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## RAPID COMMUNICATION

# CD8<sup>+</sup> T Lymphocytes in the Lung of Acquired Immunodeficiency Syndrome Patients Harbor Human Immunodeficiency Virus Type 1

By Gianpietro Semenzato, Carlo Agostini, Lucia Ometto, Renato Zambello, Livio Trentin, Luigi Chieco-Bianchi, and Anita De Rossi

Human immunodeficiency virus-1 (HIV-1) infection of CD8<sup>+</sup> lymphocytes has been described in several *in vitro* culture systems, but whether CD8<sup>+</sup> cells are a target and also serve as a reservoir for infection *in vivo* as yet is unknown. We addressed this issue in patients with acquired immunodeficiency syndrome (AIDS)-related lower respiratory tract chronic inflammation, which is characterized by a massive influx of CD8<sup>+</sup> HIV-1-specific cytotoxic T lymphocytes (CTL). Proviral load in lung T lymphocytes and their subpopulations was evaluated by using the DNA-polymerase chain reaction (PCR) technique on cells retrieved by bronchoalveolar lavage. To avoid the possibility that the presence of HIV-1 DNA could be caused by contaminating CD4<sup>+</sup> cells, serial dilutions of highly purified CD8<sup>+</sup> cells were also analyzed by PCR. Our findings showed that lung CD8<sup>+</sup> cells harbor and express HIV-1. To explore the possible mechanisms leading to pulmonary CD8<sup>+</sup> lymphocyte infection, we evaluated CD4

gene expression on highly purified CD8<sup>+</sup> cells by means of reverse transcriptase PCR. Despite the lack of membrane CD4 reactivity, we could show that CD8<sup>+</sup> cells may express CD4 RNA. Coinfection of lung CD8<sup>+</sup> cells harboring proviral HIV-1 sequences by viral agents capable of inducing CD4 expression (ie, HHV-6) was not detected. Our data indicate that not only CD4<sup>+</sup> T lymphocytes and macrophages, but also CD8<sup>+</sup> cells, may represent a target and/or a reservoir for HIV-1 *in vivo*, and suggest that lung CD8<sup>+</sup> lymphocytes could derive from precursors equipped with enough CD4 molecules to become HIV-1 permissive. Aside from the cell-to-cell contact between activated HIV-1 specific CTL and relevant targets, the infection of precursors could represent an additional mechanism accounting for the infection of pulmonary CD8<sup>+</sup> cells and their functional impairment.

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CELLS BEARING the CD4 molecule, including lymphocytes and macrophages, have been identified as the most relevant targets for human immunodeficiency virus-1 (HIV-1).<sup>1</sup> Infected CD4<sup>+</sup> cells have been detected in both lymphoid and nonlymphoid organs, such as brain, skin, lungs, liver, lymph nodes, and bone marrow (BM).<sup>2-6</sup> However, recent reports indicate that HIV-1 can also enter cells lacking the CD4 receptor,<sup>7,8</sup> thus making the pathogenetic mechanisms more complex than previously thought. Despite the broad spectrum of HIV-1 targets, the mechanisms by which the virus interacts with and affects the CD4<sup>+</sup> cells, as well as the pattern of infection (latent *v* productive) in these cells, have not yet been established.

In this report we address the issue of HIV-1 spread, and its possibility of infecting CD8<sup>+</sup> lymphocytes that infiltrate different tissues, including the lung, of patients with acquired immunodeficiency syndrome (AIDS). In fact, several recent reports have shown that pulmonary lymphoid organs may serve as a reservoir for HIV-1 and that the presence of HIV-1 within the respiratory tract may elicit an inflammatory state that is initiated and sustained by CD8<sup>+</sup> major histocom-

patibility complex (MHC)-restricted and unrestricted cytotoxic T lymphocytes (CTL).<sup>3</sup> To aid in the analysis of the *in vivo* pattern of HIV-1 infection in CD8<sup>+</sup> cells we isolated lung T lymphocytes and the CD8 cell subset from the bronchoalveolar lavage (BAL) of a series of HIV-1-infected patients. We used the polymerase chain reaction (PCR) technique to analyze HIV-1 proviral load on lung CD8<sup>+</sup> cells and compare its relative distribution in the different BAL cell types. Our results showed that not only CD4<sup>+</sup> macrophages and T cells, but also CD8<sup>+</sup> lung lymphocytes harbor human immunodeficiency replicating virus. This finding further contributes to defining the virus' pattern of infectivity and pathways of tissue spread.

