

Human immunodeficiency virus glycoprotein (gp120) induction of monocyte arachidonic acid metabolites and interleukin 1

(acquired immunodeficiency syndrome/prostaglandins/leukotrienes/CD4)

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ABSTRACT This study reports on the direct effect of the envelope glycoprotein (gp120) of the human immunodeficiency virus type 1 (HIV-1) on human monocyte function. Addition of preparations of purified gp120 from the HIV-1 to human monocytes resulted in the production of interleukin 1 (IL-1) and arachidonic acid metabolites from the cyclooxygenase and lipoxygenase pathways. Quantification of prostaglandin E₂ (PGE₂) and IL-1 revealed an increase in both mediators with 50 ng of gp120 per ml and an increase of 12- and 30- to 40-fold with 200-400 ng of gp120 per ml, respectively. Unlike native gp120, the recombinant nonglycosylated gp120 fragments PB1-RF and PB1-IIIB, as well as one of the core structural proteins of HIV-1, p24, did not increase arachidonic acid metabolism or IL-1 activity. Cytofluorometric analysis revealed that gp120 blocked the binding of OKT4A to the CD4 on monocytes, whereas OKT4 binding was unaffected. Involvement of the CD4 in signal transduction was further demonstrated by the ability of OKT4 and OKT4A monoclonal antibodies to increase monocyte PGE₂, IL-1 activity, and nanogram amounts of IL-1 β .

Human T-cell lymphotropic viruses (HTLV) types I and II and human immunodeficiency virus type 1 (HIV-1) isolated from patients with T-cell leukemia (1, 2), hairy-cell leukemia (3), and acquired immune deficiency syndrome (AIDS) (4, 5), respectively, have protein and nucleic acid compositions characteristic of retroviruses. While infection of human peripheral blood cells by HTLV-I or HTLV-II has been shown to alter immune functions *in vitro* (6-9), the purified envelope proteins from retroviruses have also been shown to have immunomodulatory effects. For example, Con A-induced transformation of human lymphocytes is blocked by the envelope protein p15E of feline leukemia virus (10-12), and the proliferative response of human lymphocytes to phytohemagglutinin is inhibited by the envelope glycoprotein (gp120) of HIV-1 (13).

HIV-1 has been demonstrated to inhibit the binding of OKT4A to monocytes (14), suggesting that gp120 on the surface of the virus binds to monocytes. Studies with a lymphoid cell line, H9, infected with HIV-1 have demonstrated massive shedding of gp120 from HIV-1 (15-17). Monocytes have also been shown to be infected by HIV-1 and may serve as a reservoir for the virus (14, 18, 19), providing another possible source of shed gp120. Thus, the monocyte may be exposed and interact with gp120 on the intact virus or free gp120 shed by the maturing HIV-1, which may have a direct effect on the function of immunocompetent cells. This study reports on gp120-mediated transduction of an activation signal in monocytes, which results in the production of arachidonic acid (AA) metabolites and interleukin 1 (IL-1).

MATERIALS AND METHODS

Purification of Viral Proteins. The gp120 used in this study was isolated from the culture fluids or cell extracts of HIV-IIIB- or HIV-RF-infected H9 cells by immunoaffinity chromatography and subsequently purified by polyacrylamide gel electrophoresis to homogeneity, as demonstrated by the isolation of gp120 from a single band (20). At the highest concentrations tested, the gp120 preparations had <1 ng of endotoxin per ml, as determined by the *Limulus* amoebocyte lysate assay (21). The PB1-IIIB and PB1-RF fragments used are the recombinant, nonglycosylated truncated fragments of the two respective HIV isolates (22), which represent most of the COOH-terminal half of gp120. The PB1 fragment is a fusion protein that contains 180 of the 481 amino acids of gp120. PB1 is encoded by the DNA sequence from *Pvu* II to the second *Bgl* II site of the HTLV-III/lymphadenopathy-associated virus (LAV) envelope gene (23) and has an additional 30 and 24 non-HTLV-III amino acids on the NH₂ and COOH termini, respectively. The core protein, p24, was purified as described (24).

