Class II (DR) Antigen Expression on CD8$^+$ Lymphocyte Subsets in Acquired Immune Deficiency Syndrome (AIDS)

H. W. L. ZIEGLER-HEITBROCK,[1,7] D. STACHEL,[1] T. SCHLUNK,[2] L. GÜRTLER,[3] W. SCHRAMM,[4] M. FRÖSCHL,[5] J. R. BOGNER,[6] and G. RIETHMÜLLER[1]

In a selected group of human immunodeficiency virus (HIV)-infected patients we confirm the expansion of a CD8$^+$ T-lymphocyte subset, i.e., the CD8$^+$/Leu7$^+$ cells, which account for 30% of the lymphocytes, compared to 3% in the control donors. In addition, a CD8$^+$ T-lymphocyte subset that coexpresses class II (DR) antigens, i.e., CD8$^+$/DR$^+$ cells, is also increased from 1.5% in controls to 27% in the HIV-infected patients. Using three-color immunofluorescence and flow cytometry we can demonstrate that the CD8$^+$/Leu7$^+$ and the CD8$^+$/class II$^+$ cells are not distinct but overlapping subsets. In the HIV-infected patients 42% of the CD8$^+$/Leu7$^+$ cells were strongly positive for class II and these CD8$^+$/Leu7$^+$/class II$^+$ cells accounted for 13% of all lymphocytes. These findings indicate that the expanded CD8$^+$/Leu7$^+$ cells are activated and hence might be actively involved in immune defense in acquired immune deficiency syndrome (AIDS).

INTRODUCTION

Detailed studies on the lymphocyte subsets in human immunodeficiency virus (HIV)[8] infection have revealed that, in addition to depletion of CD4 lymphocytes, the CD8 cells can be expanded, both in percentage and in absolute numbers (2–7). The most prominent feature evolving from two-color immunofluorescence analysis of the CD8$^+$ cells in these studies was the expansion of a usually minor subset of CD8$^+$/Leu7$^+$ cells and, in addition, in some patients of CD8$^+$/class II$^+$ cells (2, 8). Thus far it remained unclear whether these two populations are separate regulatory elements evolving in the course of the disease or whether class II is expressed on the CD8$^+$/Leu7$^+$ cells in these patients. We have approached this question by using three-color fluorescence analysis in flow cytometry. Our studies show that, in fact, CD8$^+$/Leu7$^+$ and CD8$^+$/class II$^+$ are not distinct but are overlapping populations, i.e., class II is expressed on the CD8$^+$/Leu7$^+$ cells, suggesting that these cells are actively involved in the immunoregulatory events in AIDS.

MATERIALS AND METHODS

Patients and Controls

The group of 10 patients studied consisted of eight male homosexuals and two male hemophiliacs with a mean age of 37 years. All were HIV seropositive, seven had AIDS according to the Centers of Disease Control Classification, two patients had ARC, and one patient was apparently healthy (Table I). Cytomegalovirus (CMV) carrier status was demonstrated in eight of eight patients. In seven of seven no CMV-specific IgM antibody was detectable. At the time of sampling, none of the patients had acute disease, except for patient 2, who exhibited local *Herpes simplex* virus 2 (HSV-2) infection. None of the patients received antimicro-

[1]Institute for Immunology, University of Munich, 8000 Munich, F.R.G.
[2]Genzentrum, University of Munich, 8000 Munich, F.R.G.
[3]Max-von-Pettenkofer-Institute, University of Munich, 8000 Munich, F.R.G.
[4]Hemostasiology Laboratory Department of Internal Medicine, University of Munich, 8000 Munich, F.R.G.
[5]Department of Dermatology, University of Munich, 8000 Munich, F.R.G.
[6]Medizinische Poliklinik, University of Munich, 8000 Munich, F.R.G.
[7]To whom correspondence should be addressed at Institute for Immunology, Goethestr. 31, 8000 Munich 2, F.R.G.
[8]Abbreviations used: AIDS, acquired immunodeficiency syndrome; ARC, AIDS-related complex; CD, cluster of differentiation; FALS, forward-angle light scatter; HIV, human immunodeficiency virus; 90° LS, 90° light scatter; MAB, monoclonal antibody; CMV, cytomegalovirus; NK, natural killer.

