JOURNAL OF CLINICAL MICROBIOLOGY, July 1988, p. 1304–1308
0095-1137/88/071304-05$02.00/0

Vol. 26, No. 7

Entamoeba histolytica Antigen-Specific Induction of Human Immunodeficiency Virus Replication

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Received 18 November 1987/Accepted 11 April 1988

Replication of human immunodeficiency virus (HIV) may be initiated in infected lymphocytes by antigenic or mitogenic stimulation. A soluble protein derived from an invasive strain of *Entamoeba histolytica* (amoebic antigen [AA]) was used to study the lymphoblastic responses of T lymphocytes derived from 8 HIV-seronegative homosexual men (controls) and 15 HIV-seropositive homosexual men (patients). The soluble protein was also used in long-term cultures as a stimulus for HIV replication. No control or patient produced detectable lymphoblastic responses to AA in a 6-day tritiated-thymidine incorporation assay. Of 15 patients, 5 (33%) produced HIV p24 (ranging from 31 pg/ml to 151 ng/ml) in response to AA in 30-day cell cultures. HIV p24 was expressed in three of seven patients in response to AA but not to the T-lymphocyte mitogen phytohemagglutinin. Implications for managing HIV-infected patients are discussed.

The human immunodeficiency virus (HIV), a retrovirus of the Lentivirus subfamily, causes the acquired immune deficiency syndrome (AIDS) and a chronic neurologic disorder (3, 9, 10, 12, 16). The immune deficiency is characterized in part by the destruction of CD4+ (helper) lymphocytes (15, 20). Many HIV-infected persons do not develop AIDS or other symptoms of immune deficiency for prolonged periods of time. Numerous epidemiological studies have noted the high frequency of sexually transmitted diseases in AIDS patients in the United States and Northern Europe and other infectious diseases in AIDS patients in the developing countries (5, 14). A prospective study of homosexual men followed from seroconversion correlated the development of AIDS with the acquisition of other sexually transmitted diseases (26). Cell culture models have demonstrated that in vitro activation of lymphocytes by mitogen or antigen is a prerequisite for HIV replication (22, 27). These clinical and laboratory studies have prompted the basic assumption that antigenic activation of the immune system in vivo acts as a cofactor for the development of AIDS in the infected person (27).

On several occasions we have noted the development of AIDS in HIV-infected homosexual men after exposure to intestinal parasites. Protozoan parasites have long been known to activate both the humoral and cell-mediated immune system (4). Studies of biopsies of the gastrointestinal (GI) tracts of homosexual men with histories of chronic or recurrent GI parasites note lymphoid hyperplasia and chronic lymphocytic inflammatory infiltrates (13). Humoral antibodies to amoebic antigens (AA) were detected in 21% of the homosexual men studied by one group (1). Salata and Ravdin (24) have demonstrated that an *Entamoeba histolytica*-derived soluble protein has mitogenic activity for normal human lymphocytes in the presence of monocytes. On the basis of these and similar studies, several investigators have suggested that enteric parasites should be treated aggressively in the presence of HIV infection (23). Other investigators have maintained that treatment might be required for all commensal nonpathogens if the antigenic stimulation theory was followed to its "illogical conclusion" (2).

We have studied the lymphocytic responses of 22 homosexual men to a soluble protein (AA) of a pathogenic strain of *E. histolytica*, and we show that this protein did not produce detectable lymphocyte proliferation in 6-day cultures. Moreover, we studied the ability of the AA preparation to initiate replication of HIV in long-term cell cultures from these men, and we have evidence that HIV replication can be detected in response to AA.

MATERIALS AND METHODS

Patients and controls. All 23 subjects (except 1 seronegative man) were homosexual men, 26 to 43 years of age. Participants are part of a prospective group that has been followed for an average of 20 months; 11 of these men have been followed for more than 28 months. Of the 23, 15 men were HIV seropositive by Western blot (immunoblot) (patients), while 8 men were HIV seronegative (controls).

