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Alveolar macrophage populations are distorted in immunocompromised patients with pneumonitis

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ABSTRACT: Alveolar macrophages (AM) were obtained by bronchoalveolar lavage (BAL) from patients presenting with pneumonitis: 30 human immunodeficiency virus (HIV)-infected individuals and 12 transplant recipients. Nine normal volunteers acted as controls. The cells were washed and cytospins prepared. Monoclonal antibodies (MoAbs) and immunoperoxidase methods were used to analyse the expression of HLA-DR molecules as well as phenotypic macrophage markers. P values apply to the differences between medians using the Mann-Whitney test.

Median percentages of macrophages, lymphocytes and neutrophils were similar in all three groups. No differences were found in the median percentages of macrophages expressing the monocyte phenotype (MoAb UCHM1, CD14). However, in HIV-infected patients and transplant recipients a median of only 45% of macrophages expressed the pan-macrophage phenotype identified by MoAb EBM11 (CD68) in contrast with 98% in the normal volunteers. The AM population expressing the dendritic cell marker (MoAb RFD1) was also markedly reduced in both groups of immunocompromised patients (2 *vs* 28% in normal volunteers). Transplant recipients had significantly more phagocytic cells identified by MoAb RFD7 than the HIV-infected patients (25 *vs* 2%), but the numbers were still low when compared with the volunteers (48%). HLA-DR expression on BAL cells was reduced by 90% in both immunocompromised groups. For the transplant recipients, severity of pneumonitis was correlated with expression of dendritic cell marker RFD1, (Spearman's rank correlation r=0.538, p<0.05) and pan-macrophage marker EBM11 (r=0.581, p<0.05), while no such correlation was found in HIV-infected patients.

These results suggest that a defective macrophage population is probably a serious factor contributing to immunosuppression.

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Immunocompromised patients are at risk of developing episodes of acute respiratory illness. Organisms such as *Pneumocystis carinii* (PC) and cytomegalovirus (CMV), which are very rarely associated with respiratory disease in immunocompetent individuals, are commonly identified in lung samples isolated from human immunodeficiency virus (HIV)-infected patients and transplant recipients presenting with pneumonitis. In addition, bacteria which are associated with acute respiratory episodes in immunocompetent individuals may also affect these patients. Finally, there may be cases where no infectious organisms are identified in lung samples.

The mechanisms responsible for the emergence of lung inflammatory responses in individuals with a compromised immune system are poorly understood. Depletion of CD4 positive T-cells accompanied by a variety of opportunistic infections is the most striking characteristic of the degenerating immune system in HIV infection [1]. Lymphocytes are also the prime target of therapies used in transplantation. Systemic immunosuppression by irradiation and drugs such as cyclophosphamide and azathioprine reduce numbers of circulating lymphocytes [2–4], whilst treatment with cyclosporin A results in impaired lymphocyte function without affecting their numbers [5].

Macrophages play a key role in inducing local acquired immunity *via* antigen presentation to T-cells and secretion of soluble factors controlling and regulating cell interactions [6, 7]. However, the possible contribution of alveolar macrophages to the emergence of respiratory episodes in immunocompromised patients has not been investigated. The alveolar macrophage population is heterogeneous and consists of subsets of cells expressing differing phenotypes and functions [8]. Such lung macrophage subsets change significantly

in sarcoidosis [9–11], cryptogenic fibrosing alveolitis [12, 13], and asthma [14] and correlate with disease progression [15]. Steroid treatment in sarcoidosis can reverse the changes in phenotype of alveolar macrophages coincidental with clinical improvement of the patients [16]. It is not clear to what extent the macrophage population is involved in drug-induced immune compromise in transplantation. Limited data suggest that some immunosuppressive drugs can affect functions of macrophage-like cells and this may be related to their therapeutic efficacy [17, 18].

Phenotypic and functional changes that occur in various macrophage populations in the course of HIV infection are not well defined and most investigations have been limited to peripheral blood [19–21]. Recently, accumulated evidence suggests that macrophages could act as a major reservoir of HIV during all stages of HIV infection in many tissues [22]. HIV nucleic acids and proteins can be detected in 10–50% of alveolar macrophages isolated from the bronchoalveolar lavage (BAL) fluid of infected patients [23–25] but little is known of the contribution of these cells to the pathology of HIV disease.