## MATERIALS AND METHODS

**Study populations.** Eleven HIV-1 seropositive patients (8 men and 3 women, average age 34 ± 11.3 years) were studied. According to the criteria for HIV-1-related disorders of the Centers for Disease Control, 1 patient had persistent generalized lymphadenopathy (PGL; group III), whereas 10 patients were classified in group IV, which includes subjects with clinical symptoms and signs of HIV-1 infection other than, or in addition to, lymphadenopathy (4 patients in subgroup IV-A, and 6 in subgroup IV-C1). BAL was performed to obtain a specific diagnosis of opportunistic infection, according to our study protocol for HIV-1 patients with pulmonary involvement. *Pneumocystis carinii* infection was detected in the BAL or transbronchial lung biopsy of all subgroup IV-C1 patients. In accordance with the guidelines of the Helsinki declaration, this invasive procedure was performed only for diagnostic purposes when a pulmonary complication was suspected.

**Preparation of BAL cell suspensions.** BAL was performed as previously described.<sup>9</sup> Briefly, a total of 200 to 250 mL of saline solution was injected via fiberoptic bronchoscopy in 25-mL aliquots, with immediate vacuum aspiration after each aliquot. The fluid was filtered through gauze and its volume measured; 57.1% ± 6.6% of the injected fluid was recovered. BAL cells were then washed, resuspended in RPMI 1640 supplemented with 20 mmol HEPES and L-glutamine (Sigma Chemical Co, St Louis, MO), and counted. Macrophages, lymphocytes, neutrophils, and eosinophils were dif-

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**Table 1. Cell Retrieval by BAL, and the Percentage and Absolute Number of Alveolar Macrophages, Lymphocytes, CD4, and CD8 Cells in 11 HIV-1-Infected Patients**

Patients		Cell Recovery ×10 <sup>3</sup> mL	Alveolar Macrophages		BAL Lymphocytes		BAL CD4 <sup>+</sup> Lymphocytes		BAL CD8 <sup>+</sup> Lymphocytes	
Code No.	CDC Stage		%	×10 <sup>3</sup> mL	%	×10 <sup>3</sup> mL	%	×10 <sup>3</sup> mL	%	×10 <sup>3</sup> mL
1	IV-A	369	55	203	45	166	0.7	1.1	92	152
2	IV-C1	57	95	54	5	3	3.5	0.02	68	2
3	IV-C1	53	98	52	2	1	5	0.05	60	0.6
4	IV-C1	412	12	49	88	362	4	14	81	293
5	III	342	35	119	65	222	1.8	4	92	204
6	IV-A	450	83	373	17	76	3.6	0.6	70	53
7	IV-C1	777	27	209	73	567	0.8	1.5	75	425
8	IV-C1	110	52	68	48	52	2.6	0.5	87	45
9	IV-A	75	48	36	52	39	1	0.04	91	28
10	IV-A	425	62	263	38	161	0.7	1.1	78	125
11	IV-C1	817	50	408	48	392	4.7	18	94	368
Controls		150.6 ± 35.1	90.2 ± 5.1	135.2 ± 29.4	8.0 ± 1.6	13.1 ± 7.1	51.5 ± 1.8	7.2 ± 2.3	25.4 ± 1.7	3.5 ± 1.1

Abbreviation: CDC, Centers for Disease Control.

ferentially counted in cytocentrifuged smears stained with Wright-Giemsa (Table 1).

BAL T-cell subsets were characterized by monoclonal antibodies (MoAbs) of the OK (Ortho Pharm, Raritan, NJ) and Leu (Becton Dickinson, Sunnyvale, CA) series, including CD2 (OKT11), CD3 (Leu 4, OKT3), CD4 (Leu 3, OKT4), and CD8 (Leu 2, OKT8). The frequency of cells positive for the above MoAbs was determined by flow cytometry, as described.<sup>10</sup> Cells were analyzed using a FACSscan (Becton Dickinson) and fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated MoAbs; data were processed using the Consort 30 and Lysys programs (Becton Dickinson). Table 1 reports the differential counts of the T lymphocyte subsets retrieved by BAL in the individual patients.

**Purification of alveolar macrophages (AMs), lung T cells, and CD8 cells.** AMs and lung T cells were enriched from the entire mononuclear cell suspension by rosetting with neuraminidase (Sigma)-treated sheep red blood cells (SRBC) followed by repeated F/H gradient separations, as described.<sup>9</sup> The resulting nonrosetting cell population was composed of more than 95% macrophages, whereas the rosetting population contained more than 99% T cells, as determined by positivity on nonspecific esterase staining and the percentage of CD3<sup>+</sup>, CD19<sup>+</sup>, and CD16<sup>+</sup> cells.

