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Decreased T-Cell Proliferative Response to Common Environmental Antigens Could Be an Indicator of Early Human Immunodeficiency Virus-Mediated Lymphocyte Lesions

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To evaluate $CD4^+/CD29^+$ cells and their responses to different antigens in polar stages of human immunodeficiency virus (HIV) infection, we studied 26 HIV-seropositive carriers (SPCs) and 15 patients with AIDS simultaneously with 20 healthy volunteers (HVs) and 10 seronegative homosexual and bisexual men (SNH). CD3, CD4, CD29, and CD45RA phenotypes were analyzed by two-color flow cytometry. Significant depletion of $CD4^+$ T cells and both memory ($CD4^+/CD29^+$) and naive ($CD4^+/CD45RA^+$) T-cell subsets was found among SPCs and AIDS patients compared with the numbers of such cells in the HV and SNH groups. Responses to optimal doses of *Candida albicans*, streptokinase, and tetanus toxoid were explored in peripheral blood mononuclear cells and $CD4^+$- and $CD4^+/CD29^+$-enriched cell populations. In SPCs, the response to *C. albicans* in peripheral blood mononuclear cells showed a statistically significant diminution compared with the response of HVs (15,308 versus 35,951 cpm). In addition, a significantly reduced response to streptokinase was evident only when cell preparations were $CD4^+/CD29^+$ enriched (3,048 versus 10,367 cpm). Furthermore, the SPC group comprised seven responders to at least one antigen and seven nonresponders to any of the selected specific antigens. Absence of a response in these latter patients was independent of the absolute counts of memory and naive T-cell populations. The response to tetanus toxoid, although diminished in SPCs, was not significantly different from that in controls. Our results suggest that defective responses to common environmental antigens, unrelated to the absolute number of $CD4^+/CD29^+$ cells, is probably an early indicator of an HIV-induced lymphocyte lesion.

The human immunodeficiency virus (HIV) is related to a wide spectrum of immunological abnormalities in humans (2, 8). $CD4^+$ lymphocytes are particularly vulnerable to HIV, which in fact induces selective dysfunction and destruction of this population. Recently, investigative efforts have been focused on the interaction between HIV and $CD4^+$ evolutionary subsets, naive ($CD4^+/CD45RA^+$) and memory ($CD4^+/CD29^+$) cells. These subsets display different functional capabilities; naive cells respond to mitogens and autologous lymphocyte stimuli but do not proliferate in response to soluble antigens; in contrast, memory cells recognize recall antigens (14, 15). Altered proliferative responses to soluble antigens, alloantigens, and mitogens, plus depletion of both naive and memory cell subsets, have been reported in patients with HIV infection (6, 7, 10, 11, 13, 18).

We assessed the number and functional status of $CD4^+$ and $CD4^+/CD29^+$ memory cell subsets in asymptomatic HIV-infected individuals with the aim of gaining further insight into the natural history of the immunopathology of the HIV-$CD4^+$ cell subset interaction.

MATERIALS AND METHODS

Patients and controls. Twenty-six HIV-seropositive carriers (SPCs) and 15 male patients with AIDS were investigated. Ten homosexual or bisexual men were included as a high-risk seronegative reference group (SNHs); in addition, 20 healthy heterosexual volunteers (HVs) were also evaluated as controls.

Antibodies against HIV. Antibodies to HIV were assessed in all patients and controls by enzyme-linked immunosorbent assays (Abbott Recombinant HIV-1 EIA; Abbott Laboratories; Vironostika anti-HTLV-III, Organon Teknika, Boxtel, Holland), and the result was confirmed by Western blot (immunoblot) analysis (HIV-1 Western blot kit, Organon Teknika).

Phenotype analysis. Cell surface markers were determined by single- and dual-color flow cytometry (EPICS-753; Coulter Corporation, Hialeah Fla.). Fluorescein isothiocyanate- or RD1-tagged monoclonal antibodies against CD3, CD4, CD8, CD29, and CD45RA cells (Coulter Corporation) were added to EDTA-treated peripheral blood; after 10 min of incubation, the samples were processed in a Coulter Q-Prep Immunology Work Station (Coulter Corporation); total leukocytes and differential counts were assessed in the same blood sample.

