Enrichment and characterization of dendritic cells from human bronchoalveolar lavages

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SUMMARY

In the present study about 0·3% to 1·6% of human bronchoalveolar lavage (BAL) cells were identified as typical dendritic cells (DC), having an irregular outline, lobulated nucleus, and clear distinguishable acid phosphatase activity or EBM11 (anti-CD68) reactivity in a spot near the nucleus. After DC enrichment, using transient adherence to plastic, FcR-panning, and a density metrizamide gradient, a population containing 7–8% typical DC was obtained. This DC-enriched low density fraction, containing the highest percentages of DC, very strongly induced T cell proliferation in an allogeneic mixed leucocyte reaction (MLR), which was significantly higher than that induced by other partly (un)fractionated BAL cells. These data indicate that DC seem to be the major accessory cells in the BAL fluid, and therefore may be important in the regulation of T cell immune responses in the lung.

Keywords pulmonary immune responses   mixed leucocyte reaction   dendritic cells alveolar macrophages   bronchoalveolar lavages

INTRODUCTION

The majority of studies with human bronchoalveolar lavage (BAL) cells of normal subjects showed that alveolar macrophages (AM) generally function poorly as antigen-presenting cells in T cell responses compared with blood monocytes or macrophages from other tissues [1–4], although a high percentage of human AM express HLA-D region antigens [5–8]. The AM may even suppress T cell responses, as shown particularly at high AM/T cell ratios [2,9–12].

In the rat system we demonstrated that in the steady state low numbers of dendritic cells (DC) can be found in the BAL fluid. Induction of an inflammatory response resulted in an increase of monocyte-like cells and DC in the BAL fluid [13]. These DC were potent stimulators in T cell proliferation assays *in vitro*. Moreover, *in vivo* studies showed that antigen-pulsed DC, but not AM, were capable of priming antigen-specific T cells in the draining lymph nodes of the lung after intratracheal instillation [14]. This underlines the functional importance of DC present in the BAL.

Inflammatory processes may alter the antigen-presenting capacity of AM, as was implied for AM from patients with pulmonary sarcoidosis, which have increased antigen-presenting capacity [1,4,8]. Poulter [15] reported that within the BAL of patients with pulmonary sarcoidosis increased numbers of RFD1+/RFD7− cells, a dendritic cell-associated phenotype, were found. However, identification of human DC can be difficult, and is hampered by the absence of a specific DC marker.

In our laboratory we use the following morphological and phenotypical criteria to identify DC: (i) typical dendritic processes; (ii) strong MHC class II expression; and (iii) clear distinguishable acid phosphatase activity [16,17] located in a juxtanuclear position. The latter criterion may be replaced by EBM11 reactivity in the juxtanuclear position, as recently shown [18]. Induction of allogeneic T cell proliferations is used to show the potent stimulatory capacity [19–22]. Using these criteria we have identified DC in the human BAL. A partially DC-enriched fraction showed the most potent capacity for stimulating allogeneic T cells.

MATERIALS AND METHODS

Samples

BAL fluid cells were obtained from six patients, lavaged because of suspected sarcoidosis. This was done with confirmed consent according to a protocol approved by the local Ethical Committee. Details are given in Table 1.

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Table 1. Clinical characteristics of the patients

Patient*	Age	Clinical characteristics
1	42	Increased numbers of T cells and neutrophilic granulocytes; no final diagnosis
2	23	Increased numbers of T cells; increased CD4/CD8 ratio; tuberculosis
3	39	Increased numbers of T cells and neutrophilic granulocytes; lymphoproliferative disease
4	29	Increased numbers of T cells; increased CD4/CD8 ratio; active alveolitis; pulmonary sarcoidosis
5	64	Increased numbers of T cells; active alveolitis
6	29	Increased numbers of T cells (CD4$^+$ T cells); increased CD4/CD8 ratio; active alveolitis, pulmonary sarcoidosis

* All male; all patients had increased numbers of bronchoalveolar lavage (BAL) cells; patients 1, 3 and 5 were smokers.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed after premedication with thiazinamium and local anaesthesia with lidocaine, 2% spray. The bronchoscope was placed in a wedge position and four aliquots of 50 ml sterile saline were infused and immediately aspirated into either polypropylene or siliconized bottles. The BAL cells were washed twice with PBS and resuspended in complete medium which comprised: RPMI medium (Flow Labs, Irvine, UK) supplemented with 20 mM glutamine, penicillin 50 U/ml, gentamycin 60 μg/ml and 10% heat-inactivated fetal calf serum (FCS) (all from GIBCO, Paisley, UK). For culture, complete medium was supplemented with 50 μM β-mercapthoethanol (Merck, Darmstadt, Germany).