CD8<sup>+</sup> cells were further purified from the T-cell suspension by a two-step sequential depletion using magnetic separations over columns (Mini MACS; Miltenvi Biotech, Sunnyvale, CA), as described.<sup>11</sup> Briefly, the T-cell suspensions were incubated for 30 minutes at 4°C with magnetic beads coated with anti-CD4 MoAb (Leu3). The cells rosetting with the antibody-coated beads were then isolated, and removed by applying a magnetic system on the outer wall of the columns. The CD4-depleted cell population was further incubated with anti-CD8 MoAb (Leu2a)-coated microbeads for 45 minutes at 4°C, and the CD8<sup>+</sup> rosetting cells immobilized on the columns were then collected. By means of this multistep selection procedure, we obtained enough CD8<sup>+</sup> cells to perform molecular analyses in 4 cases (8 to 11 in Table 2). The purity of the CD8<sup>+</sup> lymphocyte populations thus obtained exceeded 99%, with virtually no detectable residual CD4<sup>+</sup> cells (see Fig 1); viability was more than 99%, as judged by the trypan blue exclusion test.

**HIV-1 detection and expression in BAL cell suspensions.** The amount of HIV-1 proviral copies in patient cells was determined by the PCR technique, as described,<sup>12</sup> using serial dilutions of 8E51 cells that contain one proviral copy per cell as a standard reference curve.<sup>13</sup> Briefly, 8E51 cells were serially diluted in 1 × 10<sup>5</sup> A301

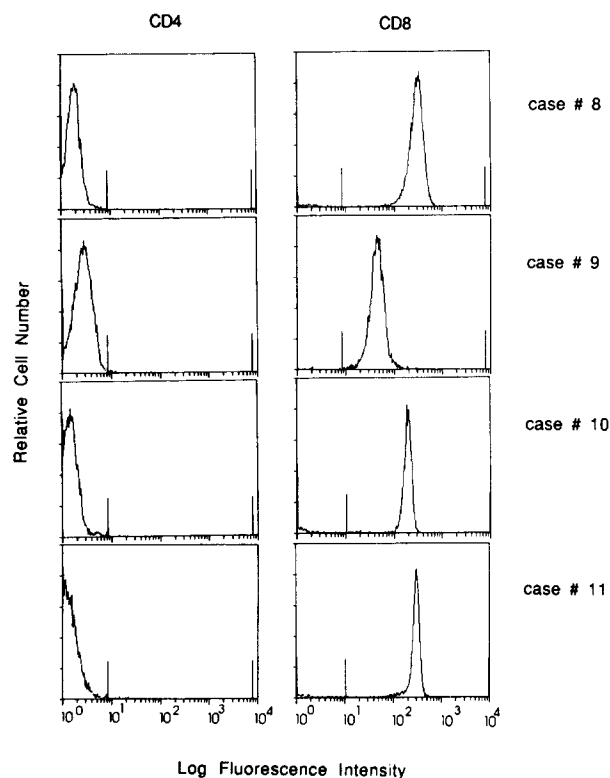
HIV-1-negative cells; amplification by PCR was accomplished directly on 1 × 10<sup>5</sup> lysed cells in 100 μL of reaction mixture containing 50 pmol of each of the primers SK29 and SK30,<sup>14</sup> 20 nmol of each deoxynucleotide triphosphate (dNTP), 2.5 U of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT), 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), and 1.5 mmol/L MgCl<sub>2</sub>. Target DNA was amplified using a DNA thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer); 30 cycles were performed, each of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C. To control the reaction and the quality of the DNA to be amplified, PCO3/PCO4 primers<sup>15</sup> specific for the β-globin gene were used. Thirty microliters of each amplified sample were electrophoresed on agarose gel and transferred to Nytran filters (Amersham Int, Little Chalfont, Buckinghamshire, UK). Hybridization was achieved with a 5' end <sup>32</sup>P-labeled SK31<sup>14</sup> oligonucleotide probe, as described<sup>16</sup>; filters were exposed to x-ray films for 24 hours. Under our experimental conditions, the

**Table 2. Viral Burden in Unfractionated BAL Cells and Highly Purified AMs, BAL T Cells, and CD8<sup>+</sup> Cells**

Patients	HIV-1 Proviral DNA			
	Copies/10 <sup>5</sup> Unfractionated BAL Cells	Copies/10 <sup>5</sup> Purified Alveolar Macrophages*	Copies/10 <sup>5</sup> Unfractionated T Cells*	Copies/10 <sup>5</sup> Enriched CD8 <sup>+</sup> Cells*
1	396	—	—	—
2	9	—	—	—
3	8	—	—	—
4	108	57	388	—
5	9	29	750	—
6	7	6	114	—
7	191	400	559	—
8	32	4	—	9
9	212	55	—	162
10	36	73	—	33
11	252	3	—	52

—, Samples not available.

