Eur Respir J
1992, 5, 717–726

Isolation of phenotypically and functionally distinct macrophage subpopulations from human bronchoalveolar lavage

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ABSTRACT: Bronchoalveolar lavage was used to obtain alveolar macrophages (AM) from the lower respiratory tract of healthy normal volunteers. Monoclonal antibody (MoAb) probes specific against macrophage determinants were then applied, in conjunction with density separation techniques, to identify and isolate three relatively homogeneous subpopulations from the AM pool. The MoAbs used, RFD1 and RFD7, have previously been shown to differentiate between "dendritic" cells and mature macrophages, respectively, in normal tissue.

In addition to these two phenotypically distinct AM subsets (RFD1+D7- and RFD1-D7+ AM), a third AM subpopulation was isolated, which appeared to express both markers (RFD1+D7+). All three separated macrophage subsets were morphologically similar but exhibited distinct differences in surface receptor expression, enzyme content and physiology. Isolated RFD1+D7- AM (the phenotype of "dendritic" cells) did not adhere to the glass, had weak expression of C3b and FcR1 receptors, low fibronectin content and lysosomal activity; only a small proportion of these cells exhibited phagocytosis. The other two isolated AM subsets adhered to glass, expressed C3b and FcR1 receptors, had high fibronectin and acid phosphatase content, and a large majority exhibited phagocytic capacity; qualitative and quantitative differences in these features existed between the two AM subtypes. Furthermore, a diverse spectrum of hexose monophosphate shunt activity was observed throughout all three AM subpopulations, with the highest activity being recorded in the non-adherent AM.

These data support the concept of a dynamic heterogeneity within the AM population. The variation in surface antigen expression and physiological capabilities observed amongst the three isolated AM subsets implies the presence of functionally distinct AM within the human lung, which, during steady-state conditions, may be critically balanced under the influence of stimuli in their local microenvironment. In support, proportional and functional shifts have been witnessed amongst these three AM subpopulations with the advent of disease.

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Keywords: Distinct phenotype and physiology
human alveolar macrophage subpopulations
isolation

Received: July 30 1991
Accepted after revision February 26 1992

This work was supported by a grant from the fellowship of postgraduate Medicine to MAS and a grant from the British Lung Foundation to LWP.

The macrophage has been observed to be involved in a dichotomy of roles in its defence of the local microenvironment, with which it closely interacts. This versatile character allows the macrophage to process and present antigens, and to secrete biologically active substances in order to set up specific immune responses. As a result, the macrophage itself becomes the target of a positive feedback loop through the production of macrophage-activation lymphokines, which heighten its capacity to ingest and terminate the offending stimulus. In the light of this, several reports have tried to elucidate whether the heterogeneity observed within the macrophage family stems from differences in the differential stage or activation state of a single highly dynamic macrophage-monocyte lineage, or the existence of multiple distinct macrophage-monocyte lineages. Animal experiments have shown that macrophage subpopulations do exist and that these arise from distinct bone marrow precursors [1]. As yet, however, there is no evidence to support the existence of similar multiple distinct macrophage-monocyte lineages in man. On the contrary, data from human studies support the view that the different functions observed within the macrophage family are performed by the same cell [2].

To solve this enigma, several workers have attempted to isolate distinct macrophage subpopulations by exploiting well-recognized differences in macrophage morphology [3], density [4], and expression of cell surface proteins [5], as well as motile capacity and immunological function [6–10]. In both animal and human studies the alveolar macrophage (AM) population has proved to be the most easily accessible for such studies by direct access through

bronchoalveolar lavage (BAL) or, indirectly, from lung tissue digests.

Anything from three [11] to 18 [12] bands of cells have been separated from BAL by the use of colloidal silica density gradients alone. However, with the possible exception of the relationship between so-called "dendritic" cells and mature phagocytes in light-density and dense bands, respectively [13], no established correlations exist between cell density and function. Furthermore, it is clearly impossible to use density alone to discriminate cells *in situ*. On the other hand, correlations between surface antigen expression (phenotype) and function in cells of the monocyte-macrophage lineage are well-documented [2, 14–18].

Therefore, there is clearly a need for improvement in the currently established techniques to enable isolation of homogeneous subpopulations of phenotypically distinct macrophages, which can then be characterized individually in sufficient detail to ascertain their functional role in the lung. By using monoclonal antibody (MoAb) probes that can distinguish "dendritic" cells (RFD1) and mature classic macrophages (RFD7) in normal tissue [19], three discrete subpop-ulations have already been identified within the AM pool of human subjects (RFD1+D7-, RFD1-D7+, RFD1+D7+) [20]. The possible significance of these subsets was revealed by the observation that their proportions within BAL altered dramatically with the advent of disease, and that T-cell responsiveness might be influenced by relative changes within this heterogeneous AM population [20, 21].

The aim of this study was to isolate these three phenotypically distinct macrophage subpopulations from normal BAL, using macrophage-specific MoAbs in conjunction with antibody-coated magnetic beads and density separation techniques. This separation allowed a detailed comparative characterization of each AM subpopulation.

Materials and methods

Subjects

A total of 10 normal healthy volunteers, all non-smokers, 8 males, 2 female, mean±SEM age 23±1.4 yrs, were recruited. All had normal chest radiographs and pulmonary function. None had a history of lung disease or any viral illness in the preceding two weeks. All subjects gave formal written consent; the study had received prior approval by the local Ethics Committee.

Recovery of AM by BAL

In each of the 10 subjects, BAL was performed using a 6 mm fibreoptic bronchoscope (Olympus model BF-IT20D) following intravenous premedication with 0.6 mg of atropine sulphate and 5–10 mg midazolam (Hypnovel; the relative dose depending on individual age and weight), 15 min prior to the procedure. The right middle lobe was anaesthetized with 2% lignocaine and lavaged with 20 ml aliquots of sterile buffered 0.9% isotonic saline to a total fluid volume of 180 ml. The lavage fluid was gently aspirated after each aliquot, and collected into a sterile siliconized glass bottle maintained at 4°C.