Preparation of PBMCs. Peripheral blood mononuclear cells (PBMCs) were prepared by gradient centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) (6). After repeated washes with Hanks phosphate-buffered saline (Whittaker/M.A. Bioproducts, Walkersville, Md.) and assessment of viability by trypan blue exclusion, the cells were suspended in RPMI 1640 supplemented with glutamine, 10% fetal bovine serum, and antibiotics (100 U of penicillin G per ml, 100 μg of streptomycin per ml, and 0.25 μg of Fungizone per ml [Whittaker/M.A. Bioproducts]).

Cultivation, harvest, and preparation of soluble AA. AA was prepared as a standard antigen from *E. histolytica* organisms (strain HK-9) as described by Mathews et al. (18). Briefly, HK-9 organisms were cultivated in TYI-S-33 medium (8) and harvested after 48 to 72 h of growth. The organisms were detached from the glass culture tubes by chilling the tubes in an ice bath for 5 to 10 min and recovering the amoebae by centrifugation at 160 × *g* for 6 min. The supernatant was removed, and the amoebae were suspended in cold 0.85% (wt/vol) NaCl and centrifuged as before. After

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being washed three times in saline, the amoebae were counted with the aid of a hemacytometer, centrifuged, suspended in a minimal volume of deionized water, and frozen. Several harvests were pooled to obtain a total of 4×10^8 organisms. The organisms were thawed and treated with ultrasound (W-375 Sonifier; Heat Systems Inc.) until no intact organisms were detected microscopically. The disrupted organisms were centrifuged at 20,000 × *g* for 30 min, and the supernatant was recovered. The volume was adjusted with water so that each 0.5 ml contained the extract from 2×10^6 organisms. This extract was lyophilized in 0.5-ml quantities and stored at −20°C. Protein content of a vial taken at random was determined by the Bradford dye-binding assay (7) and was found to contain 0.24 mg of total protein.

For use in lymphocyte proliferation studies, the antigen was dissolved in 2 ml of sterile saline, aliquoted, and stored at −20°C until use.

Lymphocyte proliferation studies. PBMCs were diluted to a viable cell concentration of 4×10^6/ml in complete medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. Dilutions of phytohemagglutinin (PHA) (10 and 5 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and AA (12, 6, and 3 μg/ml) were prepared in complete medium. Equal volumes of cells and medium with antigens or mitogens were then added to achieve a cellular concentration of 2×10^6/ml in 5.0 and 2.5 μg of PHA per ml and in 6, 3, and 1.5 μg of AA per ml. Aliquots of 250 μl were then added to wells of flat-bottom microdilution plates (Falcon; Becton Dickinson Labware, Oxnard, Calif.) in triplicate. Control wells contained cells with an equal volume of complete medium. Microdilution plates were incubated in a humidified chamber at 37°C for 6 days in 95% room air and 5% CO_2. At 18 h before harvesting, wells were pulsed with [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) at 1 μCi per well. The cells were harvested with an automatic cell harvester (Brandell Scientific, Gaithersburg, Md.) onto glass fiber strips. The strips were air dried at room temperature for 2 days and then placed in 7-ml scintillation vials (Fisher Scientific Co., Pittsburgh, Pa.) with 5 ml of scintillation fluid per vial (Scintiverse E; Fisher Scientific). After equilibration in the dark, the vials were counted in a scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Data were expressed as counts per minute per well after subtraction of the background.

Replication of HIV in cell culture. Preliminary studies indicated that proliferation of lymphocytes in the presence of AA occurred slowly. Consequently, an AA concentration of 3 μg/ml was chosen arbitrarily for use in long-term cell culture. PBMCs were diluted to a concentration of 4×10^6/ml in RPMI 1640 supplemented with 15% fetal bovine serum, glutamine, and antibiotics. (This cellular concentration was selected because of previous experience with HIV-infected lymphocyte cultures in which the concentration of cells decreased rapidly.) Aliquots (10 ml) were set up in 25-cm^2 culture flasks (Corning Glass Works, Corning, N.Y.) in duplicate. One flask was stimulated with 5.0 μg of PHA per ml (final concentration); the second flask was stimulated with 3 μg of AA per ml. After 72 h, the volume of each culture flask was reduced by 25% and replaced with fresh complete medium containing recombinant human interleukin 2 (New England Nuclear) (final concentration, 950 BRM units/ml [11]). Cultures were maintained in humidified 95% room air and 5% CO_2 at 37°C for 30 days. Every 3 days, 20% of the culture medium was replaced with complete medium containing recombinant interleukin 2. On days 10, 15, and 20, the cultures were fed an additional 5% (vol/vol) lectin-free T-cell growth factor (Electronucleonics, Inc., Silver Spring, Md.). On day 15, cultures were restimulated with either 5.0 μg of PHA or 3 μg of AA per ml.