Patient No.	Clinical staging[a]	CD4[b]	CD8	Leu7	T4/T8 ratio
1	PCP, AIDS	4.0 (77)	74.1 (1429)	58.2 (1122)	0.1
2	KS, AIDS	2.0 (24)	61.6 (729)	33.1 (391)	0
3	CE, AIDS	18.0 (523)	53.8 (1564)	36.4 (1058)	0.3
4	KS, AIDS	11.0 (326)	45.0 (1331)	31.5 (933)	0.2
5	ARC	18.0 (324)	46.7 (840)	23.3 (420)	0.4
6	CE, AIDS	9.0 (95)	68.9 (725)	33.3 (350)	0.1
7	ARC	19.0 (357)	55 1 (1036)	37.1 (697)	0.3
8	PCP, AIDS	5.0 (109)	66.0 (1432)	60.7 (1318)	0.1
9	PCP, AIDS	0 (0)	62.4 (779)	49.5 (618)	0
10	Healthy	6.0 (41)	51.2 (348)	31.7 (216)	0.1
Mean ± SD		9.2 ± 7.0* (188 ± 179)*	58.5 ± 9.7* (1021 ± 401)*	39.5 ± 12.4* (712 ± 377)**	0.2 ± 0.1*
Mean ± SD of controls (N = 11)		31.4 ± 11.5 (748 ± 314)	16.0 ± 4.5 (392 ± 189)	15.8 ± 8.3 (374 ± 206)	2.1 ± 1.1

[a]CE, candidiasis of esophagus; PCP, *Pneumocystis carinii* pneumonia; ARC, AIDS-related complex; KS, Kaposi's sarcoma.
[b]Values are given as percentages of all lymphocytes (cells/mm^3).
*$P \leq 0.001$.
**$P \leq 0.02$.

bial treatment and none received cytotoxic therapy. The 11 control donors (mean age, 30 years; all male) were apparently healthy volunteers.

Isolation of Mononuclear Cells

Heparinized blood was taken for routine diagnosis and PBM were isolated by Ficoll–Hypaque density-gradient separation (1); aliquots were stored in liquid nitrogen after controlled freezing. Samples for the present study were selected based on high percentages of CD8 lymphocytes.

Absolute leukocyte counts and differential counts were obtained from EDTA blood samples according to routine hematological procedures.

For immunofluorescence analysis cells were thawed rapidly in a 37°C water bath. After washing 10^6 cells per sample were incubated for 30 min at 4°C with saturating concentrations of a mixture of the monoclonal antibodies (MAB) Leu2a–phycoerythrin, Leu7–fluorescein (both Becton Dickinson), and either L243–biotin (anti-class II purified from the supernatant of the respective hybridoma) or M-T910–biotin CD2, kindly provided by E. P. Rieber, Munich. After two washes cells were incu-

bated with avidin Texas red (Medac, Hamburg, F.R.G.) for 30 min at 4°C, and after final washes they were fixed with 1% paraformaldehyde. Immunofluorescence for T4 was done with T151 MAB in the first and goat anti-mouse IgFITC (Tago, Burlingame, CA) in the second step.

Flow Cytometry

Analysis was done on an EPICS 753 (Coulter Electronics, Krefeld, F.R.G.) equipped with an argon laser (300 mW) for 488-nm excitation and with a dye laser (Coherent 599) containing rhodamine 6G, pumped with all lines from a second argon laser (3 W) to generate the ~600-nm excitation. The intersections of the 488- and 600-nm laser beams with the liquid stream were set 7 μsec apart. The 488-nm scattered light was used for determination of the forward-angle light scatter (FALS) and 90° light scatter (90% LS) properties and signal processing was triggered by FALS. Ninety-degree LS signals were directed to the 90° LS photomultiplier tube (PMT) using a 488-nm long-pass dicroic mirror. The passing light was blocked with an additional 457- to 502-nm laser blocking filter.