Cell preparation. Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood by centrifugation over Ficoll-Hypaque gradients (3). Adherent cells were removed by incubation in plastic petri dishes (Falcon Labware, Becton Dickinson, Lincoln Park, N.J.); 10 ml of cell suspension (2×10^6 cells per ml) was used. Nonadherent cells were carefully removed, washed, and resuspended in RPMI–20% normal human serum (NHS) for a second cycle on plastic petri dishes; viability determined by trypan blue exclusion was 99%, and the proportion of peroxidase-positive cells ranged from 1 to 3%. Nonadherent cells were B cell depleted by passage through nylon columns (12); briefly, 12-ml syringes were packed with 1 g of nylon wool (Robbins Scientific, Mountain View, Calif.), autoclaved, washed with RPMI–10% NHS, and warmed at 37°C. The cell suspension was incubated in the column for 1 h at 37°C, and nonadherent cells were recovered by elution with prewarmed RPMI–10% NHS.

A panning technique (19) was used to obtain a $CD4^+/CD29^+$-enriched cell preparation by negative selection; briefly, 10×10^6 PBMCs depleted of B and adherent cells were incubated with 50 μl of anti-$CD8^+$ monoclonal antibody for 30 min before addition of the cells to plastic petri dishes (15 by 100 mm; Falcon Labware, Becton Dickinson) previously coated with goat anti-mouse immunoglobulin G (Atlantic Antibodies, Scarbourough, Maine). The dishes were then incubated at 4°C for 1 h, after which the nonadherent cells were carefully collected. Cells were washed and adjusted, and the procedure was repeated with 2H4 monoclonal antibody in order to finally obtain a $CD4^+/CD29^+$-enriched cell subpopulation; flow cytometric analysis showed more than 90% $CD4^+/CD29^+$ cells and fewer than 3% $CD8^+$ and $CD4^+/CD45RA^+$ cells.

Cell cultures. A total of 10^5 cells (PBMCs or $CD4^+$ or $CD4^+/CD29^+$ cells) were cultured with preestablished optimal doses of the following soluble antigens: tetanus toxoid (TT; 2.5 Lf/ml), kindly supplied by the Instituto Nacional de

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TABLE 1. Phenotypic analysis of T cells and T-cell subsets in patients and controls

Cell	Amt (mm^3 [%]) of fluorescent cells in the following subjects[a]:			
	HVs (n = 20)	SNHs (n = 10)	SPCs (n = 26)	AIDS patients (n = 15)
Leukocytes	8,358 ± 2,745	8,272 ± 2,569	7,354 ± 2,949	5,610 ± 1,600[b]
Lymphocytes	3,164 ± 978 (39 ± 10)	3,352 ± 1,012 (42 ± 12)	2,676 ± 1,067 (38 ± 11)	786 ± 818[c] (31 ± 12)
$CD3^+$	2,237 ± 612 (72 ± 7)	2,586 ± 990 (75 ± 10)	2,030 ± 1,064 (74 ± 16)	1,245 ± 753[c] (68 ± 18)
$CD29^+$	2,349 ± 821 (74 ± 12)	2,919 ± 870 (87 ± 4[b])	1,924 ± 973 (71 ± 17)	1,287 ± 714[c] (73 ± 19)
$CD45RA^+$	2,243 ± 766 (72 ± 13)	2,722 ± 880 (81 ± 6[b])	1,927 ± 871 (72 ± 10)	1,138 ± 647[c] (69 ± 12)
$CD3^+/CD29^+$	1,772 ± 646 (53 ± 12)	2,210 ± 828[b] (64 ± 10[b])	1,430 ± 1,007 (51 ± 24)	893 ± 648[b] (49 ± 22)
$CD3^+/CD45RA^+$	1,538 ± 625 (50 ± 15)	2,219 ± 895[b] (62 ± 14[b])	1,333 ± 888 (47 ± 18)	789 ± 547[c] (43 ± 18)
$CD4^+$	1,335 ± 500 (43 ± 8)	1,425 ± 754 (40 ± 11)	679 ± 358[b] (25 ± 7[b])	183 ± 194[c] (10 ± 10[c])
$CD4^+/CD29^+$	998 ± 377 (31 ± 9)	1,223 ± 604 (33 ± 9)	455 ± 290[c] (16 ± 7[c])	104 ± 112[c] (5 ± 4[c])
$CD4^+/CD45RA^+$	759 ± 426 (25 ± 11)	986 ± 594 (27 ± 10)	406 ± 302[c] (14 ± 7[c])	104 ± 90[c] (5 ± 4[c])
$CD8^+$	899 ± 326 (29 ± 8)	1,126 ± 456 (34 ± 9)	1,366 ± 833[b] (49 ± 15[c])	971 ± 625 (54 ± 18)
$CD4^+/CD8^+$	1.62 ± 0.6	1.25 ± 0.6	0.6 ± 0.3[c]	0.27 ± 0.47[c]