Processing of samples

The retrieved lavage fluid was filtered through a sterile single layer of loose cotton gauze to trap any gross mucus particles, and centrifuged at 480×g, at 4°C for 5 min. The cell pellet was then washed twice in RPMI 1640 medium (Flow Laboratories), counted in a modified Neubauer haemocytometer and viability assessed by cellular exclusion of trypan blue. The final cell concentration in each sample was adjusted to 1×10^6 cells·ml^{-1} using supplemented RPMI 1640 (containing 1.25% 200 mM L-glutamine, 10% heat inactivated foetal calf serum (FCS), 100 μg·ml^{-1} streptomycin and 100 IU·ml^{-1} penicillin).

Initial separation of macrophage subpopulations

The above cell suspension was plated onto sterile plastic 85 mm diameter tissue culture Petri dishes (Nunc, Denmark), with no more than a total of 6.0×10^6 cells on each, with a medium depth of 3 mm. These were incubated for 2 h at 37°C in an atmosphere of 5% humidified CO_2. The supernatant containing the non-adherent cell population was then collected and the plate washed three times with medium to remove any further non-adherent cells. The adherent cells were gently scraped off the plates using a sterile "rubber policeman". The adherent and non-adherent cell populations were centrifuged at 480×g, 4°C for 5 min, resuspended in medium at 1×10^6 cells·ml^{-1} and 2×10^6 cells·ml^{-1}, respectively, and kept on ice until required.

Preparation of materials for macrophage subset isolation

Neuraminidase treatment of sheep red blood cells (SRBC). SRBC were used in these studies for T-cell rosetting, which was facilitated by prior treatment of the SRBC with neuraminidase, which sloughs off any sialic acid residues present on these cells [22]. Neuraminidase, supplied as a salt-fractionated, dialysed and lypophilized powder (5.6 ml solid, Sigma Chemical Co.) was dissolved in 10 ml distilled water. The solution was then divided into 0.3 ml aliquots and stored at -20°C until further use. Four ml of 25% SRBC (Tissue Culture Service, Berkshire, UK) was washed three times in Hank's buffered saline solution. Following the last wash, the red cell pellet (about 1 ml packed volume) was resuspended in 10 ml RPMI

1640 to give a 10% SRBC solution; to which 0.3 ml of neuraminidase was added. This mixture was incubated for 45 min at 37°C, after which the cells were washed three times and finally resuspended in RPMI 1640 to obtain the original 10% SRBC concentration. The cells were then stored at 4°C (for not more than one week) until use. Before each experiment the supernatant of the SRBC suspension was checked for lysis, and the viability of the cells ascertained with trypan blue.

Conjugation of magnetic beads. The MoAb RFD1 (Immunoglobulin (Ig) Class) was conjugated onto M450 uncoated, non-activated, magnetic, polystyrene beads (Dynal) by physical adsorption. The RFD1 coated beads were then used for specific separation and rosetting of any RFD1 positive cells from the heterogeneous AM suspension. The purified boric acid pre-cipitated RFD1 antibody was dissolved in 0.2 M Sörenson phosphate buffer (prepared by adding 2.72 g KH_2PO_4 dissolved in 100 ml distilled water, to a 100 ml solution of 2.84 g Na_2HPO_4, until a pH of 7.5 was reached) in a concentration of 150 μg antibody·ml^{-1} of buffer. Five hundred microlitres of this solution was then added to an equal volume of a homogeneous suspension of M450 uncoated magnetic beads in a sterile Nunc freezing tube and mixed well. This mixture achieved a ratio of 75 μg antibody per 15 mg of beads, which was incubated for 24 h at room temperature by slow end-over-end rotation. The suspension was then transferred to a 10 ml conical tube which was placed for 10 min in a Dynal magnetic particle concentrator (MPC). With the tube still in the MPC, the supernatant was decanted and discarded, whilst the MoAb coated beads were collected and washed four times at 4°C in a buffer consisting of 0.1% (w/v) phosphate buffered saline with bovine serum albumin (PBS/BSA). The first two washings were performed by end-over-end rotation for 5 min each, whilst the final two were carried out over 30 min. The RFD1 coated beads were finally resuspended in 0.1% PBS/BSA to a concentration of 2×10^8 beads·ml^{-1} (30 mg·ml^{-1}) and stored at 4°C. For storage periods of greater than 2 weeks, 0.2% sodium azide was added as a bacteriostatic agent. Adequate binding of the MoAb to the beads was tested prior to use by a modified indirect immunoperoxidase method. Cytospins of the MoAb-bead suspension were incubated for one hour with a peroxidase conjugated rabbit antimouse immunoglobulin only. Presence of adequate MoAb conjugation to the beads was indicated by clear, dark, brownish staining at the bead surface.

Preparation of metrizamide. 14.5 g of metrizamide (MW 789, density 2.17 g·cm^{-3}) (Nycomed AS, Diagnostics, Norway) was dissolved in 100 ml Dutch modified RPMI 1640 (20 mM hydroethyl piperazine ethanesulphonic acid (HEPES) buffer, 1.0 g·l^{-1} sodium bicarbonate and 6.5 g·l^{-1} sodium chloride supplemented with 2 mM L-glutamine, 100 IU·ml^{-1} penicillin, 100 μg·ml^{-1} streptomycin and 10% FCS). The suspension was then divided into 2 ml aliquots in bijoux bottles, and stored, frozen at -20°C until use.

