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Abnormal alveolar macrophage populations in bone marrow transplant recipients with pneumonitis

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Abnormal alveolar macrophage populations in bone marrow transplant recipients with pneumonitis. H.J. Milburn, H.G. Prentice, L.W. Poulter. ©ERS Journals Ltd 1993.

ABSTRACT: The purpose of this investigation was to determine whether there is any relationship between different subsets of alveolar macrophages and type of infection or survival from interstitial pneumonitis following bone marrow transplantation (BMT).

The population of alveolar macrophages found in bronchoalveolar lavage fluid (BALF) from 16 BMT recipients with 19 episodes of interstitial pneumonitis was investigated, using immunocytochemical methods. Results were compared with those from seven normal volunteers.

The results showed that patients with pneumonitis had significantly higher numbers of total cells in BALF than normals but reduced proportions of macrophages, although the absolute numbers were unchanged. Of the cells present which were morphologically macrophages, there were raised proportions of both RFD1+ cells (interdigitating cells) and RFD7+ cells (mature macrophages) in patients compared with normals, but expansion of these two subsets could be explained, in part, by a significant increase in cells positive for both markers (42% in patients compared with 9% in normals). Proportions of cells with the monocyte phenotype (CD14+, UCHM1) were also significantly raised in patients with pneumonitis (17% compared with 6% in normals). These patients, however, had significantly reduced proportions of macrophage-like cells which were positive for the DR antigen (Class II major histocompatibility complex (MHC) antigen) (47% compared with 88% in normals), and this abnormality was greater still in patients who died from pneumonitis (40%) compared with those who survived (52%).

The results of this study indicate a breakdown of local immunoregulation, thus contributing to the high incidence of, and mortality from, opportunistic pulmonary infections in this group.

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Interstitial pneumonitis is a major cause of morbidity and mortality following bone marrow transplantation (BMT) [1, 2], and is frequently associated with the presence of opportunistic organisms in the lungs. Since alveolar macrophages are thought to play a central role in the protection of the lower respiratory tract from infectious agents, it is probable that an abnormality of the macrophage population in the lungs is involved in the pathogenesis of pneumonitis. Alveolar macrophages are involved in the regulation of immune function in the lungs, and in the presentation of antigen to immunocompetent cells. They are important for the initiation, regulation and resolution of inflammation [3, 4]. As alveolar macrophages are found at the environment-alveolar interface, they have potential for daily exposure to multiple antigens, and are also involved in phagocytosis and enzymatic degradation of pathogens. Oxyen derived reagents produced during these reactions cause peroxidation of cell membrane lipid components and, therefore, cellular damage. This destructive action is necessary for microbial killing but, without appropriate inhibition, may also cause local tissue breakdown.

In the present study, we have investigated the population of alveolar macrophages in a group of patients with interstitial pneumonitis following BMT. Despite being derived from a common bone marrow progenitor cell [5–7], macrophages and monocytes exhibit both phenotypic and functional heterogeneity [8–11] and, using immunocytology with monoclonal antibodies, we have been able to determine the relative proportions of different macrophage/monocyte subsets found in bronchoalveolar lavage fluid (BALF). We have investigated whether there is any relationship between different subsets of macrophages and type of infection or survival.

Patients and methods

Patients

Sixteen patients with interstitial pneumonitis following T-cell depleted allogeneic BMT were investigated. All had been transplanted for haematological malignancies, and all received pretransplant conditioning with cyclophosphamide, and total body irradiation with a lung average dose of 670–830 cGy. Twelve patients were male and four female,

age range 12–46 yrs, (median 30 yrs). Only one patient had smoked prior to his transplant.

The majority of patients developed pneumonitis between 40 and 180 days post transplant (median 101 days), but one had pneumonitis one year after transplant and one at two years. Both of these latter patients had earlier episodes of pneumonitis, which have not been included in this study. Three other patients, however, were investigated on a second occasion, and both of these episodes have been included in this study. Thus, a total of 19 pneumonitis episodes were investigated.

