (1, 2, 35, 37) whereas others have not (4, 13, 34, 40). Recently it has been shown that after multiple cycles of structured treatment interruptions, there is recovery of historical cytotoxic T lymphocyte responses. Since these responses are the same as those that were found before induction of HAART, this suggests a persistent memory response (20). Alternatively, data showing that there is a loss of memory cell responders with successful viral load suppression over time have been presented (see, e.g., reference 33). Since the memory cell population is important for protection against persistent infection in numerous viral diseases, it is important to understand whether these reports are secondary to loss of responder cells or not. In the present study the majority of the subjects (7 of 10) with durable viral load suppression for ≥10 months responded to p24 after LPS and monocyte adherence, whereas only 3 of 10 responded to p24 alone (Table 2). Therefore, these data are consistent with a persistent memory response over the time period studied.

The data in Table 2 further suggest that those subjects with a relatively high CD4 cell nadir (>200 c/ml) can respond to p24 antigen after adherence and LPS augmentation. This can be considered, together with an earlier study (6) that found little proliferative response to p24 antigen for those infected individuals with a suppressed viral burden and a preserved elevated nadir CD4 count, to indicate that responsive CD4 cells exist but are not optimally driven to proliferate because of the cytokine environment (11) or a lack of a costimulatory signal (28).

The assay presented in this work is sensitive and specific in evaluating responses to p24 antigen. Monocyte adherence to the tissue culture wells affects both cytokine production and activation state. In addition, LPS affects cytokine production and expression of costimulatory molecules. Together these factors cooperate by engaging multiple inductive pathways conducive to proliferation. What specific coordination is occurring is a subject of further investigation.

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