\* The incompleteness of data in the table are a consequence of the fact that it is unrealistic to obtain absolutely pure populations of CD4 and CD8 cells from the lung at the same time and in the same sample. We chose to enrich for CD8<sup>+</sup> cells, because this subset represented the target of our research.



**Fig 1. Cytofluorimetric patterns of highly enriched CD8<sup>+</sup> BAL populations obtained from HIV-1<sup>+</sup> patients 8 to 11 reported in Table 2. CD4 and CD8 positivity are shown in the left and right panels, respectively. Cytofluorimetric data refer to the ungated population on which PCR analysis was later performed. As explained in Materials and Methods, virtually pure CD8<sup>+</sup> cell populations were obtained following a two-step sequential depletion procedure with magnetic separation over columns.**

sensitivity of PCR in 8E51 cell samples was the detection of as few as 1 to 10 HIV-1 copies in  $10^5$  cells; samples containing 10 HIV-1 cells (corresponding to 10 HIV-1 proviruses) were invariably positive.<sup>12,17,18</sup> The amplified products were quantified by densitometer analysis (Ultrascan LX Enhancer Laser Densitometer; LKB, Bromma, Sweden). The relationship between OD values and the number of 8E51 cells (proviruses) was tested by linear regression analysis using a biomedical computer program (Statistical Analysis System Institute, Cary, NC). In this set of experiments, optical density (OD) values were linearly related up to 5,000 HIV copies ( $r = .98$ ). By comparing the OD values of patient samples against the reference curve, we obtained the number of proviruses/ $10^5$  cells.

Total cellular RNA was extracted using the RNA fast isolation system (Molecular System, San Diego, CA). An aliquot containing  $1 \times 10^5$  and  $1 \times 10^6$  cell equivalents of RNA was incubated at 42°C for 30 minutes with 50 pmol of antisense primer SK69,<sup>14</sup> 1 mmol/L of each dNTP, 1 U of RNase inhibitor, 2.5 U of reverse transcriptase (RT) (Perkin-Elmer), 50 mmol/L KCl, 10 mmol/L Tris-HCl, and 5 mmol/L MgCl<sub>2</sub> in a total volume of 20  $\mu$ L. RT was inactivated by incubation for 5 minutes at 99°C. The total volume of each RT reaction was subjected to 30 cycles of PCR (30 seconds at 94°C, 30 seconds at 56°C, 30 seconds at 72°C) in the presence of 50 pmol of each of the primers SK68 and SK69, 20 nmol of each dNTP, 2.5 U of Taq polymerase, 50 mmol/L KCl, 10 mmol/L Tris-HCl, and 2 mmol/L MgCl<sub>2</sub> in a total volume of 100  $\mu$ L. Thirty

microliters of each amplified sample was electrophoresed and transferred to Nytran filters. Hybridization was achieved with 5' end <sup>32</sup>P-labeled SK70<sup>14</sup> oligonucleotide probe as described elsewhere.<sup>16</sup> RNA from 8E51 cells was used as a positive control of the PCR reaction.

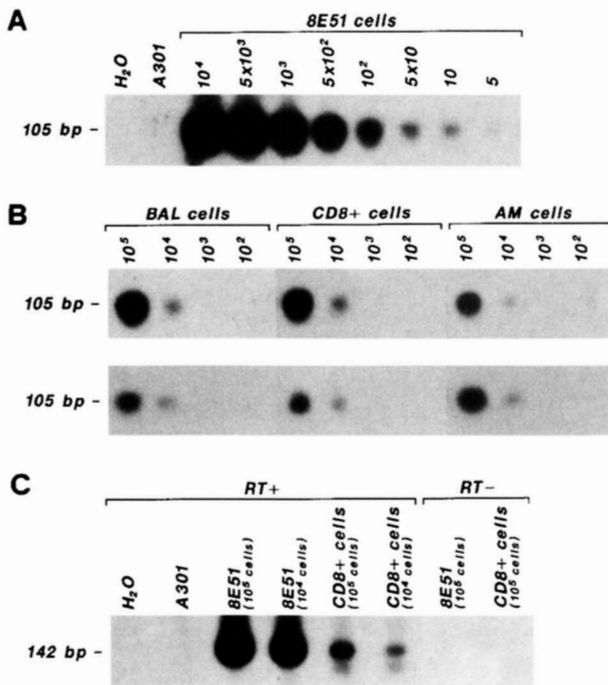
**CD4-mRNA detection by RT-PCR.** The same RT-PCR protocol described above was used to investigate the presence of CD4-mRNA in highly purified CD8<sup>+</sup> cells; the primer pair used was CD4.1 and CD4.2, and the oligonucleotide probe was CD4.3.<sup>19</sup> Samples underwent 35 PCR cycles, each of 50 seconds at 94°C, 50 seconds at 58°C, and 1 minute at 72°C. RNAs extracted from MOLT-3 CD4<sup>+</sup> and Raji B cells were used as the positive and negative controls, respectively, of the PCR.

