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Characterization of immune inducer and suppressor macrophages from the normal human lung

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SUMMARY

Monoclonal antibodies (MoAbs) that are able to discriminate between dendritic cells (MoAb RFD1$^+$) and mature macrophages (MoAb RFD7$^+$) in normal tissues were used in combination with density separation techniques to isolate relatively homogeneous subpopulations of macrophages from human bronchoalveolar lavage (BAL). A characterization of surface antigen expression, and functional capacity was then carried out on each isolated alveolar macrophage (AM) subset. One population with the phenotype RFD1$^+$RFD7$^-$ obtained from the non-adherent cell pool showed the characteristics of antigen-presenting cells having absent or poor expression of Fc and C3b receptors, a low content of lysozomal hydrolase and poor phagocytic capacity. This population strongly stimulated T lymphocytes in allogeneic mixed lymphocyte reactions (MLR). A second AM population, isolated by adherence and density centrifugation expressed the phenotype RFD1$^+$RFD7$^+$. These cells showed the same phenotypic characteristics of mature macrophages with strong expression of C3b and Fc receptors, and marked phagocytic capacity. Such AM were very poor stimulators of allogeneic MLR. Under certain circumstances the RFD1$^+$RFD7$^+$ cells were shown to actively repress the stimulatory capacity of the RFD1$^+$RFD7$^-$ subpopulation. These results suggest that variations within the functional capacity of AM subsets may be capable of influencing the strength of acquired T cell immune responses of the lung.

Keywords macrophages phenotypes function monoclonal antibodies

INTRODUCTION

Local defence mechanisms in the lung rely heavily on alveolar macrophages to remove inhaled particular materials. Macrophage-like cells are responsible for inducing acquired immunological responses by antigen presentation to T cells (Unanue & Allen, 1987) and secreting mediators central to the control of local cellular interactions (Takemura & Weib, 1984). In the light of this it is not surprising that several reports have demonstrated a heterogeneity within the macrophage populations of the lung. Separate subsets have been identified in terms of morphology (Nakstad *et al.*, 1989), cell density (Zwilling, Campolito & Reiches, 1982), and surface antigen phenotype (Baumgartner *et al.*, 1988). Investigation of cell populations isolated by colloidal silicone gradients from animal bronchoalveolar lavage (BAL) and lung tissue digests have revealed that these macrophage subsets can display a functional heterogeneity in terms of immunologic function (Shellito & Kaltreider, 1984; Holt, Schon-Hegrad & Oliver, 1988) as well as motile capacity (Brannen & Chandler, 1988). Using the same separation methods, a similar heterogeneity has been observed in human alveolar macrophages (Sandron *et al.*, 1986b). While the majority of density-fractionated macrophage-like cells function as stimulators of mixed lymphocyte reaction (MLR), some fractions have an inhibitory effect (Sandron *et al.*, 1986a). This observation is in keeping with results of similar investigations into the T cell-inducing capacity of alveolar macrophages in rats (Holt *et al.*, 1985). These studies therefore strongly suggest that within the alveolar macrophage pool exist subsets of cells that regulate the induction and strength of local T cell mediated immune responses.

By using monoclonal antibodies (MoAbs) that discriminate functionally distinct macrophage like cells in normal tissues (RFD1 and RFD7; Poulter *et al.*, 1986), previous studies in our laboratory have identified three subpopulations within the alveolar macrophage pool obtained by BAL from human subjects (Campbell, Poulter & du Bois, 1986). The possible significance of these subsets was revealed by subsequent observations that their proportions within BAL altered dramatically with the advent of disease (Spiteri *et al.*, 1988; Ainslie, du Bois & Poulter, 1989) and could also be modulated by therapeutic regimes (Spiteri, Clarke & Poulter, 1989). Preliminary investiga-

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tion of the functional capacity of these phenotypically distinct macrophage subsets suggested that RFD1$^+$RFD7$^-$ cells acted as inducers (antigen presenters) in immunological reactions, and RFD1$^+$RFD7$^+$ cells could act as suppressors of T cell responses (Spiteri *et al.*, 1988). The third subset, RFD1$^-$RFD7$^+$, exhibit the phenotype of classic mature phagocytes.