Isolation of distinct macrophage subsets

To each volume of the 2×10^6 cells·ml^{-1} non-adherent cell suspension, an equal volume of 1% (v/v) neuraminidase treated SRBC (Tissue Culture Services, Berkshire, UK) was added. This suspension was supplemented with 2.5% FCS (Sigma Chemical Co, St Louis, USA). This was then incubated for 30 min at 37°C in 5% humidified CO_2. Following incubation, the cell suspension was centrifuged at 450×g at 4°C for 5 min; then left on ice at 4°C with the supernatant intact for one hour. The cell pellet was then very gently resuspended and each 20 ml of the cell suspension carefully underlayed with 14 ml of a Ficoll-Hypaque gradient (Nycomed, Norway) using a large bore pipette. The preparation was centrifuged (650×g) at room temperature for 15 min, the resulting cell interface (free of E-rosetting cells) was harvested, washed twice, and finally resuspended in supplemented RPMI 1640 medium, labelled and kept on ice.

The adherent cell population was divided into two separate cell suspensions, each adjusted to 1×10^6 cells·ml^{-1}. To one cell suspension was added 50 μl magnetic beads (Dynabeads, Norway) conjugated to monoclonal antibody RFD1 (table 1) at 2×10^8 beads·ml^{-1}, giving a bead:cell ratio of 100:1. This bead/cell suspension was then made up to a volume of 5 ml with phosphate buffered saline containing 0.1% bovine serum albumin (v/v). This was allowed to stand on ice for 30 min (being gently agitated every 10 min). A magnet was then applied to the side of the tube for 10 min to separate out any cells bound to the antibody-conjugated beads. The supernatant was then carefully decanted into a clean tube, a further 50 μl of beads added and the incubation/magnetization steps repeated. The cells obtained in the supernatant were washed twice in medium, resuspended in supplemented RPMI 1640 and kept on ice.

Using 10 ml conical centrifuge tubes, 5 ml aliquots of the second adherent cell suspension (1×10^6 cells·ml^{-1}) were layered on a 2 ml metrizamide gradient (frozen metrizamide suspension was removed from -20°C prior to use, and allowed to equilibrate to room temperature unaided). This was spun at 650×g for 10 min at room temperature. The light density fraction was then removed, washed twice in medium resuspended in supplemented RPMI and kept on ice at 4°C.

The sequence of all steps used in isolating macrophage subsets is shown in figure 1.

Phenotype of separated cell populations

The homogeneity of each macrophage subpopulation obtained by the above techniques was assessed using monoclonal antibodies RFD1 and RFD7 [23] and analysed by immunocytological methods (see below).

Table 1. – Panel of MoAbs used in this study

Antibody	MW	Specificity	Source	References
RFDR1	28/33 kD	Identifies a framework epitope on the HLA-DR molecule	RFHSM	[23]
RFD1	28/33 kD	Identifies interdigitating cells and a small proportion of B-cells	RFHSM	[19]
RFD7	77 kD	Identifies mature phagocytic macrophages	RFHSM	[19]
RFD9	-	Identifies epithelioid cells and tingible body macrophages	RFHSM	[24]
EBM11 (CD68)	110 kD	Identifies all cells of the monocyte - macrophage lineage	Dakopatts, Denmark	[25]
UCHMI (CD14)	52 kD	Identifies antigen present on the majority of blood monocytes	PCL Beverley, University College, London	[26]
Anti C3b receptor	205 kD	Reacts with the receptor for the third component of human complement	Dakopatts, Denmark	[27]
Anti fibronectin	-	Reacts with fibronectin in human cells	Dakopatts Denmark	[28]
MoAb 10.1	71 kD	Reacts with Fc receptors, FcR1, on human mono-nuclear cells	N Hogg, Imperial Cancer Research Lab., London	[29]

RFHSM: Royal Free Hospital School of Medicine; CD: cluster designation; MoAb: monoclonal antibody; HLA-DR: human leucocyte antigen-DR.

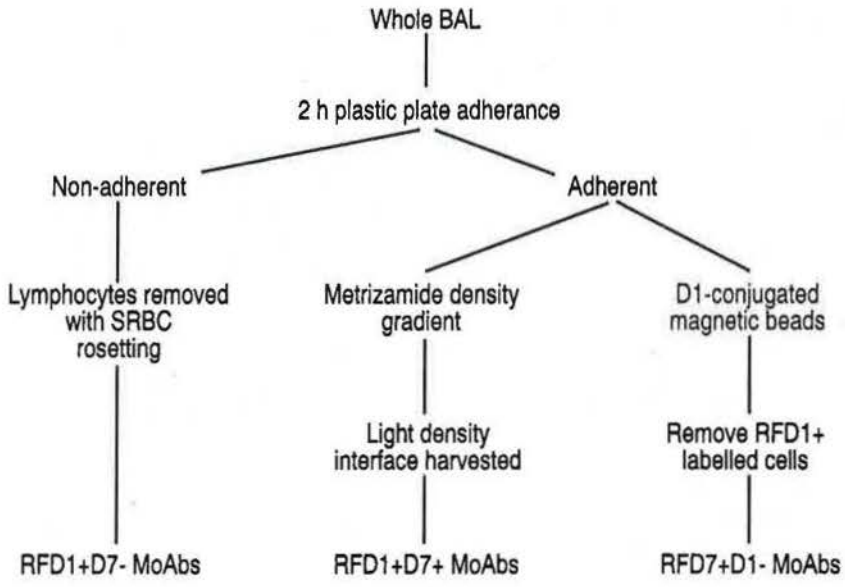

Fig. 1. – A schematic diagram of the methods used for separation of alveolar macrophage subpopulations from bronchoalveolar lavage. BAL: bronchoalveolar lavage; SRBC: sheep red blood cells; MoAbs: monoclonal antibodies.

The viability of the cells recovered by the above methods was assessed by trypan blue exclusion.

Cytospins were prepared on a Shandon Cytospin 2 using 100 µl aliquots of each of the above cell suspensions (2×10^5 cells·ml^{-1}). One cytospin from each sample was stained for morphology, whilst the remainder were air-dried for one hour at room temperature, fixed in 1:1 mixture of chloroform-acetone for 10 min, wrapped in plastic film and stored at -20°C until use.