Immunoprecipitation of gp120. Goat anti-gp120 antisera were obtained as described (20). Fifty microliters of anti-gp120 antiserum or preimmune serum was added to 50 μ l of phosphate-buffered saline containing 20 μ g of gp120 and was incubated at 4°C for 3 hr. One hundred microliters of 20% (vol/vol) protein A (Pharmacia) was added and the mixture was rotated for 60 min at 4°C. The two preparations were microcentrifuged at 10,000 \times g for 2 min and the supernatants were stored at 4°C.

Human Monocytes. Human peripheral blood mononuclear cells were obtained by leukapheresis of normal volunteers at the blood bank of the National Institutes of Health. After density sedimentation of the mononuclear cells with lymphocyte separation medium (Organon Teknika), the monocytes were purified by counterflow centrifugation elutriation (CCE) as described (25), except that pyrogen-free phosphate-buffered saline (B & B Research Laboratories, Fiskeville, RI) was used in the elutriation procedure. Monocytes were enriched to >90% as determined by morphology, nonspecific esterase staining, and flow cytometry (25).

Evaluation of gp120 Binding to Monocytes. Monocytes preincubated in the presence or absence of 400 ng of gp120 per 10⁶ cells at 37°C for 3 hr and subsequently stained with OKT4 and OKT4A monoclonal antibodies (Ortho Diagnostics) were examined by cytofluorometry on an Ortho Cyto-

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Abbreviations: HTLV, human T-cell lymphotropic virus; HIV, human immunodeficiency virus; AIDS, acquired immune deficiency syndrome; AA, arachidonic acid; IL, interleukin; CCE, counterflow centrifugation elutriation; PG, prostaglandin; TXB₂, thromboxane B₂; HETE, hydroxyicosatetraenoic acid; LT, leukotriene; CI, channel intensity.

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fluorograph System-50 with an argon ion laser. The percentage of T4- and T4A-positive cells was calculated against a background of nonspecific labeling by using normal mouse IgG (1–3%). Fluorescein isothiocyanate-conjugated F(ab')₂ goat anti-mouse IgG was obtained from Tago. For analysis of T4 and T4A fluorescence, emission was measured at 488 nm in relative units (axis \times channel number). As a positive control, cells from the A3.01 human T-lymphocyte line were analyzed under identical conditions.

Analysis of [³H]AA Metabolites Released by Monocytes. Two milliliters of serum-free Dulbecco's modified Eagle's medium (DMEM; Biofluids, Rockville, MD) containing 5×10^6 monocytes was cultured in 35-mm wells for 1 hr at 37°C, and then 10% AB serum and [³H]AA (1.0 μ Ci/ml; 1 Ci = 37 GBq; Amersham) were added to the cultures for 18 hr. The monocyte cultures were washed three times with DMEM without phenol red containing 0.02% fatty acid-free human serum albumin (Sigma) and cultured in 2 ml of this medium for 24 hr in the presence or absence of gp120. The culture media were harvested, centrifuged at $400 \times g$ for 5 min, and the supernatants were stored under argon at -70°C in 1-dram (≈ 4 ml) vials. Prior to analysis, a 2-ml sample was thawed and the pH was adjusted to 3.6 with H₃PO₄ and microcentrifuged for 1 min. The sample was applied to a C18 reverse-phase guard column (Brownlee Labs), located in the sample loop of a Valco injector (Valco Instruments, Houston), which had been equilibrated with solvent A (0.1% H₃PO₄ in water adjusted to pH 3.6 with triethylamine). The guard column was washed with 2 ml of solvent A and 3 ml of solvent A containing 15% acetonitrile. The AA metabolites extracted by the guard column were then injected onto two AXXI-chrom C18 (3 μ m; 4.6×100 mm) columns (Cole Scientific, Calabasas, CA) connected in series at a flow rate of 1 ml/min. HPLC analysis was performed with a Perkin Elmer series 4 liquid chromatography system and involved the use of four solvents: solvent A (described above), solvent B (0.1% H₃PO₄ in water with 2 mM EDTA adjusted to pH 5.3 with triethylamine), solvent C (acetonitrile), solvent D (methanol). A flow rate of 1 ml/min was used for the chromatographic run, which included the following steps: 1, 0–8 min, 85% A/15% C to 67% A/33% C; 2, 8–33 min, 67% A/33% C (isocratic); 3, 33–35 min, 67% A/33% C to 67% B/33% C; 4, 35–65 min, 67% B/33% C to 58% B/42% C; 5, 65–70 min, 58% B/42% C to 100% A; 6, 70–77 min, (flow rate, 0.7 ml/min) 100% A to 26% A/74% D; 7, 77–117 min, 26% A/74% D (isocratic); 8, 117–127 min, 26% A/74% D to 100% D; 9, 127–140 min, 100% D (isocratic).