Replication of HIV was assessed by two measures. On days 10 and 13, cells from spent medium were recovered by centrifugation (400 × *g* for 10 min). Cell pellets were suspended in phosphate-buffered saline, deposited on glass slides, and allowed to air dry. The cells were fixed in ice-cold methanol-acetone and stained with murine monoclonal antibody to HIV p24 (*gag* gene product, internal capsid protein) (Biotech Research Labs, Inc., for Du Pont Co., Billerica, Md.). Sections were stained with a solution (2.0 μg/ml) of anti-p24 in phosphate-buffered saline for 1 h at 37°C in a humidified chamber. Secondary labeling was performed with fluoresceinated goat anti-mouse immunoglobulin G (Tago, Burlingame, Calif.) diluted 1:100 in phosphate-buffered saline. Sections were examined by epifluorescence microscopy (E. Leitz Inc., Rockleigh, N.J.). Quantitative HIV p24 protein production was assessed by antigen capture enzyme immunoassay (19) (Biotechnology Systems, New England Nuclear, for Du Pont Co., Bethesda, Md.) according to instructions of the manufacturer with minor modifications.

Cell culture supernatants were clarified by centrifugation at 500 × *g* for 20 min. Samples (200 μl) of the supernatants were introduced into duplicate wells of immunoplates sensitized with a polyclonal rabbit anti-HIV-p24. After overnight incubation at room temperature, the wells were washed with ice-cold buffer and incubated with biotinylated rabbit anti-p24 diluted 1:50 for 2 h at 37°C. After washes, the wells were incubated with a 1:50 dilution of streptavidin-horseradish peroxidase for 15 min at room temperature. After further washes, OPD (*o*-phenylalanine diamine) substrate was developed for 30 min at room temperature in the dark and stopped with 4 N H_2SO_4, and the A_{490} was read in a spectrophotometer (Bio-Tek Instruments, Burlington, Vt.). Data were handled by an enzyme immunoassay data reduction package (Biotechnology Systems). All samples were run in duplicate on two occasions. Several samples required multiple dilutions before coming into assay range.

Statistics. Statistical analysis of the lymphocyte proliferation studies and p24 antigen capture studies was performed by using the Number Cruncher Statistical Software package (Jerry Hintz, Keysville, Utah). Tests of significance were the unpaired Student *t* test and one-way analysis of variance with Fisher's least significant difference test, Duncan's test, and the Newman-Keuls multiple comparisons test. The criterion for significance was $P \leq 0.05$.

RESULTS

Parasitology. All seronegative controls were free of GI symptoms; two reported amoebic enteritis by history but were negative for ova and parasites on stool examination. Of 15 HIV-seropositive men, 14 (93%) reported amoebic histories. Most seropositive men reporting positive amoebic histories noted one or more episodes of amoebiasis, although the exact numbers and types of parasites were not specified. Six seropositive men (40%) reported GI symptoms suggestive of current parasitism, including chronic diarrhea, poorly formed stool, flatulence, urgency, and GI discomfort; four of these men (27%) were parasitized at the time of study. (For results of stool examinations at the time of lymphocyte culture for each of the seropositive patients, see Table 2.)

Lymphocyte proliferation studies. Lymphoblasts of all 23 men responded to the lectin PHA but failed to respond to AA

TABLE 1. Lymphoblastic transformation responses in HIV-seronegative and HIV-seropositive homosexual men by clinical diagnosis[a]