Immunocytological analysis

The proportion of the three macrophage subsets in all unfractionated BAL samples was assessed using MoAbs RFD1 and RFD7. Other MoAb probes (table 1) [19, 23–29] were used to characterize the surface receptor features of the three macrophage subpopulations. Immunocytological analysis was carried out using the standard staining techniques of immunoperoxidase for single antigen expression [30], and immunofluorescence for double expression [31]. The latter incorporated Ig class-specific second layer reagents conjugated with fluorescein isothiocyanate (FITC) and tetra-methylrhodamine isothiocyanate (TRITC), respectively, (Southern Biotechnology, Birmingham, AL, USA). Background staining was identified by comparison with negative control cytospins on which the monoclonal antibody was omitted; positive specificity controls were always prepared using sections of human palatine tonsil. A Zeiss fluorescence microscope, equipped with epi-illumination and barrier filters appropriate for FITC and TRITC was used for recording RFD1+D7+ cells. Background fluorescence was identified using controls as described above. A total of at least 150 cells was counted in each cytospin, and the percentage of positive cells to the tested MoAbs recorded.

Lysosomal enzyme activity. Lysosomal enzyme activity in each of the isolated macrophage subpopulations was investigated using a standard histochemical reaction for acid phosphatase [32]. The reaction mixtures were prepared immediately prior to use. Two ml of 4% sodium nitrate in distilled water was mixed with 2 ml hexa[3] pararosaniline. Of this mixture 2 ml was added to 0.1 M acetate buffer at pH 5 and the solution was then gently stirred into 0.5 ml naphthol AS-BI phosphate. The appropriate cytospins were then incubated in the above mixture at 37°C for one hour and counterstained with haematoxylin. The percentage of total morphologically identifiable macrophages that gave a positive result in each test sample was read using an Olympus light microscope with high magnification (×600).

Hexose monophosphate shunt activity. The level of hexose monophosphate shunt activity (HMS) in each separated AM subset was determined by a histochemical method that identified glucose-6-phosphate dehyrogenase (G-6-PD) [33]. The reaction mixtures were prepared immediately prior to use. To 5 ml of glycyl glycine buffer (0.1 M; pH 8.0; warmed to 37°C) was added 15 mg glucose-6-phosphate disodium, 7.5 mg nicotinamide adenine dinucleotide phosphate (NADP) and 15 mg of nitroblue tetrazolium (NBT). A negative control mixture consisting of 3 mg NADP and 6 mg NBT measured into 2 ml glycyl glycine buffer was also prepared. Unfixed cytospins of each macrophage subpopulation were incubated with 50 µl of the reaction mixture and control mixture, respectively, for 90 min at 37°C.

Quantification of G-6-PD reactivity

The density of G-6-PD reactivity in each cell is reflected in the "reaction product" (*i.e.* the insoluble formazan) that precipitates following the reduction of the NBT salt (which acts as an H^+-acceptor). To facilitate the quantification of the amount of formazan deposited, use was made of a Vickers M85 scanning and integrating microdensitometer set at 585 nm, the isosbestic wavelength of the formazans of NBT [34] with a ×40 objective. The mask was positioned so that only one cell was within the masked area at each reading. One hundred consecutive readings of individual AM were made in each cytospin by a single observer (MAS) who completed all the recordings in the study repeat sets of measurements were conducted to a total of six cytospins from each separated macrophage subset. Each reading was recorded as relative density of "reaction product" per unit area of cell. The area of each cell was determined simultaneously by setting the threshold value of the microdensitometer to the background absorbance value.

Phagocytosis

Macrophages from each separated subpopulation (1×10^5 cells) were incubated with fluorescein-coated latex beads of 1.0 µm diameter (Polysciences, Northampton, UK) in a concentration of 100 beads·cell^{-1} for 2 h at 37°C in an atmosphere of 5% humidified CO_2. Control plates were set up with the inclusion of cytochalasin B (Imperial Chemical Industries Ltd, Alderly Park, UK) at a concentration of 2.0×10^{-5} M, prepared fresh in a solution of 0.2% dimethylsulphoxide to block phagocytosis [35]. This would facilitate differentiation of the cells that have actually phagocytosed the beads, from those in which the beads are just stuck to their surface. Following incubation, each cell suspension was washed twice to remove excess beads. Cytospin preparations were made and latex beads within the cells were identified by the presence of fluorescein on their surface using a Zeiss standard microscope fitted with epi-illumination and appropriate filters for FITC. A minimum of 100 cells was counted on each cytospin preparation, and the presence of five or more latex beads in a cell was considered to constitute phagocytosis.

Statistics

Quantitative data from the 10 subjects were expressed as the mean±SEM. Where appropriate, significance between results was determined by student's t-test for non-paired data.

Results

Using a standard technique for BAL in each subject, the mean percentage return of lavage fluid was 72.0±11.0% of the instilled volume.

Differential cell counts

The mean±SEM total BAL cell yield in the ten subjects was 9.0±1.4×10⁶ cells, of which the absolute number of AM was 8.3±1.0×10⁶ cells. The mean proportion of lymphocytes and polymorphonuclear cells was less than 9% and 2%, respectively.

The viability of unfractionated macrophage-like cells and separated AM subsets was >90% by trypan blue exclusion. All cells in the separated populations were morphologically identified as macrophages.

Immunocytological analysis

In the unfractionated lavage, 13.2±3.5% of the macrophages expressed positivity for RFD1, and 28.2±10.6% for RFD7. Only 6.0±1.5% of lavage macrophages expressed both these surface markers. The remaining AM were not identified by these two probes, but they all reacted with EBM11. Only 5% of the total unfractionated AM population were UCHM1 positive. The phenotypic homogeneity in each macrophage subset was over 85% in each case (table 2). Data of the phenotypic features of each AM subpopulation are summarized in table 3. The three isolated AM subsets exhibited distinct phenotypic features. Over 95% of each subpopulation was RFDR1 and EBM11 positive; but UCHM1 negative. Only 5% of RFD1+D7+ macrophage was positive to RFD9; the other cell types were negative.