Green fluorescent light was directed with a 550-nm long-pass dicroic mirror to the green PMT equipped with a 525-nm band-pass filter. The red and yellow fluorescent light passing the 550-nm long-pass dicroic mirror was then split by a 600-nm short-pass dicroic mirror that reflected the red fluorescent light to the red PMT equipped with a 635-nm band-pass and a 610-nm long-pass filter. The yellow fluorescent light passing the 600-nm mirror reached the yellow PMT, which was equipped with a 575-nm band-pass plus a 550-nm long-pass filter.

To control for spectral overlap and for proper temporal correlation of signals, control beads (Texas red, 1/16 bright) were used. TR beads gave red signals only (calibrated to channel 130 on the 256-channel scale). The 1/16 bright beads (Coulter Electronics) gave green plus yellow staining (calibrated to channel 190 each).

Analysis was done gated on lymphocytes and at least 40,000 cells were analyzed per sample with log amplification. Evaluation of acquired histograms was done using a SAM 68 K computer (KWS, Ettlingen, F.R.G.) with software written by P. Rohwer, Erlangen, F.R.G.

Percentages of positive cells were determined by setting cursors around distinct populations such as CD8 and Leu7 or they were determined by using a subtract program (Coulter Electronics) for fluorescence distributions that overlap with the isotype control staining, as is the case for class II. Absolute cell counts from the respective populations were determined by multiplying the absolute lymphocyte count by percentages/100. Fluorescence intensity is expressed as the mean channel that marks the intensity level at which 50% of the cells are to the right. The mean specific fluorescence intensity is the mean channel of specific staining minus the mean channel of staining with the isotype control.

RESULTS

All patients studied had percentages of $CD4^+$ lymphocytes ranging from 0 to 20% and all but one had absolute $CD4^+$ lymphocyte counts of less than $360/mm^3$ (Table I). The $CD8^+$ lymphocytes in absolute numbers were increased, with more than 600 cells/mm^3 in 9 of 10 patients and normal in 1, but in percentages they were always increased, with an average of 59%. The resulting CD4/CD8 ratio therefore was abnormal in every patient, with values of 0.1 or less in six patients (Table I). The $Leu7^+$ cells
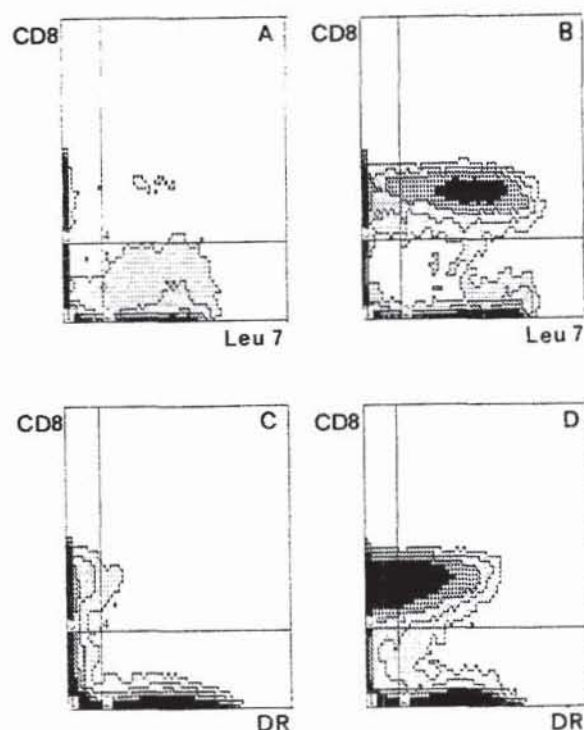

Fig. 1. Expansion of $CD8^+/Leu7^+$ and $CD8^+/DR^+$ cells in a patient with HIV infection. Shown are two-color immunofluorescence plots, always giving CD8 staining on the y axis. The x axis gives Leu7 staining (A, B) or DR staining (C, D). Samples from a control donor are in A and C; those from a patient (No. 10) in B and D. The patient exhibits 20.8% $CD8^+/Leu7^+$ cells and 35.3% $CD8^+/DR^+$ cells, compared to 3.2% and 2.6%, respectively, for the control donor.

showed a pattern similar to that of $CD8^+$ cells, with increased values of more than $600/mm^3$ in 6 of 10 patients.