The elution was monitored with a radioactive flow-through detector (Ramona-LS, IN/US, Fairfield, NJ) containing a

1-ml flow cell with an eluant/scintillation (Tru-Count, Tru Laboratories, Libertyville, IL) ratio of 1:2. The radioactive counts are expressed as the sliding average of the previous 4 sec with the current 1-sec count. Identification of the eluted metabolites was determined by comparison with the elution times of known ³H radioactive standards, which included prostaglandin E₂ (PGE₂), 6-keto-PGF_{1 α} , thromboxane B₂ (TXB₂), PGF_{2 α} , PGE₁, PGD₂, 5(s)-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE, 15-HETE, 12-hydroxy-5,8,10-heptadecatrienoic acid, leukotriene B₄ (LTB₄), LTC₄, LTD₄, AA (Amersham), and LTE₄ (New England Nuclear).

PGE₂ Assay. PGE₂ levels in the media supernatants from monocyte cultures were determined by radioimmunoassay as described (26) using rabbit anti-PGE₂ antiserum obtained from Lawrence Levine (Brandeis University, Waltham, MA).

IL-1 Assay. Media supernatants from monocyte cultures were assayed for IL-1 activity using thymocytes from 6- to 8-wk-old CeH/HeJ mice as described (27). The thymocyte cultures were pulsed with [³H]thymidine for the final 5 hr of incubation and the incorporated radioactivity was transformed into units by comparison to the IL-1 standard of 100 units/ml. One unit represents the amount of IL-1 required to double the proliferative response of mouse thymocytes exposed to a suboptimal dose (1 μ g/ml) of phytohemagglutinin. The thymocyte assay has a sensitivity of 1 unit of IL-1; however, it does not differentiate between IL-1 and IL-2 or other factors that may inhibit or enhance proliferation. IL-1 levels were also determined with an ELISA kit for IL-1 β (Cistron, Pine Brook, NJ), which can detect IL-1 β at 20 pg/ml. The antiserum used in the assay is specific for IL-1 β with no cross-reactivity for IL-1 α , IL-2, tumor necrosis factor, or interferon- γ .

Addition of Monoclonal Antibodies to Monocytes. Monocytes (5×10^6 cells per ml) were cultured in suspension in polypropylene tubes (12 \times 75 mm; Falcon, Becton Dickinson) in 1 ml of DMEM containing 10% AB serum for 20 min to block the Fc receptors. Unconjugated OKT4, OKT4A, and OKDR (Ortho Diagnostics), which had been dialyzed against three changes of DMEM, were then added to some of the cultures in various concentrations. The cells were cultured for 24 hr at 37°C and the supernatants were assayed for PGE₂, IL-1 activity, and IL-1 β .

RESULTS

Specific Binding of gp120 to Monocytes. Expression of CD4 receptors on the surface of monocytes (28) suggested that the HIV-1 envelope protein gp120 should bind to CD4. This possibility was examined with cytofluorometry by comparing