Table 2. – Homogeneity of AM subsets isolated from 10 normal volunteers

Subset	RFD1+D7-	RFD1-D7+	RFD1+D7+
	97.0±2.41	90±4.16	97.4±1.60

The homogeneity is expressed as the mean±SE percentage of macrophage-like cells (within each isolated sample) that express positivity to the MoAb probe(s) that identify the appropriate AM subpopulation.

RFD1+ D7- cells had poor C3b and FcR1 receptor expression, and low fibronectin content, in contrast to the other two AM subsets. Fibronectin content was positively stronger in RFD1 +D7+ AM.

A minimal degree of intra-subset contamination did occur in some of the samples (<5% of all cells in each subset). A proportion of morphologically identifiable macrophages (a mean of 6.22±3.0%) was found in all the isolated subpopulation samples. These cells were EBM11+ UCHM1-, but unreactive to the specific macrophage probes.

Acid phosphatase reaction

Only 21.2±2.36% of RFD1+D7- cells were acid phosphatase positive; in contrast, lysosomal enzyme activity was significantly higher in both RFD1+D7+ (75.4±2.80%) and RFD1-D7+ macrophages (59.1±1.43%) ($p<0.001$).

Phagocytosis

Phagocytic capacity using fluorescent latex beads was observed in all three isolated AM subpopulations. 64.1±9.12% of RFD1-D7+ and 73.0±8.11% RFD1+ D7+ cells showed a significantly higher phagocytic capacity, when compared to only 39.3±4.01% of RFD1+D7- cells ($p<0.001$). Changes in cell morphology were accompanied by total inhibition of phagocytosis in those cultures containing cytochalasin B.

Hexose monophosphate shunt activity

The scatter of G-6-PD levels in each AM subpopulation is shown in figure 2. Analysis revealed a diverse spectrum of HMS activity. In all cases the high activity was observed in the RFD1+D7- AM (2.45±0.12). G-6-PD levels were minimal in RFD1-D7+ cells (0.83±0.08). HMS activity in RFD1+D7+AM was 1.19±0.35. In all test samples, the background control was 0.85±0.02.

Table 3. – Phenotypic features of macrophage subsets isolated from normal 10 volunteers

AM Subset#		RFDR1	RFD9	EBM11	UCHM1	Anti C3b	Anti FcR1	Anti fn
D1	Mean	98.3	<1	95.4	<1	<1	20.3	30.3
	SE	1.21		0.61			1.21	0.21
							weak*	weak*
D7	Mean	95.7	<1	98.2	<1	65.3	63.4	90.5
	SE	4.21		0.14		0.82	4.63	1.22
								weak*
D1D7	Mean	95.4	5.44	96.3	<1	35.7	85.3	95.3
	SE	3.51	1.12	0.21		0.45	1.24	3.41
								weak*

The above values represent the mean percentage and standard error (SE) of total morphologically identifiable macrophages within each separated AM subset that express positivity with the monoclonal antibodies RFDR1, RFD9, EBM11, UCHM1, anti-C3b and anti-Fc receptor (FcR1), and antifibronectin (fn). *: degree of the intensity of staining; # for clarity, D1= RFD1+D7- macrophages, D1D7=RFD1+D7+ macrophages; AM: alveolar macrophages.

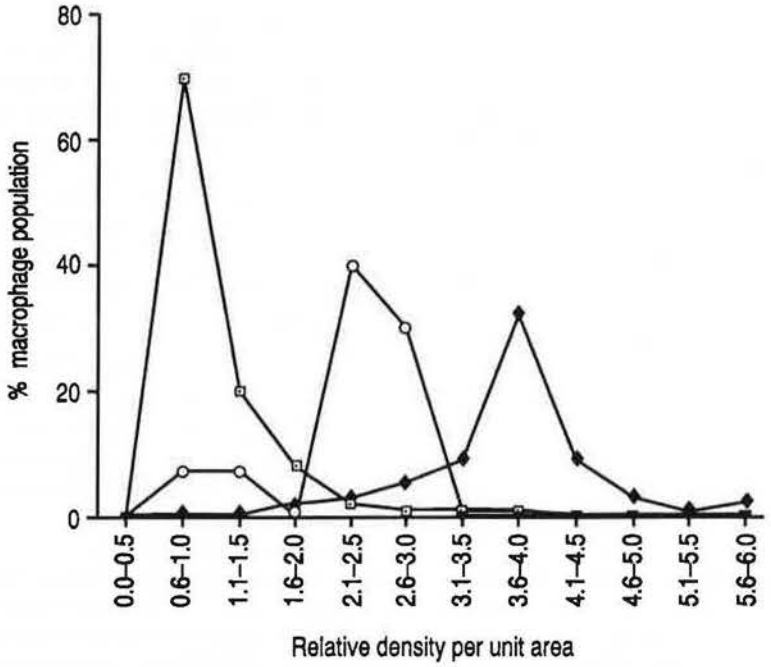

Fig. 2. – Hexose monophosphate shunt activity in each of the three isolated macrophage subsets. Each graph shows the percentage proportion of readings of relative density of reaction product per unit area for each macrophage subset that fell within selected limits on the bottom axis. —◆—: RFD1+D7-macrophages; —□—: RFD1-D7+macrophages; —○—: RFD1+D7+macrophages.

Discussion

This study has shown that phenotypically distinct macrophage subpopulations can be identified and isolated from BAL of the human lower respiratory tract. Although these cells share a similar macrophage-like morphology, they have been conclusively shown to exhibit a diverse spectrum of surface membrane receptors and physiological features. In addition, these distinct characteristics can be related to specific macrophage phenotype. In particular, a discrete macrophage subset can now be identified in human BAL, which reacts with both MoAb probes previously described as discriminating between "dendritic" cells and "classic" mature macrophages.