Episodes of pneumonitis are a clinical feature common to both transplant patients and HIV-infected individuals. This study was, thus, designed to characterize the phenotypic subsets of alveolar macrophages isolated from the lungs of HIV-infected patients and transplant recipients presenting with pneumonitis, in order to assess the possible role that these cells play in the development of lung pathology in these patients.

Methods

Subjects

According to a prospective protocol, all patients within the hospital who were immunocompromised by HIV infection by drugs or conditioning for tissue transplantation and who presented with an acute respiratory episode suggesting a pneumonitis were referred for diagnostic bronchoalveolar lavage to one team of chest physicians.

BAL was carried out at 33 separate episodes of pneumonitis in 30 HIV-infected patients and 15 episodes in 12 recipients of allogeneic transplants. No endobronchial lesions were seen at bronchoscopy in any subject. The median age among the HIV-infected patients was 40 yrs, compared with medians of 33 and 23 yrs in the transplant and volunteer groups, respectively. Eighteen of the 30 HIV-infected patients smoked 1–20 cigarettes per day compared with 6 of the 12 transplant recipients, who smoked 15–20 cigarettes per day.

At BAL, information on the severity of the respiratory episode was recorded to enable ranking of episodes as shown in table 1. For HIV-infected patients, peripheral blood CD4 counts, serum HIV antigens, and Center for Disease Control (CDC) ranking (I–IV) were noted in order to assess the severity of HIV disease (table 2).

Table 1. – Criteria for ranking severity of respiratory episode

	Score
Fever >38°C prior to lavage	1
O_2 sat. >95% prior to lavage and desat. on exertion	1
O_2 sat. <95% >90%	2
O_2 sat. <90% >85%	3
O_2 sat. <85% or requiring O_2 therapy	4
CXR shadowing 1 zone	1
CXR shadowing 2 zones	2
CXR shadowing 3 zones/confluent/generalized	3
Requiring ventilatory support	2
Death from respiratory episode (max. possible)	12

CXR: chest X-ray; sat.: saturation; desat.: desaturation.

Table 2. – Criteria for ranking severity of HIV disease

CDC Group	Peripheral blood CD4 count $\times 10^9 \cdot l^{-1}$	Serum HIV antigens	Score
CDC II/III	>0.2	-	1
	>0.2	+	2
	<0.2	-	3
	<0.2	+	4
1 prior CDC IV	>0.2	-	5
diagnosis	>0.2	+	6
	<0.2	-	7
	<0.2	+	8
>1 prior CDC IV	>0.1	-	9
diagnosis	>0.1	+	10
	<0.1	-	11
	<0.1	+	12

CDC: Center for Disease Control; HIV: human immunodeficiency virus.

For transplant recipients, the median time from transplantation to BAL was 4 months. Bone marrow transplant recipients received cyclophosphamide (2×60 mg·kg^{-1}) 4 days before transplantation and total body irradiation (15 cGy·min^{-1}, mean lung dose=726 cGy). Recipients of liver and kidney transplant were treated continuously with methylprednisolone, azathioprine and cyclosporin A starting on the day of transplantation.

In addition, BAL was carried out on nine normal volunteers following written consent and approval of the local Ethics Committee. All of these volunteers had no past history of lung disease and had not suffered any symptoms suggesting viral infection in the two weeks prior to BAL. One of the volunteers was a smoker and smoked up to 10 cigarettes per day.

Bronchoalveolar lavage

A standard diagnostic BAL procedure was carried out on all subjects using an Olympus P20 fibreoptic bronchoscope. Following sedation with 10 mg of midazolam *i.v.*, the cords were anaesthetized under direct vision with 4% lignocaine. Further anaesthetization of the airways was achieved as necessary with 2 ml aliquots of 2% lignocaine as the bronchoscope was advanced. The tip of the bronchoscope was wedged in a subsegmental bronchus of the right middle lobe in those subjects where radiographic shadowing was generalized or the radiographic appearances were normal. In those patients where the radiographic shadowing was localized, the tip was wedged in the relevant lobe. One hundred and eighty millilitres of sterile, buffered 0.9% normal saline were instilled in 20 ml aliquots and aspirated into a sterile, siliconized glass bottle aiming for a return of between 80–100 ml. A maximum of 240 ml was instilled. BAL samples were collected onto ice and maintained at 4°C throughout processing.