Analysis of the expression of the Leu7 and the DR antigens on the $CD8^+$ cells confirmed the expansion of both the $CD8^+/Leu7^+$ and the $CD8^+/DR^+$ cells (Fig. 1). In this example 21% of the lymphocytes were $CD8^+/Leu7^+$ and 35% were $CD8^+/DR^+$. In the entire group of HIV-infected patients, $CD8^+/Leu7^+$ accounted for 28%, compared to 3% for the controls (Table II). The $CD8^+/DR^+$ cells were 25% in the patients and 1.5% in the controls. Since in the patients studied, the sum of $CD8^+/DR^+$ cells is less or only a few percent higher than the total number of $CD8^+$ lymphocytes, it is possible that the two double-stained cell populations are, by far and large, mutually exclusive, distinct lymphocyte subsets. Alternatively, they could represent overlapping populations. To approach this question we performed three-color immunofluorescence and we analyzed the expression

Table II. Class II Expression on Lymphocyte Subpopulations

Patient No.	Clinical staging[c]	CD8$^+$/Leu7^{+a}	CD8$^+$/DR^{+a}	Class II-positive cells[b]		
				CD8$^+$/Leu7$^-$	CD8$^+$/Leu7$^+$	CD8$^-$/Leu7$^+$
1	PCP, AIDS	49.8 (960)	31.1 (600)	54	36	50
2	KS, AIDS	26.0 (308)	30.8 (365)	54	44	39
3	CE, AIDS	30.7 (893)	12.9 (375)	27	22	28
4	KS, AIDS	16.4 (485)	22.0 (652)	55	39	29
5	ARC	16.6 (299)	22.9 (412)	54	40	30
6	CE, AIDS	31.1 (328)	43.4 (457)	64	62	77
7	ARC	29.7 (559)	14.9 (280)	39	17	52
8	PCP, AIDS	40.1 (869)	23.8 (515)	41	33	21
9	PCP, AIDS	42.5 (531)	31.8 (397)	55	49	35
10	Healthy	20.8 (142)	35.3 (240)	64	76	59
Mean ± SD		30.4 ± 11.1* (537 ± 285)*	26.9 ± 9.4* (429 ± 131)*	50.7 ± 11.8*	41.8 ± 17.5*	42.0 ± 17.3*
Mean ± SD of controls (N = 11)		2.7 ± 1.8 (70 ± 58)	1.5 ± 1.0 (38 ± 31)	7.1 ± 4.8	16.7 ± 6.8	7.0 ± 7.1

[a]Values are given as percentages of all lymphocytes (cells/mm³).
[b]Values are given as percentages of the specified subpopulation.
[c]CE, candidiasis of esophagus; PCP, *pneumocystis carinii* pneumonia; ARC, AIDS-related complex; KS, Kaposi's sarcoma.
*P ≤ 0.001.

of DR antigens on CD8$^+$/Leu7$^-$, CD8$^+$/Leu7$^+$, and CD8$^-$/Leu7$^+$ lymphocyte subsets.