By utilizing specific macrophage probes in conjunction with plastic plate adherence, antibody-conjugated magnetic beads and metrizamide density gradients, we have developed a reproducible, novel approach that isolates relatively homogeneous subpopulations of AM from human BAL. Whilst we accept that our macrophage markers (RFD1 and RFD7) were only able to identify <50% of macrophages in the normal lavage, this supports their potential specificity. The significance of these identified AM subsets is reflected in their marked changes in proportion to the advent of disease [20, 21]. The two macrophage probes used have been extensively studied.

RFD1 recognizes a unique class II major histocompatability complex (MHC) cell membrane antigen related to human leucocyte antigen DR (HLA-DR) [17]. This observation is substantiated by its normal tissue distribution, which is limited within the macrophage-like cell family to the "dendritic" interdigitating cells of the T-cell zones of secondary lymphoid tissue. Whilst all RFD1+ cells are HLA-DR positive, not all HLA-DR cells co-express RFD1 (*e.g.* Langerhan's cells). This suggests that the RFD1 antibody recognizes an epitope with restricted expression, or an associated but distinct class II antigen as yet ill-defined. The functional role of the RFD1 epitope in antigen presentation is supported by studies, in which the addition of the RFD1 MoAb inhibited *in vitro* lymphocyte transformation [36].

RFD7 identifies a 77 kD cytoplasmic antigen, that is found in mature, acid phosphatase positive, phagocytic tissue macrophages. This antigen is not present in dendritic cells [17]. Studies of foetal tissue reveal that RFD1 and RFD7 antigens are separate in the developing foetus [23], and are mutually exclusive on cells in normal secondary lymphoid tissue, skin and cultured peripheral blood monocytes. Other observations note that when peripheral blood monocytes (RFD1-D7-) are cultured *in vitro,* two distinct populations of RFD1+D7- and RFD1-D7+ cells emerge [19].

A very small proportion of macrophages in normal BAL appear to express both antigens [20, 37]. Similar dual phenotype cells with macrophage-like morphology have also been identified within human colonic mucosa [38]. The functional significance of this dual antigen expression on macrophages, which appear to be situated at sites constantly exposed to environmental stimuli, has yet to be fully elucidated. It is, however, of particular interest to note that these RFD1+D7+ macrophages markedly increase in proportion in the presence of lung inflammation [20, 21].

The rationale behind our separation techniques essentially made use of well recognized properties of macrophages. By culturing on a plastic substrate, AM with the phenotype of "dendritic" cells (non-adherent) were easily separated from other macrophage-like cells present in BAL (adherent); associated lymphocytic cells being removed by standard SRBC rosetting. On the other hand, previous workers have demonstrated that the adherent AM population can be separated into subpopulations using a variety of density gradients [4, 9, 10]. It could, therefore, be assumed that the two identified adherent AM subpopulations in this current study (RFD1-D7+ and RFD1+D7+ macrophages) would have different densities; if the adherent AM obtained from BAL were to be passed through an appropriate density gradient, the heavier cells would precipitate out leaving the lighter of the two subsets at the interface. A number of different iodinated density gradient media have been used for isopyknic cell separation [39]. However, these hypertonic solutions can give rise to changes in cell volume which, in turn, affect the buoyant density of the cells to be separated [40, 41]. In contrast, the medium that we used (metrizamide) is completely non-ionic and for any given density its osmolarity is only one quarter that of its ionic counterparts [42]. Metrizamide has, thus, been repeatedly reported to be non-toxic to cells. Our study shows that following metrizamide density fractionation of adherent AM, the light interface is made up of double phenotype macrophages, with the heavier RFD1-D7+ cells forming a precipitate. These denser RFD1-D7+ AM can also be isolated from BAL by RFD1 conjugated magnetic beads. Other studies have also reported the separation of mononuclear cell subsets with immunomagnetic particles [43–45], but not from BAL. Such a method is preferred for its speed and preservation of cell viability.

A range of MoAb probes was used to analyse specific cell surface determinants on our isolated macrophage subsets. Whilst all cell subpopulations were EBM11 positive [25], none expressed positivity to UCHM1 [26]. This implies that the separated AM subpopulations were relatively mature, and had been resident for a time in the local environment from which they were obtained, rather than recently recruited from the circulating pool of blood monocytes. These results would suggest that, whilst a continual recruitment of monocytes occurs into the lungs, the characteristics of AM after they settle locally do not just occur by a simple differentiation from monocytes. Rather, as suggested by Riches and Hanson [46], the maturing macrophage adapts to its particular environment, within which stimuli may be present that could influence expression of unique phenotypes. Indeed, a wide variation in C3b and FcR1 receptor expression, fibronectin content and lysosomal enzyme activity was observed amongst our macrophage subsets. Such heterogeneity in AM characteristics has been observed previously, and reinforces the hypothesis that AM features can be influenced by local factors such as the lipid content in the alveolar lining [47].

As the homogeneity in each isolated macrophage subset was at best 97%, it could be argued that the observed phenotypic and physiological results were possibly influenced by contaminating cells. As intra-subset contamination was minimal, and other morphologically identifiable macrophages were EBM11+ UCHM1- (implying that they had not yet undergone full differentiation), we feel that the influence that this small proportion of cells could have on the overall results is negligible. Furthermore, a possible controversial observation in our study was that a proportion of cells with the phenotype of "dendritic" cells (RFD1+D7-) were as efficient as other macrophage types in phagocytosing inert latex beads. In fact, such phenomena have been observed in other studies where "dendritic" cells have been found to be capable of phagocytosing inert material as well as micro-organisms [48].

An important correlate that has emerged from the detailed analysis of these isolated AM subpopulations is that the observed spectrum of phenotypic and physiological features possibly reflects separate functional capabilities. In support of this hypothesis, parallel studies to the current one have conclusively shown these AM subpopulations to have distinct functional potential: whilst RFD1+D7- AM strongly support T-cell proliferation, RFD1+D7+ AM specifically suppress the induction of T-cell responses 18]. The presence of such functionally discrete AM subsets in the lung is supported by similar observations in animals [13]. In addition, Nicod *et al.* [49] have harvested loosely adherent macrophage-like cells from minced lungs and found them to be FcR negative and poorly phagocytic. Could these be similar to our RFD1+D7- AM isolated from BAL?