Seven normal volunteers were also investigated to act as controls. All were nonsmokers with no history of upper respiratory tract infections in the six weeks immediately before bronchoscopy.

A diagnosis of pneumonitis was made on the basis of symptoms (shortness of breath and fever) and a fall in the transfer factor of the lungs for carbon monoxide (T_{LCO}) (corrected for haemoglobin) of >20% when compared with earlier post-transplant measurements. In addition, the chest radiograph showed local changes in 11 cases, diffuse changes in six, and was normal in two. Clinical symptoms and signs, with or without radiographic changes, are well accepted as indicators of pneumonitis by the major transplant centres both in Europe and the USA. A recent multicentre study on the treatment of cytomegalovirus (CMV) interstitial pneumonitis [12] used a definition which accords with our own. Furthermore, measurements of lung function, in particular the T_{LCO}, are sensitive indicators of pneumonitis following BMT [13]. The fall in T_{LCO} usually occurs before chest radiographic abnormalities are seen and before the appearance of some clinical signs.

With one exception (a patient who had been transferred from another hospital), all patients were investigated within 24 h of the onset of symptoms. Fourteen of these patients had significant infective organisms in the lower respiratory tract. The diagnoses made in all episodes of pneumonitis are outlined in table 1.

Sample collection

Fibreoptic bronchoscopy was performed as described previously [14] using a BFT1 fibreoptic bronchoscope. Care was taken not to use suction above the vocal cords to minimize bacterial contamination of the suction channel of the bronchoscope. BAL was performed using 3×60 ml aliquots of normal saline, warmed to 37°C, and buffered to pH 7.4 by the addition of 175 μEq of sodium bicarbonate to 500 ml normal saline. Each aliquot was aspirated immediately after its instillation and collected in silicon-coated glass bottles, maintained at 4°C to reduce problems of macrophage adherence.

Processing lavage samples

All lavage samples were investigated for the presence of bacteria (including mycobacteria), fungi, protozoa and viruses by standard microscopy and culture. CMV was detected using both conventional cell culture and the detection of early antigen fluorescent foci (DEAFF) test [15]. Using an aliquot of untreated lavage fluid, the total cell count was determined in a modified Neubauer haemocytometer. Mucous strands were aspirated from BALF which was then centrifuged at 350×g for 10 min, and the supernatant decanted. The cell pellet was washed twice in phosphate buffered saline (pH 7.2) and the cell suspension adjusted to a final concentration of 3×10^5 cells·ml^{-1}. Aliquots of this suspension containing about 3×10^4 cells were used to make cytospin preparations in a Shandon Cytospin II (Shandon Instruments, Runcorn, UK). The cytospins obtained were air-dried at room temperature for 1 h, fixed in a chloroform:acetone mixture (1:1) for 10 min, air-dried again, and then wrapped in cling film and stored at -20°C for future use.

Immunocytochemistry

Differential cell counts were performed on cytospin

Table 1. – Patient characteristics

Patient no.	Underlying disease	BAL diagnosis	Days from transplant	Survival
1	CGL	CMV	73	No
2	AML	CMV	45	No
3 ep. 1	AML	CMV	85	Yes
ep. 2		CMV	101	No
4	CGL	CMV + *P. carinii*	105	Yes
5 ep. 1	AML	CMV	58	Yes
ep. 2		CMV + Cryptosporidium	121	No
6	AML	CMV	98	Yes
7	AML	CMV	69	No
8	CGL	Idiopathic	84	No
9 ep. 1	ALL	*S. aureus*	112	Yes
ep. 2		*S. Aureus*	175	No
10	ALL	*C. albicans*	212	Yes
11	CGL	Idiopathic	357	Yes
12	CGL	*P. carinii*	101	Yes
13	ALL	Idiopathic	150	Yes
14	AML	Idiopathic	44	Yes
15	CGL	Measles	730	No
16	CGL	Intrapulmonary haemorrhage	128	Yes

ep.: episode; BAL: bronchoalveolar lavage; CMV: cytomegalovirus; CGL: chronic granulocytic leukaemia; AML: acute myeloid leukaemia; ALL: acute lymphoblastic leukaemia.