**HHV-6 and HTLV-I/II detection by PCR.** We investigated the presence of HHV-6 or HTLV-I/HTLV-II sequences in patient cells using the PCR technique. Sample preparation and reagent concentrations for the PCR were the same as described for HIV-1 DNA PCR. Briefly, to amplify HHV-6 sequences, 30 cycles of PCR were performed using the HHV-6.1/HHV-6.2 primer pair,<sup>20</sup> each cycle consisted of 1 minute at 95°C, 1 minute at 55°C, and 1 minute and 30 seconds at 72°C. HHV-6.3 was used as the oligonucleotide probe for the detection of amplified samples. The pZVH14 plasmid<sup>21</sup> was used as the positive control of the PCR. Amplification of HTLV-I and HTLV-II sequences was performed exactly as reported elsewhere.<sup>22</sup>

## RESULTS AND DISCUSSION

HIV-1 expression in unfractionated BAL cell populations, AMs, and pulmonary T cells is reported in Table 2. Although HIV-1 DNA sequences could be demonstrated in all unfractionated BAL cell samples, the degree varied, with the viral burden ranging from 7 to 396 proviral copies/100,000 cells. Analysis of AMs and T-cell-enriched populations (Table 2, cases 4 to 7) disclosed that HIV-1 DNA was present in both, but the difference in the proviral load among the cell subsets was striking because in all cases  $10^5$  BAL T cells harbored higher quantities of HIV-1 DNA copies than  $10^5$  AMs. This finding was unexpected because, as seen in Table 1, BAL T cells accounting for the alveolitis in the lungs of AIDS patients are mainly T lymphocytes that express the CD8<sup>+</sup> phenotype.<sup>3,23</sup>

The presence of proviral DNA in circulating CD8<sup>+</sup> cells was previously reported in *in vitro* models only, and attempts to infect highly purified populations of CD8<sup>+</sup> cells directly have been unsuccessful to date.<sup>24-27</sup> To investigate whether lung CD8<sup>+</sup> cells might represent an *in vivo* target for HIV-1 infection, highly enriched cell suspensions of BAL CD8<sup>+</sup> lymphocytes were analyzed for the presence of proviral HIV-1 DNA. After repeated CD4<sup>+</sup> cell depletions by rosetting with magnetic microspheres, the CD8<sup>+</sup> T cells obtained from the BAL of four HIV-1-infected subjects (cases 8 to 11; two from subgroup IV-A and two from subgroup IV-C) were more than 99% pure, and contained less than 0.01% residual CD4<sup>+</sup> cells (Fig 1). We found (Table 2, cases 8 to 11) that lung CD8<sup>+</sup> cells harbor detectable amounts of HIV-1 DNA sequences (9, 162, 33, and 52 proviral copies/100,000 cells, respectively). When values obtained in enriched cell subsets were calculated per  $10^5$  unfractionated BAL cells according to the cell percentage in BAL suspension, we found that the sum of HIV-1 DNA copies in AMs and T-cell populations was always higher than that obtained directly on the unfractionated  $10^5$  BAL cells. Conversely, the sum of values ob-



**Fig 2.** HIV-1 detection and expression on BAL cells. (A) Quantitative determination of HIV-1 proviral copies by PCR. Serial dilutions of 8E51 cells, which contain 1 provirus/cell, were processed by PCR; the amplified products (105 bp) were hybridized with a specific <sup>32</sup>P-labeled probe, and quantified by densitometer analysis. A standard curve was obtained by plotting OD values against the cell (provirus) number. The negative control was represented by HIV-1 uninfected A301 cells. (B) Distribution of HIV-1 DNA in BAL cell subset of two representative patients, case 9 (upper panel) and case 10 (lower panel). Serial dilutions of total BAL cells, AMs, and CD8<sup>+</sup> cells were analyzed by PCR as reported in (A). The number of HIV-1 copies/10<sup>5</sup> cells was obtained by interpolation of OD values with the reference curve. (C) HIV-1 expression in CD8<sup>+</sup> cells. RNA was extracted from CD8<sup>+</sup> cells of patient 9; 1 × 10<sup>5</sup> and 1 × 10<sup>4</sup> cell equivalents of RNA were retrotranscribed *in vitro*, and then amplified with SK68/SK69 primers. The amplified products (142 bp) were hybridized with a specific SK 70 probe. RT+, RT-PCR performed using the RT enzyme in the reverse transcription step; RT-, RT-PCR performed without the RT enzyme to rule out contaminating DNA in the RNA samples. The negative and positive controls were A301 and 8E51 cells, respectively.