We conclude that under normal circumstances in man, the alveolar macrophage population is a dynamic cellular system comprising phenotypically and functionally distinct subpopulations, the delicate and critical balance of which produces an effective immunologically-mediated host defence in the lung. Of significant interest, the identification and isolation of these AM subsets can now be extended to the inflamed lung [20, 21]; future investigations should provide a better understanding of the possible influence of specific AM subpopulations on local T-cell function and *in situ* cytokine production in disease.

References

1. Bursuker I, Goldman R. – Distinct bone marrow precursors for mononuclear phagocytes expressing high and low 5'-nucleotidase activity. *J Cell Physiol*, 1982; 112: 237–243.

2. Hofman FM, Lopez D, Husmann L, Meyer PR, Taylor CR. – Heterogenicity of macrophage subpopulations in human lymphoid tissue and peripheral blood. *Cell Immunol*, 1984; 88: 61–74.

3. Nakstad B, Lyberg T, Skorten F, Boye N. – Subpopulations of human lung alveolar macrophages: ultrastructural feature. *Ultrastruct Pathol*, 1989; 13: 1–13.

4. Zwilling BS, Campolito LB, Reiches NA. – Alveolar macrophage subpopulations identified by differential centrifugation on a discontinuous albumin density gradient. *Am Rev Respir Dis*, 1982; 125: 448–452.
5. Baumgartner I, Scheiner O, Holzinger C, Bolztnitulescu G, Klech H, Lassmann H, Rumpold H, Forster O, Kraft D. – Expression of the VEP 13 antigen (CD16) on native human alveolar macrophages and cultured blood monocytes. *Immunobiology*, 1988; 177: 317–326.
6. Shellito J, Kaltreider HB. – Heterogeneity of immunologic function among subfractions of normal rat alveolar macrophages. *Am Rev Respir Dis*, 1984; 129: 747–753.
7. Brannen AL, Chandler DB. – Alveolar macrophage subpopulations' responsiveness to chemotactic stimuli. *Am J Pathol*, 1988; 132: 161–166.
8. Holt PG, Degebrodt A, O'Leary C, Krska K, Plozza T. – T-cell activation by antigen presenting cells from lung tissue digests; suppression by endogenous macrophages. *Clin Exp Immunol*, 1985; 62: 586–593.
9. Sandron D, Reynolds HY, Laval AM, Venet A, Israel-Biet D, Chretien J. – Human alveolar macrophage subpopulations isolated on discontinuous albumin gradients. Cytological data in normals and sarcoid patients. *Eur J Respir Dis*, 1986; 68: 177–185.
10. Sandron D, Reynolds HY, Venet A, Laval AM, Israel-Biet D, Chretien J. – Human alveolar macrophage subpopulations isolated on discontinuous albumin gradients: functional data in normal and sarcoid patients. *Eur J Respir Dis*, 1986; 69: 226–234.
11. Zeidler RB, Flynn JA, Arnold JC, Conley NS. – Subpopulation of alveolar macrophages inhibits superoxide anion production by macrophages. *Inflammation*, 1987; 1: 371–379.
12. Chandler DB, Fuller WC, Jackson RM, Fulmer JD. – Fractionated rat alveolar macrophages by isopycnic centrifugation: morphological, cytochemical, biochemical and functional properties. *J Leukocyte Biol*, 1986; 3: 371–383.
13. Holt PG, Schon-Hegrad M, Oliver J. – MHC Class II antigen-bearing dendritic cells in pulmonary tissues of the rat. Regulation of antigen presentation activity by endogenous macrophage population. *J Exp Med*, 1988; 67: 262–274.
14. Raff HV, Picker LJ, Stobo JD. – Macrophage heterogeneity in man. A subpopulation of HLA-DR bearing macrophages required for antigen-induced T-cell activation also contains stimulators for autologous-reactive T-cells. *J Exp Med*, 1980; 152: 581–593.
15. Sun D, Lohmann-Matthes ML. – Functionally different subpopulations of mouse macrophages recognised by monoclonal antibodies. *Eur J Immunol*, 1982; 12: 134–140.
16. Hancock WW, Zola H, Atkins RC. – Antigenic heterogeneity of human mononuclear phagocytes: immunohistologic analysis using monoclonal antibodies. *Blood*, 1983; 62: 1271–1279.
17. Reittie JE, Poulter LW, Prentice HG, Burns J, Drexler HG, Balfour B, Clarke J, Hoffbrand AV, McGee JO, Brenner MK. – Differential recovery of phenotypically and functionally distinct circulating antigen-presenting cells alter allogeneic marrow transplantation. *Transplantation*, 1988; 45: 1084–1091.
18. Spiteri MA, Poulter LW. – Characterisation of immune inducer and suppressor macrophages from the normal human lung. *Clin Exp Immunol*, 1991; 83: 157–162.
19. Poulter LW, Campbell DA, Munro C, Janossy G. – Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. *Scan J Immunol*, 1986; 24: 351–357.
20. Spiteri MA, Clarke SW, Poulter LW. – Phenotypic and functional changes in alveolar macrophages contribute to the pathogenesis of pulmonary sarcoidosis. *Clin Exp Immunol*, 1988; 74: 359–364.
21. Spiteri MA, Clarke SW, Poulter LW. – Alveolar macrophages that suppress T-cell responses may be crucial to the pathogenetic outcome of pulmonary sarcoidosis. *Eur Respir J*, 1992; 5: 394–403.
22. Warren L. – The thiobarbituric acid assay of sialic acids. *J Biol Chem*, 1959; 234: 1971–1975.
23. Janossy G, Bofill M, Poulter LW, Rawlins E, Burford GD, Navarette C, Zeigler A, Keleman E. – Separate ontogeny of two macrophage-like accessory cell population in the human fetus. *J Immunol*, 1986; 136: 4354–4361.
24. Munro CS, Campbell DA, Collings LA, Poulter LW. – Monoclonal antibodies distinguished macrophages and epithelioid cells in lesion of sarcoidosis and leprosy. *Clin Exp Immunol*, 1987; 68: 282–287.
25. Kelly PMA, Bliss E, Morton JA, Burns J, McGee JO. – Monoclonal antibody EBM11: high cellular specificity for human macrophages. *J Clin Pathol*, 1988; 41: 510–515.
26. Hogg N, Macdonald S, Slusarenko M, Beverley PC. – Monoclonal antibodies specified for human monocytes, granulocytes and endothelium. *Immunology*, 1984; 53: 753–767.
27. Gerdes J, Naiem M, Mason DY, Stein H, – Human complement (C3b) receptors defined by an mouse monoclonal antibody. *Immunology*, 1982; 45: 645–653.
28. Kradin RL, Zhu Y, Hales CA, Bianco C, Colvin BB. – Response of pulmonary macrophages to hyperoxic pulmonary injury. Acquisition of surface fibronectin and fibrinogen and enhanced expression of a fibronectin receptor. *Am J Pathol*, 1986; 125: 349–357.
29. Dougherty GJ, Selvendran Y, Murdoch S, Palmer DG, Hogg N. – The human mononuclear phagocyte high-affinity Fc receptor, FcRI, defined by a monoclonal antibody 10.1. *Eur J Immunol*, 1987; 17: 1453–1459.
30. Mason DY, Abdulaziz Z, Falini B. – Double immunoenzymatic labelling. *In*: Polak J, Van Noorden S, eds. Immunocytochemistry: Practical Applications in Pathology and Biology. Bristol, John Wright, 1983; pp. 113–128.
31. Janossy G, Bofill M, Poulter LW. – Two colour immunofluorescence: analysis of the lymphoid system with monoclonal antibodies. *In*: Polak J, van Noorden S, eds. Immunocytochemistry:Practical Applications in Pathology and Biology. 2nd Edn. Bristol, John Wright, 1986.
32. Lojda Z, Vecerek B, Pelichova H. – Some remarks concerning the histochemical reaction of acid phosphatase by azo-coupling reactions. *Histochemie*, 1964; 3: 428–454.
33. Altmann FP. – The use of eight different tetrazolium salts for a quantitative study of pentose shunt dehydrogenation. *Histochemie*, 1969; 9: 363–374.
34. Butcher RG. – Precise cytochemical measurement of neotetrazolium formazan by scanning and integrating microdensiometry. *Histochemie*, 1972; 32: 171.
35. Axline S, Reaven EP. – Inhibition of phagocytosis and plasma membrane motility of the cultivated macrophage by cytochalasin B. Role of subplasmalemmal microfilaments. *J Cell Biol*, 1974; 62: 647–659.
36. Poulter LW, Duke O. – Dendritic cells of the rheumatoid synovial membrane and synovial fluid. *In*: Clot J, Sang J, eds. Montpellier Seminars. 1983; pp. 189–199.