[a] Results are expressed as the arithmetic mean ± standard deviation of the absolute amount (cubic millimeters) and percentages of fluorescent cells by flow cytometry.
[b] $P < 0.005$ when compared with HVs.
[c] $P < 0.001$ when compared with HVs.

Higiene, Caracas, Venezuela; *Candida albicans* (75 μg/ml), kindly donated by the Instituto de Biomedicina, Caracas, Venezuela; and streptokinase (SK; 62.5 IU/ml; Kabikinase; Kabivitrom, Stockholm, Sweden). Ten percent autologous adherent cells were added to $CD4^+$- and $CD4^+/CD29^+$-enriched subpopulations. Cultures were carried out in flat-bottom microtiter plates (Falcon Labware, Becton Dickinson); each well contained a final volume of 0.2 ml of RPMI 1640 medium supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% heat-inactivated NHS. Triplicate cultures of each combination were maintained at 37°C in a humidified atmosphere of 5% CO_2 for 7 days, pulsed with 1 μCi of tritiated thymidine 18 h before harvesting, and counted in a Betaplate Liquid Scintillation Counter (Wallac Oy, Turku, Finland). The results were expressed as the mean counts per minute of triplicate cultures.

Statistical analysis. The Student *t* test for two independent samples of unequal size was used, and *P* values were determined.

RESULTS

Patients and controls. Patients included 41 HIV-infected male individuals (ages, 19 to 61 years; mean age, 33 ± 9 years) whose HIV infections were classified according to the criteria of the Centers for Disease Control and Prevention (CDC) (5); 26 patients were asymptomatic (CDC group II) SPCs and 15 had AIDS (CDC group IV). All patients were confirmed to have antibodies to HIV by Western blotting.

Control individuals were also males (ages, 24 to 39 years; mean age, 31 ± 4 years); 10 were SNHs and 20 were HVs. All control subjects were screened for HIV antibodies, with negative results.

T-cell subpopulations. Results of phenotypic analysis of leukocytes, lymphocytes, T cells, and T-cell subpopulations in controls and patients are provided in Table 1. Both HVs and SNHs exhibited similar cell subpopulations with the exception of $CD3^+/CD29^+$ and $CD3^+/CD45RA^+$ T cells, which were present in significantly higher numbers in SNH individuals ($P < 0.005$).

The numbers of leukocytes, lymphocytes, and CD3 cells in SPCs were comparable to those in HVs. However, the numbers of $CD4^+$ cells (single-color flow cytometry) and both $CD4^+/CD29^+$ and $CD4^+/CD45RA^+$ (dual-color flow cytometry) appeared significantly reduced compared with the numbers of such cells in both HVs and SNHs. As expected, all lymphocyte subpopulations studied in AIDS patients were significantly diminished (Table 1).