preparations stained with May-Grünwald-Giemsa. Lymphocytes and macrophage-like cells were identified by morphology. Discrete subpopulations of mononuclear cells were identified with monoclonal antibodies by indirect immunocytochemical methods [16]. The monoclonal antibodies used and the cell types identified are listed in table 2. In addition, cytospin preparations were incubated with combinations of monoclonal antibodies followed by class-specific second layer antibodies conjugated to fluorescein isothiocyanate (FITC) or tetra-methylrhodamine isothiocyanate (TRITC) (indirect immunofluorescence) to assess double staining of mononuclear cells. These preparations were examined using a Zeiss fluorescence microscope with epi-illumination and selective barrier filters for FITC and TRITC.

Random high power fields from cytospin preparations of each reaction were examined. Three hundred cells were counted in each case, and the proportion of positive cells enumerated. If no positive cells were identified, this was recorded as <1%. The mean values of the proportion of cells in BALF from each patient was calculated. For the double stained preparations, the percentages of RFD7+ cells also expressing RFD1 were recorded. Appropriate positive tissue controls and reagent controls were used to ensure quality and reproducibility of the methods.

Statistical analysis

Results are expressed as the mean value with standard error of the mean (SEM) in parenthesis. Relationships between two sets of data were determined by the Spearman Rank Correlation test. Differences between groups were determined by the Mann Whitney U-Test.

Results

Total and differential cell counts

The total cell count varied between patients with five lavages yielding total numbers of cells within the range found in the normal lung ($1–2\times10^5 \cdot ml^{-1}$), three with counts below 1×10^5 cells$\cdot l^{-1}$ but considerably higher numbers were found in the remaining 11. Numbers of cells as high as 20 times the normal range were found in some of these patients.

The majority of patients had proportionately fewer macrophage-like cells (as identified by morphology) than normal (70% (4%); normal range 86–90%) but the absolute numbers of these cells were not significantly different. In fact, the mean recovery of macrophage-like cells was higher than normal in many of the transplant recipients. These results have been described in detail previously [19].

Table 2. – Monoclonal antibodies used and cell types identified

Reagents used	Source	Cells or molecules identified
RFDR1	RFHSM	Class II MHC molecules*
RFD1	RFHSM	Interdigitating cells**
RFD7	RFHSM	Mature macrophages**
CD14 (UCHM1)	UCHSM	Monocytes

*: JANOSSY *et al.* [17] 1986; **: POULTER *et al.* [18] 1986. RFHSM: Royal Free Hospital School of Medicine; UCHSM: University College Hospital School of Medicine; MHC: major histocompatibility complex.

Phenotype of BAL macrophages

Many of the alveolar macrophages recovered by BAL from the transplant recipients with pneumonitis were abnormal in appearance. There was an increased incidence of multi-nucleated giant cells, and there was little uniformity of size. Some cells from patients with CMV in the lavage fluid exhibited typical "owl's eye" inclusion bodies.

Using the immunoperoxidase technique, macrophage-like cells (MLC) in BAL were assessed for expression of D1 (interdigitating cells), D7 (mature macrophages), CD14 (monocytes) and human leucocyte antigen-DR (HLA-DR) molecules (cells capable of antigen presentation). Greater proportions of macrophages from patients with pneumonitis expressed the D1 molecule when compared with normal individuals, but there were also significantly greater proportions of macrophages expressing the D7 molecule in the patient group (fig. 1). The results of double immunofluorescence used to determine the proportion of D7+ cells which also expressed the D1 marker, however, indicated that this expansion was generally accounted for by the high proportion of cells expressing both of these markers. In the patients with pneumonitis, 42.2 (4.4)% of macrophages expressed both markers, compared with 9 (2.6)% of macrophages from normal volunteers ($p<0.001$).