tained in lung CD8<sup>+</sup> cells and AMs were in all but one (case 10) lower than that observed directly on the unfractionated 10<sup>5</sup> BAL cells. Although these considerations might indicate that most of the proviral DNA in the lung is contained within the CD4<sup>+</sup> lymphocytes, possibly in a CD4 subset underrepresented in the unfractionated BAL cells, PCR analysis directly performed on the CD8<sup>+</sup> cell subset showed that this population carries HIV-1 DNA (Table 2).

To avoid the possibility that contaminating CD4<sup>+</sup> cells might have accounted for this finding, PCR analysis was performed on serial dilutions of CD8<sup>+</sup> cells obtained as above, as well as of BAL and of AM cells. As shown in Fig 2B, in two representative patients, one with high (case 9) and one with low (case 10) viral burden, PCR findings were positive in unseparated BAL preparations, CD8, and AM cell samples at a 10<sup>4</sup> cell dilution. The negative PCR findings

at a 10<sup>3</sup> cell dilution of case 9's BAL and CD8 cell samples, which carried 212 and 162 HIV-1 copies/10<sup>5</sup> cells, respectively (Fig 2B), may be partially explained by the PCR sensitivity, but could also indicate a nonlinear distribution of HIV-1 provirus/cell. This last possibility was strengthened by the finding that the intensity of the PCR signals in the diluted serial samples was not linearly related to the number of amplified cells (Fig 2B). Because HIV-1 DNA was detected at a 10<sup>4</sup> cell dilution, we could rule out the possibility that our PCR results were caused by contaminating CD4<sup>+</sup> cells, which were less than 1 in this sample dilution.

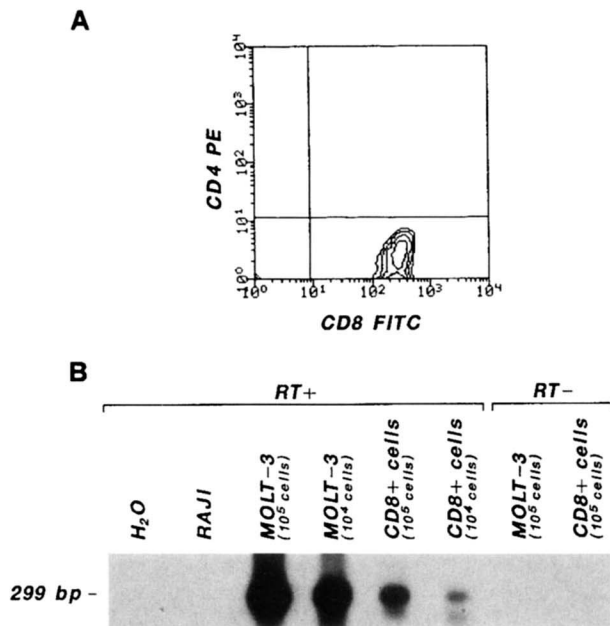
To determine whether the BAL CD8<sup>+</sup> cell infection was quiescent or productive, we studied HIV-1 expression by RT-PCR. RNA from CD8<sup>+</sup> cells of case 9 was retrotranscribed *in vitro*, and cDNA was amplified with primers specific for the gene encoding the structural protein of the viral envelope. As shown in Fig 2C, positive PCR results were observed at both 10<sup>5</sup> and 10<sup>4</sup> cell equivalents of starting RNA. Although the RT-PCR we performed was not quantitative, it is evident that the intensity of the PCR signals was consistently lower (<100-fold in OD values) than that detected in the corresponding 10<sup>5</sup> and 10<sup>4</sup> positive 8E51 cell controls. These findings were compatible with the lower number of cells carrying HIV-1 in the patient's CD8 cell sample (162 HIV-1 copies/10<sup>5</sup> cells) compared with the 8E51 cell control (1 HIV-1 copy/cell), and clearly proved that HIV-1 RNA was present in the CD8<sup>+</sup> cells.