Stimulant (final concn [μg/ml])	Incorporation (cpm) ± SEM (stimulation index)[b] in group:			
	Controls	CDC II	CDC IV A	CDC IV C1
PHA (5)	77,226 ± 8,537 (84.5)	85,117 ± 7,775 (133)	18,241 ± 4,501[c] (85)	15,186 ± 3,758[c] (39)
PHA (2.5)	81,400 ± 8,578 (98.1)	86,840 ± 9,930 (158)	17,689 ± 2,935[c] (77)	20,140 ± 15,840[c] (5.3)
AA (6)	1,106 ± 310 (1.2)	3,100 ± 2,837 (0.8)	186 ± 61 (0.9)	187 ± 50 (0.5)
AA (3)	1,122 ± 299 (1.2)	2,338 ± 655 (1.5)	237 ± 66 (1.1)	305 ± 63 (0.8)
AA (1.5)	711 ± 244 (0.7)	2,027 ± 685 (1.4)	148 ± 64 (0.7)	376 ± 8 (0.9)

[a] Responses are expressed as counts per minute of [^{3}H]thymidine incorporated in 6-day cultures (corrected for background). Centers for Disease Control clinical diagnosis groups: CDC II, asymptomatic seropositive individuals; CDC IV A, individuals with AIDS-related complex; CDC IV C1, individuals with AIDS.
[b] Stimulation index, stimulated count − background/nonstimulated count − background.
[c] Significantly different from seronegative control (defined as $P \leq 0.05$).

in the 6-day test format. The counts per minute of tritiated thymidine incorporated in response to the various concentrations of PHA and AA are shown in Table 1 along with the stimulation index.

Responses to PHA in the seropositive group varied widely. Significant differences were noted between asymptomatic seropositive men and men with symptoms of AIDS-related complex and men with AIDS. The responses of the asymptomatic patients were indistinguishable from those of controls. Men with AIDS-related complex and AIDS showed progressively decreasing responses to PHA.

The response to AA was negligible in the 6-day lymphoblast transformation assay. No significant difference was found in the response to AA between the patients and controls. When backgrounds were taken into account, only two individuals (one patient and one control) showed slight responses to any concentration of AA during the 6-day trial period. In these two cases, the response was less than a two-fold increased incorporation of tritiated thymidine over nonstimulated background. No significant differences were detectable in the responses to AA of the clinically well and clinically sick patients. No differences were detected in the level of thymidine incorporation in response to AA between men who expressed HIV p24 in response to AA and those who did not. Current infection with parasites also had no effect on the response to our amoebic preparation. Two men (one patient and one control) were also tested with a concentration of 12 μg of AA per ml without response during the 6-day proliferation period (data not shown).

Replication of HIV in cell culture. HIV cells were cultured without the use of added exogenous CD4+ target cells to approximate the in vivo condition of the host. Cultures stimulated with the T-cell lectin PHA served as controls for the AA-stimulated system.

Seven individuals (47%) were culture positive under at least one culture condition. Table 2 shows the presence and quantity of p24 viral protein produced by mitogen or antigen and relates the production to the clinical history and stool examination for ova and parasites at the time of the study. The day of assessment indicates the first day on which a positive culture was found for the subject by enzyme immunoassay. The fixed-cell fluorescence assay was performed only on days 10 and 13.

Cultures containing AA responded by minimal proliferation. By approximately day 10 (the day varied from culture to culture), foci of cells could be seen. The cells characteristically formed small clusters initially composed of three to four cells tightly associated with an adherent cell. These clusters often showed cell fusion. Although free-floating syncytia are commonly seen in HIV-infected cell cultures, the syncytia seen in these AA-stimulated cultures were firmly attached to adherent cells.

DISCUSSION

The HK-9 strain of *E. histolytica* used in this study is a laboratory-adapted strain originally isolated from a patient with invasive amoebiasis. It has been typed by isoenzyme analysis as invasive (17). This antigen preparation contains components derived from both membrane and cytosol fractions of the cell but contains virtually nothing that binds to concanavalin A (18). None of the subjects in this study responded to a wide range of concentrations of the antigen preparation in the 6-day lymphoblast proliferation assay. This contrasts with the generally pronounced proliferative responses to the lectin PHA by most individuals in both groups.