Not all MLC present in BALF had macrophage markers, and some expressed the monocyte phenotype (identified by the CD14 (UCHM1) monoclonal antibody). Patients with pneumonitis had increased proportions of monocytes in BALF, compared with normals (fig. 1). There was, however, a significant reduction in expression of HLA-DR on MLC from the patient group: 46.6 (3.6)% were

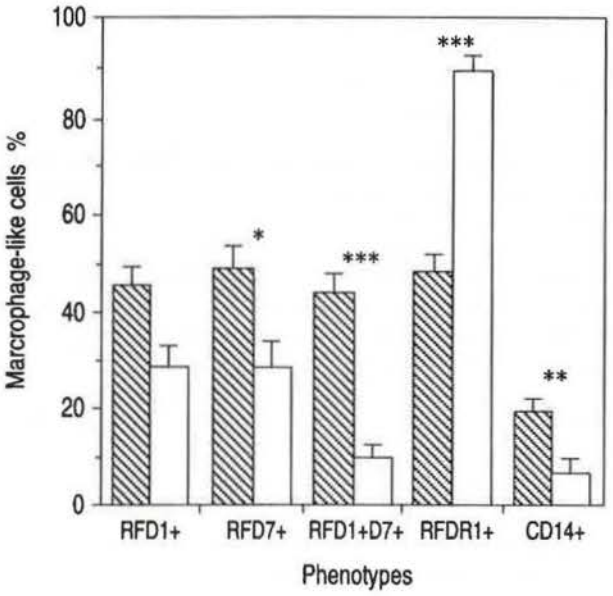

Fig. 1. – Using the immunoperoxidase technique, macrophage-like cells in bronchoalveolar lavage were assessed for expression of D1, D7, CD14 and human leucocyte antigen-DR (HLA-DR) (RFDR1) molecules. ▨: patients with pneumonitis; □: normals. Data are presented as mean and SEM. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

RFDR1+ compared with 87.7 (2.7)% in normal volunteers ($p<0.001$).

Relationship of macrophage populations to diagnosis

There was a significant decrease in the total numbers of cells recovered in BALF from those patients with CMV associated pneumonitis, compared with those in whom no CMV was detected (table 3). Patients with CMV in lavage fluid, however, had higher proportions of MLC than other patients, but this difference was not significant. Patients with CMV associated pneumonitis did, however, have significantly lower proportions of monocytes in the lavage fluid, as demonstrated by the marker CD14 ($p<0.05$). There was also a significant difference between DR expression between the CMV+ and CMV- groups: 39.0 (4.7)% of cells were positive for RFDR1 in the CMV+ group, compared with 53.2 (4.7)% in the CMV- group; $p<0.05$). There was no relationship between diagnosis and proportions of other macrophage markers used.

There was also no relationship between each different subset of MLC identified and proportion of total lymphocytes or CD8+ cells present in BALF (data not shown).

Relationship of cell populations to survival

Five patients died from an initial episode of pulmonary disease, whilst 11 patients survived. However, three of the survivors later died following a second episode. There was no significant difference between the total cell counts or proportion and number of macrophages between each group (table 4). The expression of HLA-DR, however, which was reduced on macrophages in the transplant group as a whole (*vide supra*), was significantly lower still in the patients who died (mean 40.4 (4.7)% of macrophages) compared with those who survived (52 (4.5)%) ($p<0.05$). None of the other macrophage markers used showed any differences between these two groups.

Time interval after transplantation: relationship to macrophage population and survival

The immunocytochemical profile of BALF mononuclear cells was related to the interval following transplantation, in order to see whether the cells seen simply reflected the recovery of the engrafted bone marrow. There was no correlation between time from transplant that pneumonitis developed and individual subsets of macrophages found in BALF. The time from transplant when pneumonitis developed also bore no relationship to survival. Those patients who survived their pneumonitis became ill 108 days (median) (range 44–357 days) after transplant, whilst those who died developed pneumonitis at 93.5 days (median) (range 45 days to 2 yrs).