HIV assays

All HIV-infected patients had HIV-1 antibodies detectable in peripheral blood on three assays: Wellcozyme HIV Recombinant Assay (Wellcome Diagnostics), Abbott Recombinant Non-competitive HIV-1 Enzyme Immunoassay (Abbott Diagnostics), Serodia HIV Particle Agglutination Test (Mast Diagnostics). HIV antigens were measured in peripheral blood samples by Enzyme Immunoassay (Abbott Diagnostics) [26]. All transplant recipients and donors had no detectable serum HIV-1 antibodies by Wellcozyme HIV Recombinant Assay prior to transplantation. Blood samples were taken at the time of BAL from all HIV-infected patients and absolute CD4 cell counts assessed using a whole blood method as described previously [27].

Identification of infectious agents in BAL fluid

In 20 ml of the BAL fluid, infectious agents were identified as follows: CMV was identified using a combination of Detection of Early Antigen Fluorescent Foci (DEAFF) testing and conventional cell culture [28]; *Pneumocystis carinii* was identified by an experienced cytologist on a combination of Papanicolaou and Grocott stained slides; bacteria and mycobacteria were identified using standard microbiological techniques.

Cytospin preparation

The remaining BAL fluid (60–80 ml) was filtered through a single layer of coarse gauze to remove excess mucus and the cells were washed three times in phosphate buffered saline (PBS). Cell concentration was adjusted to between $3–5 \times 10^5 \cdot ml^{-1}$ and cytocentrifuge preparations were made (Cytospin II, Shandon Instruments, UK). The cytospins were dried at room temperature for 20 min, fixed in a 1:1 mixture of chloroform and acetone, wrapped in plastic film and stored at -20°C for less than 12 months prior to immunoperoxidase staining. One slide from each BAL was also stained with a modified May-Grünwald Giemsa stain in order to record the percentages of macrophages, lymphocytes and granulocytes.

Immunoperoxidase staining and monoclonal antibodies

The cytospin preparations were stained with monoclonal antibodies (MoAb) to identify macrophages (MoAb EBM11\CD68) [29], monocytes (MoAb UCHM1\CD14) [30], dendritic cells (MoAb RFD1) [31], mature phagocytes (MoAb RFD7) [31] and framework epitope on HLA-DR (MoAb RFDR1) [32]. An indirect immunoperoxidase method, was used as described previously [33]. Briefly, the preparations were incubated with mouse anti-human MoAb for 45 min at room temperature, rinsed with PBS and then incubated with a rabbit anti-mouse peroxidase conjugate for 45 min. After rinsing with PBS, the preparations were developed in a solution containing 0.01% hydrogen peroxide and 0.6% 3,3'-diaminobenzidine tetrahydrochloride. All preparations, except those stained with MoAb for HLA-DR, were counterstained in Harris' haematoxylin, dehydrated and mounted in DPX mountant (BDH) for microscope viewing by one investigator, who was blind to the clinical histories of the subjects.

In all cases, negative controls were included omitting the primary antibody. For EBM11 (CD68), UCHM1 (CD14), MoAbs RFD1 and RFD7, any macrophages exhibiting identifiable staining differing from that of the negative control were scored as positive. At least 150 macrophages were counted from each preparation and the percentage of positive macrophages was recorded. HLA-DR expression was quantified on each preparation stained with MoAb RFDR1 *via* measurement of optical density using Seescan black and white camera attached to a microscope with a ×40 objective and a Seescan image analyser (Seescan Ltd). Optical density measured per unit area was directly proportional to the intensity of the staining, *i.e.* MoAb binding. The measurements were carried out in at least 10 fields of each cytospin, including negative controls. The density of HLA-DR antigens was expressed as the difference between the mean optical density of a given sample and its negative control.

Statistics

Median percentages of positive cells for each monoclonal antibody were compared among the different groups of subjects using the Mann-Whitney test and 95% confidence interval analysis. Spearman's rank correlation was used to assess the correlation between the expression of macrophage markers and severity of pneumonitis or HIV disease.