Enrichment of DC from BAL fluid

The DC enrichment procedure was done according to Betjes *et al.* [23]. Briefly, the BAL cells were separated into an adherent cell (AC) and a non-adherent cell (NAC) population after overnight culture on plastic tissue culture Petri dishes (Greiner, Alphen a.d. Rijn, The Netherlands) at 37°C in a humidified 5% CO$_2$ atmosphere.

The NAC cell population was depleted of Fc-receptor-positive cells by two consecutive pannings with human IgG (Organon Teknika Corp., West Chester, PA). The non-adherent cells after the second panning were designated as FcR⁻ population.

A metrizamide gradient (14·5 g Nycodenz dissolved in 100 ml RPMI 1640 medium; Nycodenz; Nycomed As, Oslo, Norway) was performed to further enrich the FcR⁻ population for DC. Both the high-density fraction (HiD) and the low-density fraction (LoD) were collected. As a control, a part of the BAL cells were cultured overnight in Teflon beakers (37°C, 5% CO$_2$) (cBAL). Cell viability, determined by trypan blue exclusion, was low in the AC (61±14%) and HiD (70±16%) fractions, whereas for the other fractions viability was about 80%.

All cell fractions were tested for their accessory function in a range of concentrations using an allogeneic mixed leucocyte reaction (MLR). Cytocentrifuge preparations were made of all populations and evaluated by immuno- and enzymecytochemistry.

Preparation of T lymphocytes

Human T lymphocytes for the allogeneic MLR were isolated from one buffy coat according to Betjes *et al.* [23]. Briefly, after 16 h of culture, non-adherent cells were separated into sheep erythrocyte-rosette-positive (E$^+$) and erythrocyte-rosette-negative fractions by separation over Ficoll–Hipaque. The E$^+$ cells were >95% CD2$^+$ and were cryopreserved in medium containing 50% complete medium, 10% dimethyl sulphoxide (J. T. Baker B. V., Deventer, Holland) and 40% FCS, in liquid nitrogen until use. Cell viability of the enriched T cell population was above 95% as determined by trypan blue exclusion.

Allogeneic MLR

The MLR was used to monitor the accessory function of the different cell fractions, serving as stimulator cells. These stimulator cells were irradiated with 30 Gy (^{60}Co). The MLR was done according to Mottolese *et al.* [24]. The stimulator cells were added in graded doses to 5×10^4 T lymphocytes in 96-well round-bottomed plates (Greiner) in a final volume of 200 μl. T cell proliferation was demonstrated by ^{3}H-thymidine (^{3}H-TdR, Amersham, Aylesbury, UK) incorporation after pulsing the cells for 16 h with 1 μCi ^{3}H-TdR (0·05 μg total thymidine/ml) at day six. Data, mean values of triplicate wells, are expressed in ct/min.

Immuno- and enzymecytochemistry

Antigens on (un)fractionated BAL cells were detected by either an immunoenzymatic labelling using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes; Dakopatts, Glostrup, Denmark) according to Li *et al.* [25] or an immunoperoxidase method using diaminobenzidinetetrahydrochloride (Sigma Chemical Co., St Louis, MO) as substrate as described in Havenith *et al.* [13]. The MoAbs 9.3F10 (anti-HLA-DR/DQ; ATCC; [26]) and EBM11 (anti-CD68; Dakopatts; [27]) were used. Cytocentrifuge preparations were air dried and stored at −20°C until staining. Acid phosphatase activity (APh) was demonstrated according to Burstone [28] with naphtol AS-BI phosphate (Sigma) as substrate and hexazotized pararosaniline as diazoniumsalt (90 min at 37°C). Control preparations showed no staining with an irrelevant antibody. A minimum of 300 cells were examined for the presence of the different antigens.

Statistical analysis

Data were expressed in mean±s.d.; where this is done differently this is reported in the text. The Wilcoxon test was used for statistical analysis [29].

RESULTS

Morphological and cytochemical characterization of BAL cells

Alveolar macrophages, DC and monocyte-like cells could be distinguished based on morphology, the MHC class II antigen expression combined with acid phosphatase activity and the staining pattern of the MoAb EBM11 (anti-CD68). Most of the round nucleated AM expressed class II antigens on the surface and had APh activity, throughout the whole cytoplasm. The MoAb EBM11 also stained throughout the whole cytoplasm. Typical DC were found with an irregular outline, an eccentric,

Table 2. Cellular differentiation of bronchoalveolar lavage (BAL) fluid cells and the different fractions based on MHC class II/acid phosphatase activity (APh) staining