Proliferative response to soluble antigens. Functional lymphocyte studies were performed in 11 controls, 10 SNHs, 14 SPCs, and 6 AIDS patients. When PBMC proliferation was evaluated, SPCs showed diminished responses to *C. albicans*, TT, and SK, reaching significance only in the case of the *C. albicans* antigen. A similar proliferation pattern was found in the responses of $CD3^+/CD29^+$-enriched cells from SPCs. However, when the responses from $CD4^+/CD29^+$ cells were studied, not only was there a diminished proliferation in response to the three antigens but significance was also again detected in the case of the *C. albicans* and SK antigens. As expected, in all instances, the responses among patients with AIDS were basically absent (Table 2).

When analyzing the proliferative responses among the SPCs, a remarkable individual variability was observed; this allowed us to divide the whole group into responders (R), when proliferative responses to one or more antigens were found, and nonresponders (NR), when the absence of a response to the three antigens was detected (Table 3). Proliferation among the R group was similar to that among the controls (HVs). Furthermore, to assess whether quantitative depletion of $CD4^+$ cells and/or memory cells ($CD4^+/CD29^+$) was responsible for the impaired responses, phenotypic analysis of cells from the R and NR groups was done. Table 4 shows no differences among T-cell subsets in the R and NR groups. Moreover, the numbers of both $CD4^+$ and $CD4^+/CD29^+$ cells were significantly depleted in both groups compared with the numbers in the HV group.

DISCUSSION

Depletion and functional abnormalities of CD4 lymphocytes are probably the most significant immunopathological lesions induced by HIV infection in humans (7, 11). In recent years, the emphasis has been placed on trying to further dissect the HIV-CD4 cell subset interaction to determine the natural history of the involvement of both naive and memory CD4 cells.

Our research protocol was mainly designed to explore the proliferative responses of memory ($CD4^+/CD29^+$) T lymphocytes in SPCs when they were challenged with two common environmental stimulants (*C. albicans* and SK) and TT, an antigen to which adults are rarely exposed by natural contact.

All of our SPCs showed a significant depletion in the number of $CD4^+$ T cells and $CD4^+/CD29^+$ and $CD4^+/CD45RA^+$ subsets, as initially reported by Vuillier et al. (18). Taken as a whole group, the $CD4^+$ cells from our SPCs were low-level responders to the three soluble antigens, reaching significance

TABLE 2. Proliferative responses to soluble antigens by PBMCs and purified $CD3^+/CD4^+$ and $CD4^+/CD29^+$ cells in patients and controls

Cell preparation	Antigen[a]	Proliferative response[b] in the following subjects:			
		HVs (n = 11)	SNHs (n = 10)	SPCs (n = 14)	AIDS patients (n = 6)
PBMCs	SP	980 ± 384	888 ± 166	1,097 ± 245	730 ± 276
	TT	78,512 ± 28,907	60,312 ± 11,254	30,413 ± 12,416	438 ± 96[c]
	CA	35,951 ± 11,308	19,759 ± 7,115	15,308 ± 5,620	765 ± 348[c]
	SK	15,751 ± 6,017	13,734 ± 7,345	7,062 ± 2,555	1,073 ± 771[c]
$CD3^+/CD29^+$	SP	1,181 ± 711	1,262 ± 291	576 ± 136	682 ± 276
	TT	89,393 ± 33,915	104,718 ± 28,030	42,290 ± 19,523	959 ± 400[c]
	CA	42,802 ± 15,710	20,489 ± 5,270	10,586 ± 3,219[c]	824 ± 281[c]
	SK	12,882 ± 2,153	20,339 ± 12,534	8,287 ± 5,412	606 ± 256
$CD4^+/CD29^+$	SP	1,097 ± 671	1,769 ± 555	749 ± 257	454 ± 241
	TT	65,138 ± 32,033	123,726 ± 34,219	42,301 ± 23,846	421 ± 172[c]
	CA	24,107 ± 8,258	13,224 ± 2,515	9,065 ± 4,667[c]	964 ± 609[c]
	SK	10,367 ± 4,357	16,562 ± 9,316	3,048 ± 1,626[c]	348 ± 113[c]