Results

Characteristics of episodes of pneumonitis

The nature of infectious agents identified in BAL fluid from HIV-infected patients and transplant recipients is shown in table 3. PC was identified in 16 out of 33 episodes in the HIV infected group compared with 4 out of 15 episodes in the transplant group. The equivalent frequencies for CMV were 13 out of 33 and 7 out of 15, respectively, whilst bacteria were identified in 6 out of 33 in the HIV-infected group and 2 out of 15 in the transplant group. No mycobacteria were isolated in either group and no infectious agents were identified in BAL samples from the volunteers.

There was no difference in the median severity of pneumonitis between the HIV-infected patients and transplant recipients (median of 4 in both groups). No correlation was observed between the severity of pneumonitis and presence of infectious organisms in BAL fluid.

Differential cell counts

There were no statistically significant differences in the median percentages of macrophages, lymphocytes and neutrophils between the three groups of subjects (data not shown). However, markedly raised lymphocyte and neutrophil percentages above levels seen in the normal volunteers were found during individual

Table 3. – Nature of organisms isolated from BAL fluid in HIV-infected patients and transplant recipients

HIV-infected patients				Transplant recipients		
Lab no.	CD4 count $\times 10^9 \cdot l^{-1}$	Serum HIV Ag	Organisms in BAL	Lab no.	Category of transplant	Organisms in BAL
1	0.27	+	PC, CMV	34	Allogeneic	
2*	0.208	+	PC, CMV		bone marrow	PC
3*	0.208	+	PC, CMV, *St. pn*	35	Allogeneic	
4	0.172	-	PC, CMV		bone marrow	*H. in*
5	0.085	-	PC	36*	Liver	CMV
6	0.08	-	CMV	37	Liver	CMV
7	0.072	-	PC	38	Renal	PC
8	0.028	-	CMV	39+	Allogeneic	
9	0.02	-	CMV		bone marrow	CMV
10	0.02	+	PC	40*	Liver	PC
11	0.02	-	PC	41	Liver	*B. cat*
12	0.013	+	CMV	42	Liver	CMV
13	0.012	-	PC	43#	Renal	PC, CMV
14	0.01	-	PC	44	Renal	CMV
15	0.007	-	PC	45#	Renal	CMV
16	0.004	-	PC	46	Allogeneic	
17	0.004	+	-		bone marrow	-
18	0.003	-	PC, CMV	47	Liver	-
19+	0.002	+	CMV, *St. pn*	48+	Allogeneic	
20	0.002	-	*H. in*		bone marrow	-
21+	0.001	+	PC, CMV, *S. aur*			
22	0.001	+	PC, *H. in*			
23	ND	+	PC, CMV			
24	0.825	-	-			
25	0.6	-	-			
26	0.462	-	-			
27	0.147	-	-			
28	0.13	-	-			
29	0.104	-	-			
30#	0.042	-	-			
31#	0.042	-	-			
32	0.018	-	-			
33	0.002	-	-			

PC: *Pneumocystis carinii*; *H. in*: *Haemophilus influenzae*; CMV: cytomegalovirus; *B. cat*: *Branhamella catarrhalis*; *St. pn*: *Streptococcus pneumoniae*; *S. aur*: *Straphylococcus aureus*; ND: not done; * + #: patients who suffered two episodes of pneumonitis; BAL: bronchoalveolar lavage; HIV: human immunodeficiency virus; Ag: antigen.

episodes of pneumonitis in both immunocompromised groups. This reflected the identification of infectious agents from the lungs of these patients, whilst no infectious agents were identified in the samples from the normal volunteers. No correlation was found between BAL lymphocytosis and severity of pulmonary disease.