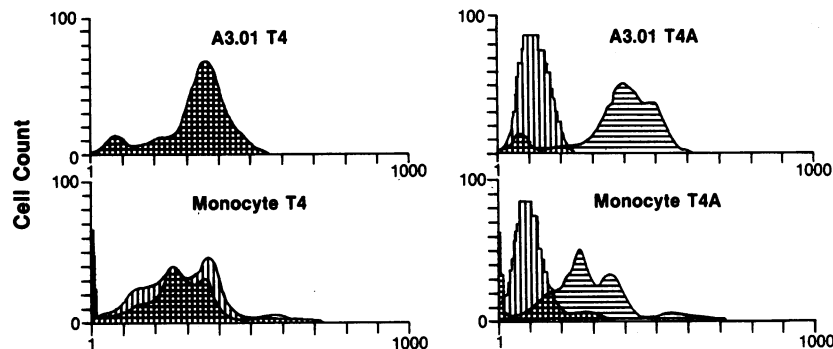


FIG. 1. Effect of gp120 on the binding of anti-T4 and anti-T4A to lymphocytes and monocytes. The A3.01 human T-lymphocyte line and human monocytes purified by CCE were stained with fluoresceinated OKT4 and OKT4A monoclonal antibodies after a 3-hr preincubation with gp120 (400 ng/ 10^6 cells) at 37°C. Cells were analyzed on a fluorescence-activated cell sorter with the emission measured in relative units (axis \times channel number). A comparison of fluorescence intensity was made by using the mean channel number for different histograms. The percentage of T4- and T4A-positive cells was calculated against a background of nonspecific labeling by using normal mouse IgG (1–3%). Horizontal lines, untreated; vertical lines, gp120 treated.

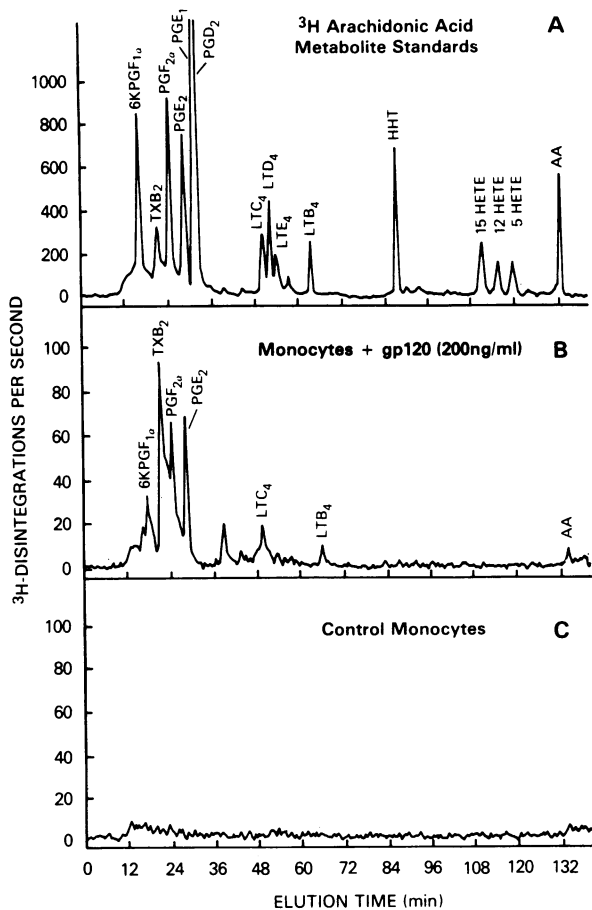


FIG. 2. Effect of gp120 on the release of AA metabolites from human monocytes. (A) HPLC profile of [³H]AA metabolite standards. (B) HPLC profile of [³H]AA metabolites released from CCE-purified monocytes cultured in the presence of gp120 (200 ng/ml) or in the absence (C) of gp120. 6KPGF_{1α}, 6-keto-PGF_{1α}; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid.

the relative binding of OKT4 and OKT4A monoclonal antibodies to CD4 in the presence or absence of purified gp120 (Fig. 1). The CD4-positive T-cell line A3.01 was used as a control. Untreated A3.01 cells displayed 98% OKT4-positive cells with a channel intensity (CI) value of 408, represented by the horizontal histogram. Likewise, A3.01 cells were 91% OKT4A positive with a CI of 351. After a 3-hr preincubation with 400 ng of gp120, the A3.01 cells were stained with OKT4A or OKT4 antibodies. A pronounced shift to the left in fluorescence intensity of OKT4A binding indicated that gp120 was binding to this epitope of the CD4 molecule. The CI changed from 305 to 151 and the percent OKT4A-positive

Table 1. Immunoprecipitation with anti-gp120 antiserum blocks induction of monocyte PGE₂ by gp120

| Monocyte treatment | PGE ₂ , ng per 10 ⁶ cells per 24 hr |
|-------------------------|---|
| Control | 0.21 ± 0.34 |
| Preimmune serum + gp120 | 5.57 ± 0.32 |
| Immune serum + gp120 | 0.11 ± 0.05 |

gp120 was added to goat serum (preimmune serum + gp120) or goat anti-gp120 antiserum (immune serum + gp120). Protein A was added and the immune complexes were removed by centrifugation. Supernatants, equivalent to 200 ng of gp120 in the preimmune serum, from these preparations were added to monocyte cultures and the media levels of PGE₂ were determined by radioimmunoassay. Results represent the mean ± SD of triplicate cultures.