[a] SP, spontaneous proliferation; CA, *C, albicans.*
[b] Expressed as the arithmetic mean ± standard deviation of counts per minute.
[c] $P < 0.005$.

only in the case of *C. albicans*. However, highly enriched $CD4^+/CD29^+$ memory cells from these patients showed a significantly decreased proliferation in response to both *C. albicans* and SK. Initially, Lane et al. (13) demonstrated that in patients with AIDS, $CD4^+$ lymphocytes failed to respond to TT but were able to express mitogen- and/or alloantigen-driven blast transformation. Subsequently, Giorgi et al. (10) showed that $CD4^+$ subsets were not selectively depleted at any stage of HIV infection and suggested that the loss of the responses to soluble antigens was not related to a particular CD4-cell subset depletion. However, Clerici et al. (6) and Shearer et al. (17) showed among asymptomatic HIV-seropositive patients four distinct patterns of proliferative responses when PBMCs were tested with recall antigens, alloantigens, and mitogens; none of these patterns was related to a critical reduction in the numbers of $CD4^+$ cells.

In our experiments, the qualitative defect (diminished proliferation) in SPCs is most probably related to memory cell dysfunction without the participation of abnormalities in the antigen presentation process, since a similar amount of autologous monocytes was added to each cell preparation. This particular finding was previously suggested by Fauci (9) in studies with accessory cells from identical twins. Furthermore, when each of the SPC individuals was analyzed, we noted that half of the patients responded to one or more of the selected antigens, representing perhaps a very early stage of the HIV infection, while the others did not respond to either antigen. The numbers of $CD4^+$ T cells and $CD4^+/CD29^+$ and $CD4^+/CD45RA^+$ subsets were similar in both R and NR individuals. Our two SPC subgroups (R and NR) may parallel the short-term and long-term HIV-seropositive asymptomatic individuals reported in 1987 by Giorgi et al. (10).

In relation to the susceptibility of $CD4^+$-cell subsets to HIV, Schnittman et al. (16) showed a preferential defect of memory $CD4^+/CD29^+$ T cells in their response to TT, being the abnormality directly related to a greater burden of HIV among these cells. Within this context, Cayota et al. (4) demonstrated that both naive and memory $CD4^+$-cell subsets are impaired in patients with HIV infection.

Therefore, with the data accumulated in several laboratories, including ours, it seems plausible to further advance the hypothesis that HIV indeed interacts not only with $CD4^+$ T

TABLE 3. Proliferative responses to soluble antigens by PBMCs and purified $CD3^+/CD4^+$ and $CD4^+/CD29^+$ cells among SPCs in the R and NR groups

Cell preparation	Antigen[a]	Proliferative response[b] in the following subjects:		
		HVs (n = 11)	SPCs	
			R (n = 7)	NR (n = 7)
PBMCs	SP	980 ± 384	1,654 ± 377	539 ± 117
	TT	78,512 ± 28,907	59,830 ± 19,469	995 ± 673[c,d]
	CA	35,951 ± 11,308	29,734 ± 8,209	881 ± 314[c,d]
	SK	15,751 ± 6,017	12,550 ± 3,636	659 ± 331[c,d]
$CD3^+/CD29^+$	SP	1,181 ± 711	762 ± 253	398 ± 64
	TT	89,393 ± 33,915	82,953 ± 33,166	1,626 ± 653[c,d]
	CA	42,802 ± 15,710	20,566 ± 3,413	606 ± 218[c,d]
	SK	12,882 ± 2,153	14,759 ± 9,657	1,350 ± 574[d]
$CD4^+/CD29^+$	SP	1,097 ± 671	1,144 ± 476	353 ± 188
	TT	65,138 ± 32,033	83,124 ± 43,682	1,479 ± 668[c]
	CA	24,107 ± 8,258	17,548 ± 8,388	581 ± 249[c,d]
	SK	10,367 ± 4,357	5,165 ± 208	578 ± 252[d]

[a] SP, spontaneous proliferation; CA, *C. albicans.*
[b] Expressed as the arithmetic mean ± standard deviation of counts per minute.
[c] $P < 0.05$ (R versus NR).
[d] $P < 0.05$ (R and NR versus HVs).