Immunological analysis

Although no differences were found in the median percentage of macrophages expressing the phenotype of monocytes (MoAb UCHM1, CD14) between the three groups (fig. 1), in HIV-infected patients and transplant recipients a median of only 45% of macrophages identified by morphology expressed the pan-macrophage phenotype as identified by MoAb EBM11 (CD68). In the normal volunteers this marker was present on a median of 98% of alveolar macrophages ($p<0.0001$, fig. 2). Median percentages of alveolar macrophages expressing the dendritic cell marker identified by MoAb RFD1 were also markedly reduced in both groups of immunocompromised patients (28% in normals, 2% in the immunocompromised patients, $p<0.004$, fig. 3). The median percentage of alveolar macrophages expressing the marker for mature phagocytes (MoAb RFD7) was greater in the transplant group (15%) than in the HIV-infected patients (2%, $p<0.02$). The median percentages of cells expressing this marker was still significantly lower in both patient groups than in the volunteers (48%, $p<0.006$, fig. 4). Median HLA-DR expression on BAL cells, as measured by the optical density readings, was reduced by 90% in both immunocompromised groups ($p<0.004$, fig. 5).

The data were analysed within each group of immunocompromised patients according to whether or not any infectious organism was identified at BAL. The expression of each of the phenotypic markers at episodes where no infectious organism was identified was no different from those where one or more infectious agents were found (data not shown). No significant differences in phenotypic characteristics of AM were observed between smokers and nonsmokers. Only one of the volunteers smoked and the results of AM analysis in his BAL sample were not different from other volunteers.

Finally, Spearman's rank correlation coefficients were used to check for correlations between the expression of the markers, blood CD4 count, severity of pneumonitis (table 1) and severity of HIV disease (table 2). For the HIV-infected population there was a significant negative correlation between blood CD4 count and median percentage expression of RFD1 on alveolar macrophages ($r=-0.397$, $p<0.05$) which was not, however, reflected in a negative correlation with severity of HIV disease, and no correlations were found with severity of pneumonitis. For the transplant recipients, severity of pneumonitis was significantly correlated with median expression of RFD1 ($r=0.538$, $p<0.05$) and EBM11 ($r=0.581$, $p<0.05$).

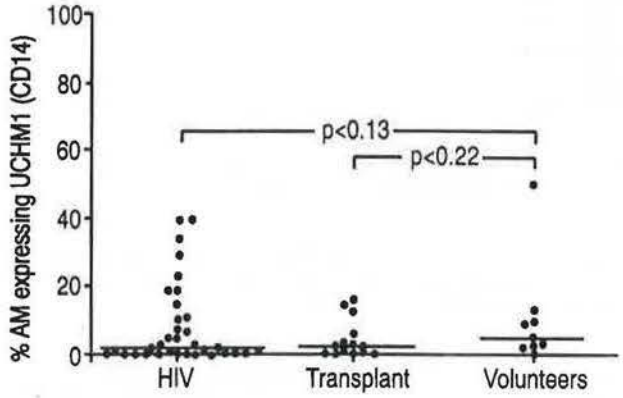

Fig. 1. – Expression of monocyte phenotype marker (CD14) on alveolar macrophages (AM). HIV: human immunodeficiency virus.

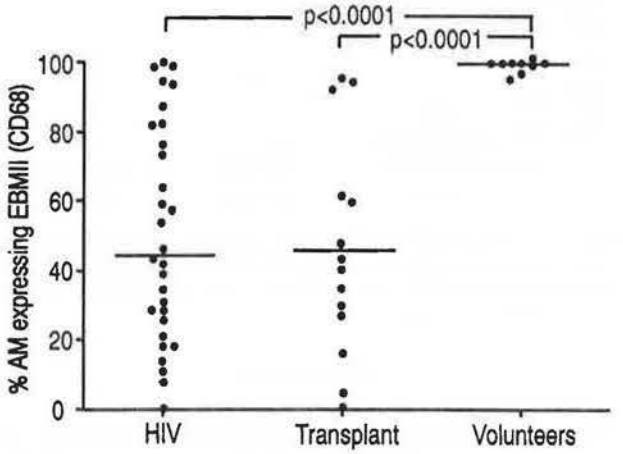

Fig. 2. – Expression of pan-macrophage marker (CD68) on alveolar macrophages (AM). HIV: human immunodeficiency virus.