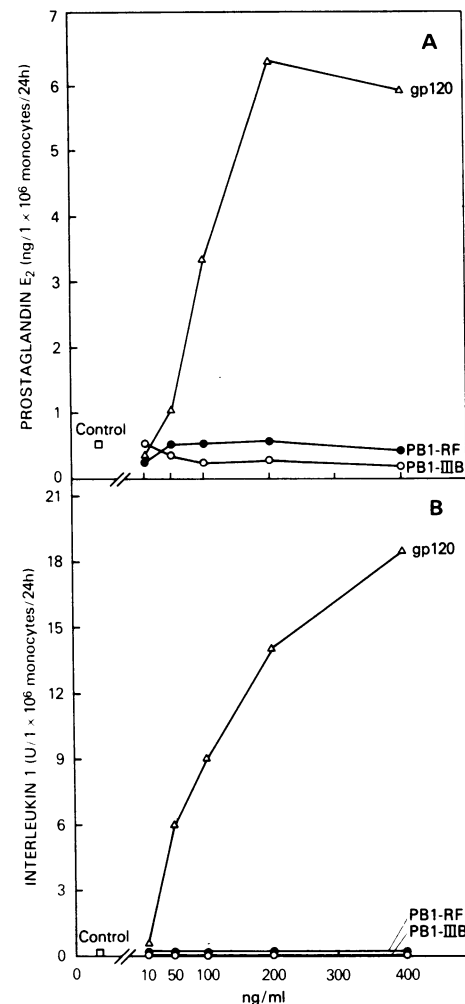


FIG. 3. Dose-dependent effect of gp120 and the recombinant gp120 fragments, PB1-RF and PB1-IIIb, on PGE₂ and IL-1 production by monocytes. Monocytes were cultured in DMEM in 24-well plates at 1 × 10⁶ cells per ml per well. (A) PGE₂ levels. (B) IL-1 activity in the media supernatants of CCE-purified monocytes exposed to the indicated concentrations of gp120 and gp120 fragments.

cells changed from 91% to 44% in the presence of gp120 (vertical histogram). OKT4 fluorescence remained virtually unchanged in gp120-treated A3.01 cells. Cytofluorometric analysis of monocytes, which were purified to >90% by CCE, revealed that these cells expressed the relative values of 23% positive/437 CI OKT4 and 23% positive/414 CI OKT4A staining (Fig. 1). After treatment of the monocytes with gp120, OKT4 staining was 26% positive/411 CI and OKT4A staining was 7% positive/160 CI. As shown by the histogram, a marked shift in fluorescence intensity is observed only with the OKT4A antibody.

Induction of Monocyte AA Metabolism by gp120. HPLC analysis of the products released from [³H]AA-labeled monocytes after exposure to gp120 (200 ng/ml) revealed inductive synthesis of cyclooxygenase and lipoxygenase pathway enzymes (Fig. 2B). Based on HPLC elution times of ³H standards (Fig. 2A), the metabolites produced via the cyclooxygenase pathway were 6-keto-PGF_{1α}, TXB₂ (the breakdown products of prostacyclin and thromboxane A₂, respectively), PGF_{2α}, and PGE₂. LTC₄ and LTB₄, metabolites of the 5-lipoxygenase pathway, were also identified. An additional unidentified radioactive peak between PGE₂ and LTC₄ may represent a leukotriene degradation product. The total ³H radioactive disintegrations for each AA metabolite released from gp120-stimulated monocytes were as follows: 6-keto-PGF_{1α}, 3059; TXB₂, 10,094; PGF_{2α}, 5738; PGE₂, 5283;