As shown in Fig. 2 there is a strong expression of DR antigens on all three subsets in the patient, while minimal staining is detected with the cells of the control donor. Staining with the CD2 MAB gave positive staining in the majority of cells for both patient and control, as exemplified for the CD8$^+$/Leu7$^+$ cells (Fig. 2). Data for the entire group of patients are compiled in Table II, which indicates that for all patients increased DR expression can be observed on all three subsets. The average percentage of DR$^+$ cells among the CD8$^+$/Leu7$^+$ cells was 42% for the patients and 17% for the controls. Taking into account, however, the low representation of CD8$^+$/Leu7$^+$ cells, with 1.5% in the control group (Table II), the triple-stained cells amount to only 0.4%, in contrast to about 13% in the patients. Further support for the minor significance of DR antigen expression on CD8$^+$ lymphocytes in healthy control donors comes from analysis of the specific fluorescence intensity, which is reflective of antigen density (Fig. 2). Whereas the patients' CD8$^+$/Leu7$^+$ cells exhibit a mean specific fluores-

cence intensity corresponding to 24.0 channels, the control donors' cells have intensity values of only 5.0 channels on the log scale. Thus compared with the weak expression of DR on a few CD8$^+$/Leu7$^+$ cells in controls, the DR antigen density is eightfold higher in HIV-infected patients.

DISCUSSION

The majority of studies concerned with the immune dysfunction of HIV infection has focused on the CD4$^+$ lymphocyte since this cell appears to be the primary target cell of HIV infection. Less attention has been given to CD8$^+$ lymphocytes, although these cells might play a major role in the pathophysiology of HIV infection, be it as cells involved in protective immunity or as cells that further suppress immune reactivity. CD8$^+$ lymphocytes were found to be increased in the different stages of HIV infection in several reports (2–7).

In patients with definite AIDS some studies report a persistent increase in absolute CD8$^+$ numbers (7), while others report that CD8$^+$ cells become normal again (4). Due to the decrease in CD4$^+$
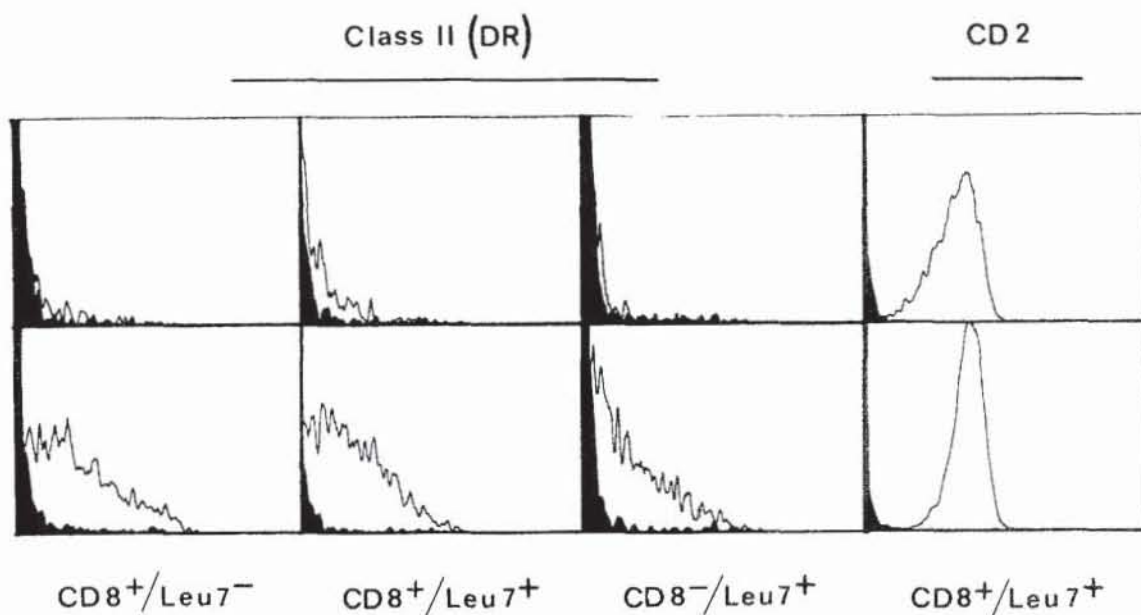
Fig. 2. Expression of DR antigens on CD8$^+$ cells in AIDS. Given is the fluorescence intensity (x axis) on a log scale versus the cell number (y axis) for DR compared to the isotype control for the first three panels, while in the fourth panel staining for CD2 is shown as the positive control. The upper row gives results from the control donor (average specific fluorescence intensity for DR, 3.5 channels); the lower row gives results for the patient (No. 10; average specific fluorescence intensity, 32.0 channels).