Fractions*	AM	Mo	DC	Lymphocytes	Neutr
cBAL	81·3 (13·1)†	1·8 (0·4)	0·3 (0·4)	15·5 (12·7)	1·0 (0·7)
AC	82·0 (12·5)	1·2 (0·4)	0·4 (0·5)	16·2 (12·0)	0·2 (0·4)
NAC	58·8 (31·9)	2·0 (0·2)	0·6 (0·5)	38·2 (29·0)	0·4 (0·5)
FcR⁻	63·8 (24·8)	2·0 (1·1)	2·2 (3·9)	31·8 (27·5)	0·2 (0·4)
HiD	63·2 (26·6)	0·5 (0·5)	0·8 (0·7)	35·5 (26·5)	0·0 (0·0)
LoD	58·0 (18·1)	7·8 (2·5)	7·2 (7·5)	26·8 (21·9)	0·2 (0·4)

* Fractions: cBAL, cultured bronchoalveolar lavage cells in Teflon beakers; AC, five adherent BAL cells; NAC, non-adherent BAL cells; FcR⁻, FcR-positive depleted six cells; HiD, high-density fraction; LoD, low-density fraction.
† Mean percentages (s.d.); $n = 6$.
AM, Alveolar macrophages; Mo, monocyte-like cells; DC, dendritic cells; Neutr, neutrophilic granulocytes.

Table 3. Cellular differentiation of bronchoalveolar lavage (BAL) fluid cells and the different fractions based on EBM11 staining

Fractions*	AM	Mo	DC	Lymphocytes	Neutr
cBAL	76·2 (17·2)†	3·6 (2·0)	1·6 (1·9)	17·6 (17·4)	1·0 (0·6)
AC	74·8 (19·2)	2·2 (1·8)	1·6 (1·3)	21·2 (18·5)	0·2 (0·4)
NAC	65·3 (25·1)	3·7 (1·6)	1·9 (1·1)	28·6 (25·0)	0·6 (1·0)
FcR⁻	64·3 (27·1)	3·6 (2·1)	2·1 (1·3)	29·8 (28·7)	0·0 (0·0)
HiD	62·8 (26·4)	4·1 (2·4)	1·6 (0·9)	33·3 (28·2)	0·2 (0·4)
LoD	62·5 (18·4)	9·9 (0·7)	7·8 (2·8)	19·8 (19·7)	0·0 (0·4)

* Fractions: cBAL, cultured BAL cells in Teflon beakers; AC, adherent BAL cells; NAC, non-adherent BAL cells; FcR⁻, FcR-positive depleted cells; HiD, high-density fraction; LoD, low-density fraction.
† Mean percentages (s.d.); $n = 6$.
AM, Alveolar macrophages; Mo, monocyte-like cells; DC, dendritic cells; Neutr, neutrophilic granulocytes.
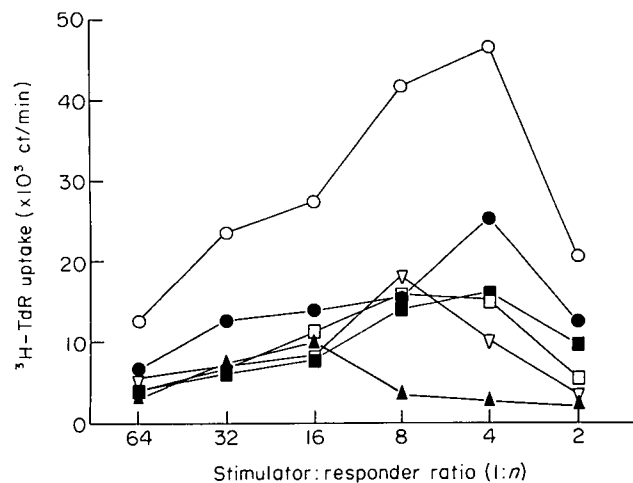
Fig. 1. Allogeneic T cell responses induced by the different cell fractions obtained by the dendritic cell (DC) enrichment procedure of the bronchoalveolar lavage (BAL). The median of six separate experiments (BAL cells of six different patients) of the different cell fractions of BAL cells are shown. ■, cBAL, overnight cultured BAL cells in Teflon beakers; ▲, AC, adherent BAL cells; ▽, NAC, non-adherent BAL cells; ●, FcR⁻, FcR-positive cell-depleted cells; □, HiD, high-density fraction; ○, LoD, low-density fraction. All experiments were performed in triplicate with s.e.m. <15% of the mean. The purified blood T lymphocytes, cultured for 6 days alone, gave an incorporation of ^{3}H-TdR of 770 ± 440 ct/min.

lobulated nucleus and clearly distinguishable acid phosphatase activity or EBM11 reactivity in a spot near the nucleus. Monocyte-like cells, with a bean-shaped to lobulated nucleus and only little of EBM11 staining or APh activity in the cytoplasm, could be distinguished.