Table 2. CD4 receptor-dependent stimulation of monocyte PGE₂ and IL-1 production

| Monocyte treatment | PGE ₂ , ng per 5 × 10 ⁶ cells per 24 hr | IL-1, units per 5 × 10 ⁶ cells per 24 hr | IL-1β, ng per 5 × 10 ⁶ cells per 24 hr |
|--------------------|---|---|---|
| Control | 0.03 ± 0.02 | <1 | 0.12 ± 0.06 |
| gp120 (400 ng/ml) | 58.22 ± 7.0 | 12.3 ± 4.5 | 3.7 ± 0.32 |
| OKT4 | | | |
| 0.1 μg/ml | 0.93 ± 1.04 | <1 | 0.67 ± 0.48 |
| 1.0 μg/ml | 6.37 ± 2.78 | 1.95 ± 1.9 | 1.9 ± 0.82 |
| 5.0 μg/ml | 62.22 ± 9.54 | 13.67 ± 1.2 | 2.6 ± 0.81 |
| OKT4A | | | |
| 0.1 μg/ml | 0.76 ± 0.45 | <1 | 0.45 ± 0.12 |
| 1.0 μg/ml | 5.76 ± 2.16 | 2.2 ± 1.7 | 1.3 ± 0.18 |
| 5.0 μg/ml | 61.11 ± 12.10 | 18.0 ± 2.65 | 3.4 ± 0.08 |
| OKDR | | | |
| 0.1 μg/ml | 0.36 ± 0.13 | <1 | 0.48 ± 0.37 |
| 1.0 μg/ml | 0.38 ± 0.40 | <1 | 0.67 ± 0.26 |
| 5.0 μg/ml | 2.43 ± 0.28 | <1 | 0.67 ± 0.57 |

Monocytes were cultured in suspension at 5 × 10⁶ cells per ml in DMEM containing 10% AB serum for 20 min prior to the addition of the indicated unconjugated monoclonal antibodies or gp120. The supernatants were harvested 20 hr later and analyzed for PGE₂ by radioimmunoassay, for IL-1 activity by the thymocyte assay, and for IL-1β by ELISA. Data represent the mean ± SD of triplicate cultures.

unidentified peak, 1992; LTC₄, 1973; LTB₄, 794; AA, 652. Monocytes not exposed to gp120 released low levels of radioactivity (Fig. 2C).

Concentration-Dependent Effects of gp120 and gp120 Fragments on Monocyte PGE₂ Production. The media from monocyte cultures exposed to various concentrations of gp120 were analyzed for PGE₂ by radioimmunoassay to determine the dose range over which gp120 stimulated AA metabolism. As shown in Fig. 3A, an increase in PGE₂ was initially detected with 50 ng of gp120 per ml. After an increase of ≈12-fold, PGE₂ levels reached a plateau at 200 ng of gp120 per ml. The amount of PGE₂ produced in response to gp120 was comparable to that obtained with stimulants such as lipopolysaccharide or Con A. Immunoprecipitation with anti-gp120 antiserum blocked the gp120-induced PGE₂ production (Table 1). The gp120 concentration-dependent effects on PGE₂ reflected proportional changes in the other AA metabolites as determined by HPLC (data not shown). The PB1-IIIB and PB1-RF fragments did not stimulate PGE₂ synthesis (Fig. 3A), nor did they increase any of the other AA metabolites, as analyzed by HPLC. Similarly, p24 also failed to induce AA metabolism (data not shown).

Concentration-Dependent Effects of gp120 and gp120 Fragments on Monocyte IL-1 Activity. The ability of gp120 to stimulate other monocyte products, such as IL-1, was examined in the medium from the same experiment shown in Fig. 3A. As shown in Fig. 3B, the production of IL-1 by monocytes in response to increasing amounts of gp120 paralleled that of PGE₂. As in the case of PGE₂, the recombinant fragments PB1-IIIB and PB1-RF had no effect on IL-1 release. The steady-state level of IL-1β mRNA in monocytes was also increased by gp120 (data not shown).

Effect of Monoclonal Antibodies to CD4 on Monocyte PGE₂ and IL-1 Production. In light of the interaction of gp120 with the CD4 on monocytes, we examined the effect of monoclonal antibodies directed against this antigen on monocyte PGE₂ and IL-1 production. As shown in Table 2, the addition of either OKT4 or OKT4A to monocytes resulted in a dose-dependent induction of PGE₂ synthesis, IL-1 biological activity, and IL-1β. The levels of PGE₂ and IL-1 released by the addition of these monoclonal antibodies (5 μg/ml) to CD4 were equivalent to those obtained from the monocytes treated with gp120 (400 ng/ml). In contrast, OKDR, which is in the same immunoglobulin subclass (IgG2a) as OKT4A, failed to substantially increase PGE₂ or IL-1.