Relationship of recovery of peripheral white cell count and development of pneumonitis

Peripheral white cell counts appeared at a level of 1.0×10^9 cells·l^{-1} between days 11–42 (median 22 days). The mean total white cell count in the patients studied was 3.5×10^9 cells·l^{-1}. Six patients had normal total peripheral white cell counts ($4–10\times10^9$ cells·l^{-1}). The remaining patients had counts between 1.0 and 4.0×10^9 cells·l^{-1}. It was noticeable that none of the 16 patients developed pneumonitis until there was evidence of engraftment and the peripheral white cell counts were recovering and approaching normal levels.

Table 3. – Cell studies in BMT recipients with pneumonitis: with and without CMV in the lung

Cells	CMV+ n=9	CMV- n=10
Total cell counts $\times10^5$·ml^{-1}	3.2 (0.7)	10.4 (2.8)*
RFD1+ % macrophages	40.0 (7.0)	47.1 (7.2)
RFD7+ % macrophages	54.0 (6.0)	40.2 (4.6)
RFD1+D7+ % macrophages	42.4 (7.1)	41.9 (5.7)
CD14+ % macrophages	13.0 (2.3)	21.8 (2.5)*
RFDR1+ % macrophages	39.0 (4.7)	53.2 (4.7)*

Data are presented as mean and SEM in parenthesis. *: $p<0.05$ by Mann Whitney U-test. n values are number of episodes in 16 patients (see table 1). BMT: bone marrow transplant; CMV: cytomegalovirus.

Table 4. – Cell studies in BMT recipients with pneumonitis: relationship to survival

Cells	Survived	Died
Total cell counts $\times10^5$·ml^{-1}	9.5 (3.3)	6.8 (3.0)
RFD1+ % macrophages	48.0 (5.7)	42.0 (9.0)
RFD7+ % macrophages	45.0 (5.7)	52.7 (5.4)
RFD1+D7+ % macrophages	41.7 (5.7)	42.0 (6.2)
CD14+ % macrophages	16.0 (2.1)	20.6 (4.2)
RFDR1+ % macrophages	52.0 (4.5)	40.4 (4.7)*

Data are presented as mean and SEM in parenthesis. *: $p<0.05$ by Mann Whitney U-test. BMT: bone marrow transplant.

Discussion

This study demonstrates a number of interesting abnormalities in the population of MLC found in the lungs of patients with pneumonitis following BMT, compared with normals. Although ideally these results should be compared with results from BMT recipients without pneumonitis, it was not considered ethical to bronchoscope asymptomatic patients. Normal volunteers were, therefore, used as controls.

Firstly, a greater proportion of cells bearing the monocyte marker CD14 were present, compared with normal. Secondly, in addition to mature tissue macrophages (RFD7+) and interdigitating cells (RFD1+), a significant proportion of cells expressed both RFD7 and RFD1 markers. Finally, significantly reduced numbers of cells expressing DR were found in the BMT recipients. This abnormality was greater in patients who died from pneumonitis compared with

those who survived, suggesting that the ability of alveolar macrophages to present antigen is important for survival in this group. Some of these results differ from those of BRAY *et al.* [20], but this earlier study included a mixture of cases with solid organ transplants, where patients had also received different pretransplant conditioning. Furthermore, unlike the present study, 50% of the transplant recipients reported by BRAY *et al.* [20] were smokers.