Enrichment of DC from BAL fluid

On the basis of MHC class II expression combined with APh activity, on average 0·3% of the cultured BAL cells could be identified as DC (Table 2). The EBM11 staining showed on average 1·6% of DC in the cultured BAL cells (Table 3). It was possible to enrich DC up to about 7–8%, if countings were performed based on either MHC class II/APh or EBM11 staining (Tables 2 and 3).

The cell recovery after overnight culture was about 55%. On average 25 ± 15% of the BAL cells adhered to plastic. More than 80% of the DC from the BAL cells were recovered in the NAC fraction. On average 30 ± 13% of the cells in the NAC fraction were removed by FcR⁻ panning. Panning for FcR did not efficiently remove AM, because after panning no differences in cell composition between the NAC and FcR⁻ fraction were found (Tables 2 and 3). A metrizamide gradient was required for further enrichment of the DC from the BAL cells. After this procedure $21 \pm 7·9$% of the cells loaded on the gradient was recovered from the interphase, giving a LoD fraction which contained about 7–8% DC (Tables 2 and 3).

Capability of the fractions to induce an allogeneic MLR

The DC-enriched LoD fraction very strongly induced T cell proliferation in the allogeneic MLR. At all stimulator to responder ratios tested the ^{3}H-TdR incorporation of the T cells induced by LoD fraction was significantly higher than induced by the other fractions (Fig. 1) ($P < 0.05$). T cell responses became partially and in some cases even totally suppressed at higher numbers of stimulator cells added (Fig. 1).

DISCUSSION

In the present study about 0·3–1·6% of the human BAL cells were identified as typical DC, having an irregular outline, an eccentric, lobular nucleus, and clear distinguishable APh activity or EBM11 reactivity in a spot near the nucleus. A population with about 7–8% typical DC was obtained after enrichment. The DC-enriched fraction most efficiently induced T cell proliferation in an allogeneic MLR, compared with the other (un)fractionated BAL populations.

A major problem in the DC enrichment was the removal of the AM. In accordance with the literature [23,19–21,30], DC were mainly present in the non-adherent fraction. However, transient adherence to plastic and panning for FcR did not significantly reduce the number of AM. After the metrizamide gradient a successful further enrichment was obtained for DC, although the contamination with AM was still relatively high. Attempts undertaken to eliminate the AM with other techniques (e.g. immunoglobulin-coated dynabeads or silica; data not shown) did not give any improvement.

Taking into account that about 7% of the DC-enriched LoD fraction are DC, we found that at ratios of about 1 DC:1000 T cells, an MLR can still be induced. These results are similar to those of Steinman *et al.* [31], who showed that at DC to T cell ratios as low as 1:1000, an allogeneic MLR can be induced. The contaminating, irradiated lymphocytes in the different fractions probably do not play a significant role in inducing resting T cells to proliferate in an MLR [32–34]. Although the presentation or modulating capacity [35,36] of AM can not be ruled out, our findings strongly suggest that the DC present in the BAL fluid of these patients play an important role in initiating immune responses.

In the rat system after an inflammatory response an increase of monocyte-like cells and DC can be found [13]. Similar to this, in the human system monocyte-like cells, with a bean-shaped nucleus and only little EBM11 staining or APh activity, were found. These monocyte-like cells were enriched, together with the DC, in the LoD fraction.

As already stated above, the exact role of AM in an allogeneic MLR and in antigen-specific T cell responses is unclear. AM from patients with sarcoidosis are more powerful accessory cells, and several possibilities have been considered for this enhanced antigen-presenting capacity, e.g. increased HLA-DQ, DP and DR expression on AM [5,7], increased IL-1 and interferon-gamma (IFN-γ) production [37,38], differences in density of adhesion molecules [39], or increased recruitment of monocytes into the lung [5,40]. Spiteri & Poulter [15,41] were the first to show with MoAbs that in normal human BAL cells a potent stimulator cell population (about 10–30% RFD1$^+$/RFD7$^-$) for MLR responses could be found in the non-adherent fraction. In sarcoidosis the RFD1$^+$/RFD7$^-$ cell population was increased [15]. Whether this RFD1$^+$/RFD7$^-$

subpopulation consists totally of DC, according to our definitions, remains unclear.

High numbers of lavage cells were found in the relatively small group of patients used in this study, suggesting that inflammatory processes were present. Because no normal subjects have been studied, we can not exclude the possibility that due to the inflammatory process the numbers of DC are increased.

Taken together, we conclude that DC, defined according to our criteria and present in relatively small numbers in the human BAL, seem to be the major accessory cell in BAL fluid. Therefore DC in human BAL may play an important role in the regulation of T cell immune responses, as has already been shown in the rat system *in vivo* [14].

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