DISCUSSION

Previous reports have shown that HIV-1 (29–31) or purified gp120 (32) binds to an epitope on the CD4 molecule on lymphocytes, which can be blocked by the monoclonal antibody OKT4A, as demonstrated in this study with the lymphocyte cell line A3.01. Our observations that the direct binding of gp120 to the CD4 molecule displaces OKT4A on human monocytes are in agreement with the findings that HIV-1 inhibits OKT4A binding to monocytes (14). The interaction between gp120 and CD4 may involve the manose-containing carbohydrate moieties on the viral envelope glycoprotein that have been reported to affect, either directly or indirectly, the interactions of the envelope protein with cell-surface CD4 on lymphocytes (33). The inability of the nonglycosylated truncated HIV-1 recombinant fragments, PB1-IIIB and PB1-RF, to induce monocytes to produce PGE₂ or IL-1 is in agreement with the finding that this fragment fails to bind to CD4 (24).

The ability of gp120 to directly transduce an activation signal in monocytes resulting in the production of AA metabolites may affect the immune system in several ways. PGE₂ is primarily a suppressor of immune function as demonstrated *in vitro* by its ability to inhibit the production of lymphokines, natural killer or cytotoxic T-cell-mediated cytotoxicity, and T- and B-cell proliferation (35). PGE₂ has been implicated in the immunosuppression associated with malignancies such as Hodgkin disease (36). In addition, PGE₂ also functions as a feedback inhibitor of monokine production, monocyte-mediated tumor cell cytotoxicity, and phagocytosis (35). Thus, the initial exposure of monocytes to either soluble or intact virion gp120 *in vivo* may result in a PGE₂-mediated suppression of immune surveillance by the lymphocytes. Furthermore, the ability to combat secondary opportunistic infections may be compromised because of the reduction in monocyte or lymphocyte function by PGE₂. The lipoxygenase product LTB₄ can have either negative or positive effects on lymphocyte function depending on which subset of lymphocytes is treated with LTB₄ (37). Of particular interest is the ability of LTB₄ to inhibit lymphocyte proliferation through the induction of active suppressor T cells (OKT8⁺) (38–40). Moreover, LTB₄ has been reported to induce suppressor T cells, which can be derived from either OKT4⁺ or OKT8⁺ cells but are phenotypically OKT8⁺ (41). Thus, in addition to the cytopathic effects of HIV-1 on

OKT4⁺ cells, the immune system may be further suppressed by the induction of active OKT8⁺ cells by LTB₄.

The IL-1 produced by monocytes in response to gp120 may have several effects in patients with AIDS. While IL-1 was initially described as a molecule that interacted with lymphocytes to enhance the immune response, it is now apparent that IL-1 can affect the function of many cell types (42). Particularly germane to the pathobiology of AIDS is the well-described pyrogenic activity of IL-1 and its ability to stimulate the growth of cells of differing tissue specificity. Recently, IL-1 has been reported to stimulate the proliferation of Kaposi's sarcoma cells *in vitro*.[¶]

An added complication of the induction of an activation signal in monocytes *in vivo* by gp120 may be the inability of these cells to respond to a subsequent stimulant. This refractory period may compromise the host defense mechanisms particularly against the secondary opportunistic diseases associated with AIDS.

The ability of gp120 to block binding of OKT4A to monocytes implicates the involvement of CD4 in signal transduction leading to monocyte activation. Further evidence for the mediation of a signal through CD4 was demonstrated by the ability of the monoclonal antibodies OKT4 or OKT4A to induce IL-1 activity, IL-1 β , and PGE₂ synthesis in monocyte cultures. The stimulation of AA metabolism by ligand-receptor interaction implies a receptor-mediated activation of phospholipase A₂ as a mechanism for mediating signal transduction. As a consequence of monocyte activation by gp120, a variety of physiological metabolites (such as PGE₂ and LTB₄) and mediators (IL-1) are produced that may affect the biology of the target cell and perhaps contribute to the physiological complexities of HIV-1 pathology.

[¶]Nakamura, S., Ensoli, B., Salahuddin, Z. & Gallo, R., Third International Conference on AIDS, June 1-5, 1987, Washington, D.C., abstr., p. 15.

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