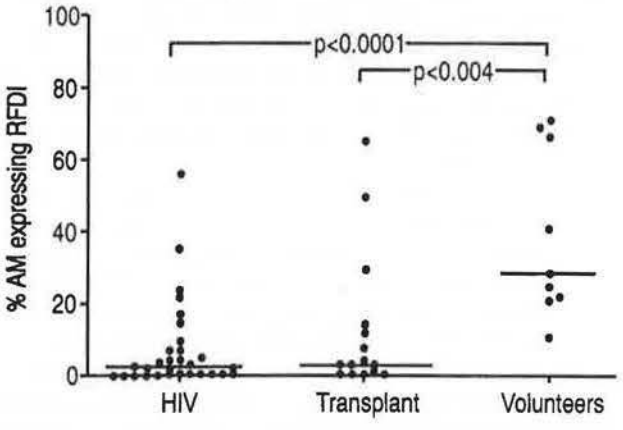

Fig. 3. – Expression of dendritic cell marker (MoAb RFD1) on alveolar macrophages (AM). MoAb: monoclonal antibody.

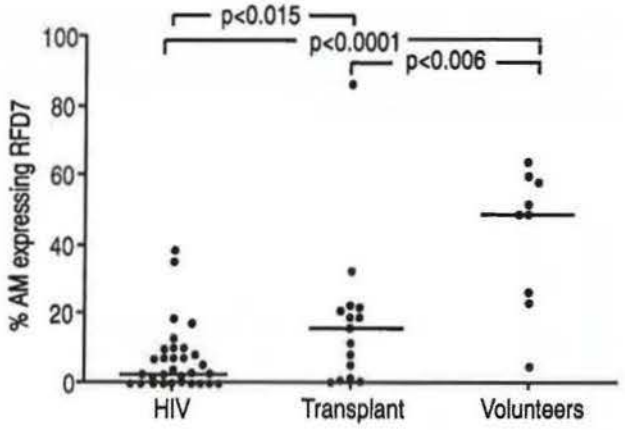

Fig. 4. – Expression of phagocytic cell marker (MoAb RFD7) on alveolar macrophages (AM). MoAb: monoclonal antibody; HIV: human immunodeficiency virus.

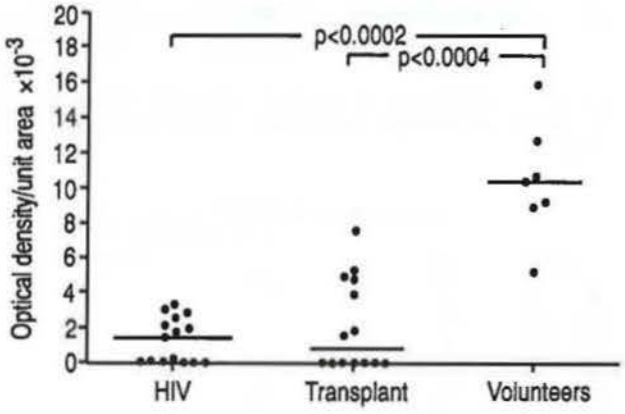

Fig. 5. – Expression of HLA-DR molecules (MoAb RFDR1) on BAL cells as measured by optical density readings. MoAb: monoclonal antibody; BAL: bronchoalveolar lavage; HIV: human immunodeficiency virus.

Discussion

We have demonstrated a dramatic decrease in the expression of HLA-DR molecules, as well as phenotypic macrophage markers, in lungs of two clinically different groups of immunocompromised patients: transplant recipients and HIV-infected patients. It is not clear to what extent these changes are associated with pneumonitis present in all the patients undergoing BAL. We found no correlation between the low levels of expression of cell markers studied and the presence of infectious organisms isolated from BAL fluid in both groups.

The severity of respiratory episode was correlated with the expressions of two macrophage markers: CD68 and RFD1 in transplant recipients but not in HIV-infected patients. Phenotypic changes in macrophage subsets have been previously described in transplant patients. Analysis of antigen-presenting cells in peripheral blood of bone marrow transplant recipients [34] has shown that RFD1 positive dendritic cells appeared in levels comparable to normal in two phases after transplantation: in the early 0–3 month period, and after 12 months. During the period of 3–12 months, after bone marrow transplant, dendritic cells were, to some extent, replaced by phagocytic cells, which rose to normal levels after 3–6 weeks post-transplantation but had reduced expression of HLA-DR antigen [34]. In our investigation, the median time from transplantation to BAL was 4 months. Only 5 out of 12 patients included in the study were bone marrow recipients but it is possible that some of the changes in alveolar macrophage subsets and HLA-DR expression could reflect modulation of the immune system in the post-transplantation period by the graft itself and/or drug/radiation treatment. Although there is compelling evidence for the concept that inhibition of T-cell functions (*i.e.* impaired production of interleukin 1 and 2 (IL-1 and IL-2)) plays a central role in the immunosuppression caused by cyclosporin [35, 36], it has been shown that the effectiveness of this drug is partially due to blocking the accessory functions of macrophage-like cells [18, 37].