37. Campbell DA, Poulter LW, duBois RM. – Phenotypic analysis of alveolar macrophages in normal subjects and in patients with interstitial lung disease. *Thorax*, 1986; 41: 429–434.
38. Allison MC, Cornwell S, Poulter LW, Dhillon AP, Pounder RE. – Macrophage heterogeneity in normal colonic mucosa and in inflammatory bowel disease. *Gut*, 1988; 29: 1531–1538.
39. Rickwood D, Birnie GD. – Centrifugal Separation *In*: Birnie D, Rickwood D, eds. Molecular and cell Biology. London, Butterworths, 1978.
40. Grdina DJ, Milas L, Hewitt RR. – Buoyant density separation of human blood cells in renografin gradients. *Expl Cell Res*, 1973; 81: 250–254.
41. Meistrich ML, Trostle PK. – Separation of mouse testis cells by equilibrium density centrifugation in renografin gradients. *Expl Cell Res*, 1975; 92: 231–244.
42. Peters DL, Dahmus ME. – A method of DNA quantitation for localization of DNA in metrizamide gradients. *Analyt Biochem*, 1979; 93: 306–311.
43. Lea T, Smeland E, Funderud S, Vartdal F, Davies C, Beiske K, Ugelstad J. – Characterization of human mononuclear cells after positive selection with immunomagnetic particles. *Scand J Immunol*, 1986; 23: 509–519.
44. Lea T, Vartdal F, Davies C, Ugelstad J. – Magnetic monosized polymer particles for fast and specific fractionation of human mononuclear cells. *Scand J Immunol*, 1985; 22: 207–216.
45. Vartdal F, Gaudernack G, Funderud S, Bratlie A, Lea T, Ugelstad J, Thorsby E. – HLA Class I and II typing using cells positively selected from blood by immunomagnetic isolation: a fast and reliable technique. *Tissue Antigens*, 1986; 28: 301–312.
46. Riches DWH, Hanson PH. – Functional aspects of mononuclear phagocyte involvement in lung inflammation. *Ann NY Acad Sci*, 1986; 465: 6–14.
47. Lipscomb MF, Lyons RC, Nunez G, Ball EJ, Stastny P, Vial W, Lem V, Weissler J, Miller LM. – Human alveolar macrophages; HLR positive macrophages that are poor stimulators of primary mixed leukocyte reaction. *J Immunol*, 1986; 136: 497–504.
48. Coonrad JD, Yoneda K. – Effect of rat alveolar lining material on macrophage receptors. *J Immunol*, 1983; 130: 2589–2596.
49. Nicod LP Lipscomb MF, Weissler JC, Lyons CR, Albertson J, Toews GB. – Mononuclear cells in human lung parenchyma. Characterization of a potent accessory cell not obtained by bronchoalveolar lavage. *Am Rev Respir Dis*, 1987; 136: 818–823.