The high proportion of monocytes recovered by BAL from the patients with pneumonitis is consistent with a state of acute or chronic lung injury, when more monocytes cross the alveolar wall [21, 22]. These data suggest that, despite systemic immunosuppression, patients with pneumonitis following BMT are able to recruit monocytes to the lung. This may contribute to the pathology of pneumonitis. Monocytes accumulate in the lungs of patients with cryptogenic fibrosing alveolitis [23], and sarcoidosis [10, 24]. In sarcoidosis, it has been suggested that the influx of monocytes results from the release of chemotactic factors by activated CD4+ lymphocytes [25]. Indeed, the percentage of monocytes recovered from the lungs may be directly proportional to the number of T-cells present [10]. A more recent report, however, casts doubt on this association [24]. Similarly, no correlation between proportions of monocytes and lymphocytes was found in the present study. BARBOSA *et al.* [24] also demonstrated an accumulation of monocytic cells in the lungs of patients with tumours of diverse histology. Thus, monocytic infiltration could either be a general response to lung disease or could be involved in the pathology. This issue is not resolved here.

Alveolar macrophage function remains abnormal during the first four months post-transplantation [26]. In this study, proportions of cells of both the mature macrophage phenotype (RFD7+), and the dendritic cell phenotype (RFD1+), were increased in lavage fluid from the BMT recipients when compared with normal. This expansion was generally accounted for by a significant increase in the macrophage-like cells expressing both these markers. Around 40% of macrophages became RFD1+RFD7+. These cells which express positivity with both monoclonal antibodies have also been demonstrated in the lungs of patients with sarcoidosis and cryptogenic fibrosing alveolitis [11, 27, 28]. These cells may be exerting a suppresser influence on immune reactivity, and could be the equivalent of the suppresser macrophages reported in studies on rat lavage cells [29]. Indeed, suppressive activity has been well described for alveolar macrophages [30–33], and such suppression has been postulated to "down regulate" immune responses in the alveoli [34, 35]. Furthermore, AINSLIE *et al.* [36] found decreased levels of activation markers on CD4+ T-lymphocytes with increased proportions of RFD1+RFD7+ macrophages in sarcoid lavage.

The transition from recipient to donor phenotype for alveolar macrophages takes up to three months [5]. Thus, it is likely that the MLC reported in this study represent a mixture of donor and recipient HLA phenotypes. The relative proportions of these cells would be difficult to determine, except in sex mismatched transplants.

Only 40–50% of the total macrophages present in BALF from the transplant recipients expressed the DR antigen compared with 88% in normals. This proportion was even smaller in patients who died, compared with those who survived. Thus, alveolar macrophages in these patients may have a reduced ability to present antigen, and this could be important for survival. BRAY *et al.* [20] also found reduced DR expression on alveolar macrophages in patients with the acquired immune deficiency syndrome (AIDS). Reduced ability of MLC to present antigen in the lung could be responsible for increased susceptibility to opportunistic pulmonary infections. In the absence of class II+ accessory cells, T-cells fail to respond to viruses and other antigens [37], and it is possible from the results presented here that CMV itself suppresses DR expression. CMV down regulates Class I expression on the surface of infected cells but cytoplasmic expression is increased [38]. About half of the patients in the present study had CMV present in their lungs, and the proportion of macrophages expressing the DR antigen was lower in this group. Human CMV is able to infect monocytes and suppress antigen presentation [39].

Specific antigenic or mitogenic stimulation of lymphocytes normally results in the production of γ-interferon, which in turn induces Class I or Class II histocompatibility molecules on macrophages [40]. Stimulation with γ-interferon *in vitro* also enhances anti-Candida activity of alveolar macrophages [41]. In previous studies of BMT recipients with pneumonitis, however, no γ-interferon could be detected in BALF [42]. This may provide some explanation for the increased mortality associated with reduced DR expression on lung macrophages in this patient group.

In conclusion, this study demonstrates the emergence of abnormal populations of MLC within the lungs of patients with pneumonitis following BMT, with raised proportions of monocytes and doubly labelled cells, and reduced proportions of macrophages exhibiting the DR antigen. This latter abnormality appeared to be associated with increased mortality, and could represent a significant factor in the increased susceptibility of BMT recipients to opportunistic pulmonary infections.

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