Induction of class I and class II antigens is also known to be blocked by treatment with cyclosporin [18] and in animals the drug inhibits acquisition and presentation of antigen by dendritic cells [38]. Patients with kidney grafts who received cyclosporin and steroids as immunosuppressive agents showed a rapid decrease in the numbers of skin Langerhans' cells expressing HLA-DR [39]. An effect on antigen-presenting cells of other immunosuppressive drugs was also indicated by evidence of a changed morphology and decreased numbers of Langerhans' cells in patients treated with azathioprine and prednisone [40]. Our results confirm that down-regulation of major histocompatibility complex (MHC) class II antigens occurs in transplant recipients and suggest that the functions of macrophage population in the lung may be seriously changed. Correlation between the expression of alveolar macrophage markers and severity of pneumonitis may reflect significant contribution of these cells to immunoparesis in the post-transplantation period.

Our data show the levels of expression of HLA-DR molecules and phenotypic markers on alveolar macrophages to be similar in both groups of immunocompromised patients studied, except for the expression of RFD7. HIV-infected patients had significantly lower numbers of RFD7 positive phagocytic cells than transplant recipients. This difference may indicate some degree of recovery of the immune system in transplant recipients in contrast to the progression of disease in HIV-infected patients. Analysis of phenotypic characteristics of HIV-infected, macrophage-like cells has been performed by many authors, frequently yielding conflicting results. *In vitro* studies on human monocytic U937 cell line using six anti-class II monoclonal antibodies provide strong evidence that HIV infection of these cells down-regulates expression of HLA class II antigen [41]. Belsito *et al.* [42] showed that the number of HLA-DR positive epidermal Langerhans' cells was reduced by 63% in patients with acquired immune deficiency syndrome (AIDS). Heagy *et al.* [43] and Braun *et al.* [44] found significant decreases in the percentages of HLA-DR

antigen-bearing monocytes in peripheral blood of AIDS patients. TSANG *et al.* [45] also reported defective HLA-class II dependent accessory function of monocytes in these patients. ROY *et al.* [46] showed class II antigen reduction not only on peri-pheral blood monocytes but also on alveolar macrophages. We found not only decreased overall expression of HLA-DR on alveolar macrophages in HIV-infected patients but also a marked reduction in phenotypic macrophage markers. The inverse correlation between the expression of dendritic cell marker (RFD1) and peripheral blood CD4 count could indicate possible involvement of antigen-presenting cells in the pathology of underlying HIV disease in these patients.

In both humans and animals, class II positive accessory cells determine the abilities of T-cells to respond to antigen [47, 48]. In the absence of these cells, T-cells fail to respond to viruses and other antigens [49, 50], rendering the host vulnerable to opportunistic infections. Immunological abnormalities in both transplant recipients and HIV-infected patients have been convincingly linked to the profound impairment of T-cell function.

It is not known whether the phenotypic changes in macrophage subsets are secondary to T-cell dysfunction. It will be of interest to confirm whether such changes are limited to lungs only and whether they have a transitional character or represent permanent damage. While this study provides some evidence that defective macrophage population in lungs may reflect the immunosuppression, further investigation, involving clinically matched patients without pneumonitis will be crucial to determine whether phenotypic changes in AM contribute to lung pathology.

Currently, some therapeutic approaches are attempting to correct immunodeficiency by enhancing T-cell activities. Clinical trials investigating benefits of interferon γ (IFNγ) and administered together with zidovudine in early stages of HIV disease are in progress. Although the involvement of the macrophage population in the mechanism of immunosuppression requires further investigation, it is certain that its overall influence cannot be excluded when therapeutic modulation of the immune system is to be considered.

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