Our data definitely show that lung CD8<sup>+</sup> cells harbor and express HIV-1, and thus indicate that not only CD4<sup>+</sup> but also CD8<sup>+</sup> cells may represent an *in vivo* reservoir for HIV-1. This finding prompts several considerations regarding the pattern of infectivity of HIV-1 and the pathways of tissue spread. Like other tissue macrophages, AMs represent a reservoir for monocytotropic HIV-1 strains from which new viral variants may be generated.<sup>28-31</sup> This event and the consequent *in situ* release of cytokines<sup>32</sup> is likely to cause a slow, but progressive impairment in local immune surveillance, ultimately leading to the development of pulmonary complications.<sup>3</sup> Besides confirming the hypothesis that HIV-1 infection of AMs plays a central role in the pathogenesis of lung involvement, our data also suggest an unexpected *in vivo* infectivity of HIV-1 toward the pulmonary CD8<sup>+</sup> cell population.

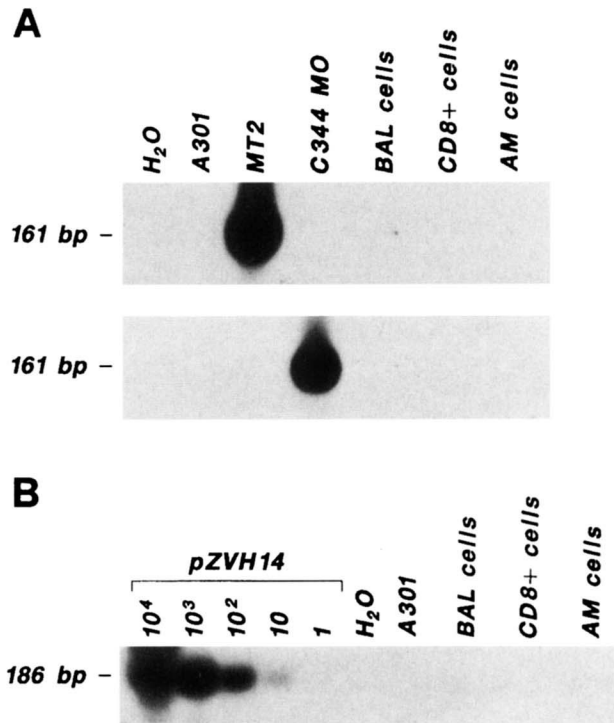
Among the mechanisms that make pulmonary CD8<sup>+</sup> cells susceptible to HIV-1 infection *in vivo*, an obvious explanation stems from recent *in vitro* studies showing that HIV-1 transmission may occur through cell-to-cell contact between persistently infected CD4<sup>+</sup> cells and CD8<sup>+</sup> lymphocytes.<sup>25</sup> In this regard, Plata et al<sup>30</sup> demonstrated that pulmonary MHC-restricted CTL recognize and lyse HIV-1 infected CD4<sup>+</sup> target cells, including autologous AMs and lung CD4<sup>+</sup> fibroblasts.<sup>30</sup> Because it was recently shown that HIV-1 can spread to CD8<sup>+</sup> CTL during the process of killing *in vitro*,<sup>33</sup> it is thus conceivable that the repeated contact occurring in the lung microenvironment between activated HIV-1-specific CTL and relevant targets could ultimately lead to the infection of CD8<sup>+</sup> cells. If this were the case, the net effect of the presence of HIV-1 in lung CD8<sup>+</sup> lymphocytes, as shown by our findings, could account for a progressive

decrease in pulmonary HIV-1-specific CTL activity, which characterizes the endstage disease.<sup>3,34</sup> Further longitudinal studies on a series of patients in different stages of the HIV infection are needed to define whether the intensity of viral burden in CD8<sup>+</sup> cells plays a central role in the progressive loss of the in situ cytotoxic activity, and thus in the progression of the pulmonary disease.

An additional, and not necessarily alternative, hypothesis to explain the susceptibility of CD8<sup>+</sup> lymphocytes to HIV-1 infection rests on the fact that these cells derive from precursors that coexpress CD4 and CD8 antigens at a distinct stage of differentiation in vivo. In view of the recent suggestion that secondary lymphoid follicles of the lung act as reservoirs for HIV-1,<sup>35</sup> it is possible that HIV-1-infected CD8<sup>+</sup> lymphocytes might originate from virgin T cells that transiently coexpress CD4 and CD8 in the secondary lymphoid tissues before relevant antigenic stimulation takes place. In this light, and prompted by the observation that the double-positive CD4<sup>+</sup>/CD8<sup>+</sup> peripheral T-cell subset in an HIV-infected patient was increased,<sup>36</sup> we evaluated the frequency of double-positive BAL lymphocytes in 35 HIV-1-infected patients; no increase in this subset was found, and