lymphocytes in blood as well as in lymphatic tissue, the CD8$^+$ cells, in any event, remain the predominant population with progression of HIV infection. An expansion of circulating CD8$^+$ cells is a common phenomenon in various viral infections (4, 9). Using two-color immunofluorescence of CD8$^+$ lymphocytes, HIV infection was specifically associated with an increase in the CD8$^+$/Leu7$^+$ subset (4). CD8$^+$/Leu7$^+$ cells were increased in HIV-infected patients without symptoms, with ARC, and with AIDS (3–5, 7, 8). Moderate expansions of the CD8$^+$/Leu7$^+$ cells were also reported for other disease states involving the immune system, i.e., in common variable immunodeficiency (10) and after bone marrow transplantation (11). In addition, some healthy CMV carriers exhibit increased CD8$^+$/Leu7$^+$ cells (12).

As to the function of CD8$^+$/Leu7$^+$ cells, one study could demonstrate cytotoxic activity (13), while in another study suggestive evidence supported suppressor activity (14). In patients with allogeneic bone marrow transplantation CD8$^+$/Leu7$^+$ cells were shown to exert suppressor-cell activity for B-cell differentiation (11). The CD8$^+$/Leu7$^+$ cells, however, are not NK cells, since the latter express low-density CD8 (15), while as also demonstrable in Fig. 2, the CD8$^+$/Leu7$^+$ and the

CD8$^+$/DR$^+$ cells are high-intensity CD8 cells. Furthermore, functional studies in patients with high CD8$^+$/Leu7$^+$ numbers revealed decreased NK-cell activity (3).

Thus far, it is unclear whether the CD8$^+$/Leu7$^+$ cells in AIDS are functionally active at all or whether they represent cells that are accumulated due to a blockade in differentiation. Human T lymphocytes upon activation express class II molecules on their surface. Incidentally, CD8 lymphocytes were found to express increased class II in a large proportion (50–60%) of patients with ARC and AIDS but not in seronegative homosexuals (2, 8, 16; Stachel *et al.*, unpublished).

In the present study of a group of patients selected for high CD8 percentages, we found increased CD8$^+$/Leu7$^+$ and CD8$^+$/DR$^+$ cells in every sample. The percentages of these two populations frequently add up to less than the total CD8 percentage or they exceed this figure slightly. Hence two possibilities can be discussed.

(1) CD8$^+$/Leu7$^+$ cells and CD8$^+$/DR$^+$ cells are, by far and large, two separate cell populations.
(2) CD8$^+$/Leu7$^+$ cells overlap with the CD8$^+$/DR$^+$ cells, i.e., CD8$^+$/Leu7$^+$ cells are DR positive.

Our studies using three-color immunofluoresc-

ence clearly demonstrate that the $CD8^+/Leu7^+$ cells in HIV-infected patients do express DR antigens. This is in contrast to results in otherwise healthy patients with CMV-carrier status, where no increase in DR antigens on T cells were detected (12).

None of the HIV-seropositive patients but one had clinical evidence of active infection except for infection with HIV and none received specific treatment at the time of study. While a role of other agents causing latent and inapparent infection cannot be excluded, the possibility might be considered that the induction of DR antigens on the $CD8^+/Leu7^+$ cells is a direct result of HIV infection of the patients. Furthermore, since DR expression on T lymphocytes goes along with lymphocyte activation, we suggest that the $CD8^+/Leu7^+$ cells are activated in the course of HIV infection and might be actively involved in mechanisms of immune defense in AIDS.

ACKNOWLEDGMENTS

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