TABLE 4. Phenotypic analysis of T cells and T-cell subsets in HVs and SPCs in the R and NR groups

Cell	Amt (mm^3 [%]) of fluorescent cells in the following subjects[a]:		
	HVs (n = 20)	SPCs	
		R (n = 7)	NR (n = 7)
Leukocytes	8,358 ± 2,745	9,407 ± 2,850	7.314 ± 3.465
Lymphocytes	3,164 ± 978 (39 ± 10)	3,148 ± 1,115 (35 ± 15)	2,932 ± 1,472 (39 ± 6)
$CD3^+$	2,237 ± 612 (72 ± 7)	2,348 ± 907 (75 ± 17)	2.501 ± 1.550 (81 ± 14)
$CD29^+$	2,349 ± 821 (74 ± 12)	2,150 ± 813 (69 ± 16)	2,402 ± 1,432 (80 ± 14)
$CD45RA^+$	2,243 ± 766 (72 ± 13)	2,176 ± 736 (70 ± 10)	2,273 ± 1,284 (76 ± 11)
$CD3^+/CD29^+$	1,772 ± 646 (53 ± 12)	1,531 ± 871 (51 ± 26)	1,975 ± 1,453 (61 ± 22)
$CD3^+/CD45RA^+$	1,538 ± 625 (50 ± 15)	1,487 ± 619 (48 ± 19)	1,876 ± 1,334 (59 ± 18)
$CD4^+$	1,335 ± 500 (43 ± 8)	888 ± 402[b] (28 ± 6[b])	772 ± 428[b] (22 ± 7[b])
$CD4^+/CD29^+$	998 ± 377 (31 ± 9)	472 ± 246[b] (15 ± 6[b])	502 ± 355[b] (17 ± 6[b])
$CD4^+/CD45RA^+$	759 ± 426 (25 ± 11)	485 ± 225[b] (16 ± 7[b])	499 ± 489[b] (15 ± 6[b])
$CD8^+$	899 ± 326 (29 ± 8)	1,551 ± 745[b] (49 ± 16[b])	1.821 ± 1.232[b] (57 ± 15[b])
$CD4^+/CD8^+$	1.62 ± 0.6	0.6 ± 0.3[b]	0.4 ± 0.2[b]

[a] Results are expressed as the arithmetic mean ± standard deviation of absolute amount (cubic millimeters) and percentages of fluorescent cells by flow cytometry.
[b] $P < 0.005$ when compared with HVs.

cells but also with $CD4^+$ T cells in both evolving stages of maturation, the naive and memory $CD4^+$ T-cell subsets. Two main and probably independent immunopathological lesions arise: the installation of an impaired lymphocyte proliferative response capability and a progressive depletion of the $CD4^+$ pool and its subsets. As we have shown, the decreased proliferation in response to common environmental antigens (i.e., *C. albicans* and SK) seems to be early evidence of the proliferative abnormality; this is followed in asymptomatic individuals by the loss of a response to less common soluble antigens (i.e., TT). Moreover, there seems to be no correlation in the asymptomatic stage of the infection between the diminution of the proliferative responses and the degree of depletion of $CD4^+$ lymphocytes. Both immune abnormalities probably would act as inducers of the state of secondary systemic anergy (1) which is characteristic of the clinical phases of HIV infection. In conclusion, the assessment of T-cell proliferation in response to common environmental antigens might prove useful when evaluating the impairment of immune response in HIV-infected individuals.

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