**Fig 3.** CD4-mRNA detection by RT-PCR in highly purified CD8<sup>+</sup> lung lymphocytes. (A) Expression of CD4 and CD8 antigens on the same highly purified population used for RT-PCR; cytofluorimetric data refer to the ungated population. The enriched population of CD8<sup>+</sup> cells obtained after sequential depletion with magnetic separation over columns contained 99.9% CD8<sup>+</sup> and 0.01% CD4<sup>+</sup> cells. This pattern refers to case no. 11 in Tables 1 and 2. (B) RNA was extracted from purified BAL CD8<sup>+</sup> cells (see the accompanying panel (A)),  $1 \times 10^5$  and  $1 \times 10^4$  cell equivalents of RNA were retrotranscribed in vitro and amplified with CD4.1/CD4.2 primers. Amplified products (299 bp) were hybridized with specific CD4.3 probe. RT+, RT-PCR performed using the RT enzyme in the reverse transcription step; RT-, RT-PCR performed without the RT enzyme to rule out contaminating DNA in the samples. The negative and positive controls were Raji B cells and MOLT-3 CD4<sup>+</sup> cells, respectively.



**Fig 4.** Search for HTLV-I/HTLV-II and HHV-6 in BAL cell subsets. (A) BAL cells, AM cells, and CD8<sup>+</sup> cells from patient 9 were amplified with primers PX.1 and PX.2 specific for HTLV-I and HTLV-II sequences. The amplified products (161 bp) were hybridized with two different oligonucleotide probes specific for HTLV-I (upper panel) and HTLV-II (lower panel), respectively. The negative control was HTLV-negative A301 cells; positive controls were HTLV-I-infected MT2 cells, and HTLV-II infected C344MO cells. (B) BAL cells, AM cells, and CD8<sup>+</sup> cells from patient 9 were amplified with primers HHV6.1 and HHV6.2 specific for HHV6 sequences. The amplified products (186 bp) were hybridized with specific oligonucleotide probes. The negative and positive controls were A301 cells and pZVH14 plasmid, respectively.

the mean double-positive CD4<sup>+</sup>/CD8<sup>+</sup> cells in the BAL of our case series was  $0.1\% \pm 0.04\%$ .

To further test the hypothesis that we were dealing with CD8<sup>+</sup> cells derived from double-positive CD4<sup>+</sup>/CD8<sup>+</sup> cells that have shed the CD4 molecule during their differentiation, we evaluated CD4 gene expression on highly purified CD8<sup>+</sup> cells obtained from a patient (case 11 in Table 1) with a CD8 high-intensity alveolitis (Fig 3A); we found detectable amounts of RNA for the CD4 molecule (Fig 3B). In other words, despite the lack of membrane CD4 reactivity, this CD8-enriched cell fraction expressed CD4 molecule RNA, further supporting the above concept that these cells might originate from mature T cells that had transiently coexpressed the CD4 and CD8 determinants.

We<sup>37</sup> and others<sup>38</sup> previously demonstrated that HTLV-I-transformed CD8<sup>+</sup> cell lines are susceptible to in vitro infection by HIV-1. Moreover, Lusso et al<sup>26</sup> found that HHV-6 induces CD4 antigen expression on CD8<sup>+</sup> cells, thus allowing their in vitro infection by HIV-1 strains. To evaluate whether these viral agents make the pulmonary CD8<sup>+</sup> cells susceptible to HIV-1 infection, we tested BAL T cells

for the presence of HHV-6 and HTLV-I/HTLV-II sequences. As reported in Fig 4, we were unable to find any HTLV-I/HTLV-II proviral DNA, nor any HHV-6 DNA.

In conclusion, this study provides the demonstration that CD8<sup>+</sup> lymphocytes represent a potential cell target for HIV-1 not only in vitro but also in vivo. In addition, our findings seem to indicate that a particular CD8 cell population expressed enough CD4 molecules during its in vivo development to become permissive to the subsequent HIV-1 infection. In turn, the presence of HIV-1 in the infected cells may have downregulated the expression of the CD4 molecule itself on the cell membrane.<sup>39</sup> To fully understand whether this double-positive subpopulation is implicated in HIV-1 infection spread to the lung, the HIV-1 burden should be quantified during the development of the pulmonary CTL immune response. However, our data do not exclude the possibility that coinfection of pulmonary T cells by the above cited or other viral agents before their memory differentiation step could lead to an increased CTL precursor sensitivity to HIV-1 infection.

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