Visual inspection of the long-term cultures stimulated with AA often identified a low-grade response by about day 9 to 13. The response was typified by foci of cells characteristically associated with adherent cells. The enlargement of these foci over several days appeared to be cellular prolifer-

TABLE 2. Production of HIV p24 in primary lymphocyte cultures

CDC diagnostic group[a]	Parasite history[b]	Current parasites[b]	HIV p24 (pg/ml) detected in assay[c]:				
			EIA			IIF	
			PHA	AA	Day	PHA	AA
CDC II	+ (?)	−				−	−
	+ (?)	−		54	14	−	+
	+ (?)	1, 3				−	−
	1, 2	−				−	−
	1, 2, 3	−				−	−
	−	1, 2				−	−
	1, 2, 3	−	47		28	−	−
	1, 2, 3	−				−	−
	1, 2	−	617	48	27	+	+
	+ (?)	−				−	−
CDC IV A	1, 2	−		220	14	−	+
	1, 2, 3	6		31	15	−	−
	+ (?)	−	151[d]	14[d]	23	+	+
CDC IV C1	+ (?)	1, 3, 4, 5	30		14	+	+
	+ (?)	−				−	−

[a] For Centers for Disease Control group definitions, see Table 1, footnote *a*.
[b] Parasites: 1, *E. histolytica*; 2, *Giardia lamblia*; 3, *Entamoeba hartmanni*; 4, *Entamoeba nanna*; 5, *Iodamoeba bütschlii*; 6, *Isospora belli*; + (?), parasites present but not identified.
[c] HIV p24 was detected (+) (−, not detected) in supernatants by antigen capture enzyme immunoassay (EIA) and in fixed cells by indirect immunofluorescence (IIF) by use of a stimulating mitogen (PHA) or antigen (AA). For the enzyme immunoassay, results are shown for the first day positive.
[d] Results expressed as nanograms per milliliter.

ation, although recruitment could not be absolutely excluded. Such findings are consistent with an antigen-specific T-cell response initiated by antigen-presenting cells. This response was seen in both the historically amoeba-sensitized and the amoeba-free groups. Similar clusters of lymphocytes and adherent cells were not seen in the PHA-stimulated cultures in which the proliferating clusters float freely. In two cultures, one stimulated with PHA and the other with AA, HIV replication was detected by the antigen capture immunoassay but not by the intracellular immunofluorescence assay. Because cell cultures were assayed by intracellular immunofluorescence only on days 10 and 13, low-grade responses may have been missed.

Cultures from patients responding to AA displayed syncytia adherent to the plastic culture vessel. Up to 50 nuclei could be counted in these syncytia. These adherent syncytia were not seen in cultures from controls or in PHA-stimulated cultures from patients. Although HIV-infected cells can stick more readily to plastic surfaces than normal cells can, we believe that these adherent cell structures may indicate that HIV-induced syncytia can be formed not only by lymphocytes but also by the interaction of mononuclear phagocytes and lymphocytes.

The most surprising finding was the discordant HIV replication responses to AA and PHA in several patients. Three of the seven patients (43%) who had positive cultures responded to AA only. The number of responses here was small, and these findings must be substantiated by larger series of isolations. Nonetheless, the finding that HIV can be detected following antigen-specific stimulation of lymphocytes but not by lectinic activation of lymphocytes from the same patient was unexpected. The data indicate that in some patients activation of lymphocytes does not stimulate HIV p24 production, while activation of the macrophage–T-cell system may result in demonstrable HIV replication. Such findings are consistent with theory invoking the macrophage as an important reservoir of HIV in vivo.

Two of four men responding with HIV production to AA were sensitized to amoebic parasites by a documented infection. Interestingly, none of the three men who were infected with *E. histolytica* at the time of culture responded with HIV production. It is possible, but not yet proven, that the responding clones of CD4+ cells were deleted in vivo by the interaction of antigenic stimulation and HIV infection. Alternatively, CD8+ cytotoxic responder cells, shown to be functionally apparent in some HIV-infected persons (25), may have blocked the responses in some of our infected subjects. Finally, this study identifies another antigen which initiates replication of HIV. Due to the high rate of amoebic parasitism in homosexual men, it seems prudent to regularly evaluate HIV-infected patients for the presence of enteric parasites and to treat such infections in spite of a benign clinical course.

ACKNOWLEDGMENTS

We thank Karen Foster for valuable editorial assistance.

This work was supported by the AIDS Research Fund, Beth Israel Medical Center, New York, N.Y.

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