If both 'inducer' and 'suppressor' macrophages reside in the lung and if the balance between these cells can alter in disease, it may be that such cells influence the intensity of T cell-mediated acquired immune responses. This study makes use of the MoAbs that distinguish these macrophage subsets, in conjunction with density separation techniques so that relatively homogeneous populations of putative inducer cells (RFD1$^+$RFD7$^-$) and putative suppressor cells (RFD1$^+$RFD7$^+$) can be isolated and a characterization of their phenotype, surface receptor expression, and functional capacity be performed.

MATERIALS AND METHODS

Samples

Lung macrophages were obtained from 15 healthy individuals, all non-smokers (12 men and three women, mean age 23 years). All had normal chest radiographs and pulmonary function in addition to a negative past history of lung disease or any viral illness in the 2 weeks prior to BAL. All were recruited following formal written consent and the study had received prior approval by the local Ethics Committee.

Bronchoalveolar lavage

BAL was performed using a 6-mm fibro-optic bronchoscope (Olympus model BT-IT20D) following pre-medication with 10 mg midazolam (Hypnovel). The right-middle lobe was anaesthetized with 2% lignocaine and lavaged with 20 ml aliquots of 0·9% normal saline to a total 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 lavage fluid was filtered through a single layer of coarse gauze and centrifuged at 480 ***g*** at 4°C for 5 min. The cell pellet was then washed twice in RPMI 1640 medium, and then the cells were counted in a modified Neubauer haemocytometer and viability was assessed by cellular exclusion of trypan blue. The cell concentration was then adjusted to 1×10^6 cells/ml using supplemented RPMI 1640 containing 1·25% 200 mM L-glutamine, 10% heat-inactivated fetal calf serum (FCS) and 100 μg/ml streptomycin and 100 U/ml penicillin.

Separation of macrophage subpopulations

The cell suspension prepared as above was plated onto sterile, plastic, 85-mm diameter tissue culture Petri dishes (Nunc, Roskilde, Denmark) with no more than 6×10^6 cells on each with a medium fluid depth of 3 mm. These were incubated for 2 h at 37°C in a humidified atmosphere of 5% CO_2. The supernatant containing the non-adherent cell populations 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*** at 4°C for 5 min, re-suspended in supplemented RPMI 1640 at 1×10^6 cells/ml and 2×10^6 cells/ml, respectively, and kept at 4°C until required.

Preparation of RFD1$^+$RFD7$^-$ cells

Lymphocytes were removed from the non-adherent population by rosetting with neuraminidase-treated sheep erythrocytes, followed by separation on a Ficoll–Hypaque gradient (Nycomed, Norway). The resulting cell interphase was harvested, washed twice and resuspended in supplemented RPMI 1640 medium. Subsequent analysis with anti-macrophage MoAbs demonstrated that between 80 and 90% of the cells obtained in this way were RFD1$^+$RFD7$^-$ (the phenotype of inter-digitating cells; Poulter *et al.*, 1986).

Preparation of RFD1$^+$RFD7$^+$ cells

To obtain cells with a phenotype RFD1$^+$RFD7$^+$, the adherent cell population was placed on a metrizamide (Nyegaard) gradient and spun at 650 ***g*** for 10 min at room temperature. The light density fraction was removed, washed twice in medium, and resuspended in supplemented RPMI 1640. This was kept on ice at 4°C. Subsequent staining with MoAbs RFD1 and RFD7 showed that over 80% of the cells obtained exhibited the phenotype RFD1$^+$RFD7$^+$ (see Results).

Immunocytological analysis

Immunocytological analysis of these separated populations was performed with a panel of MoAbs (Table 1) using either the immunoperoxidase method (Munro *et al.*, 1987), or double immunofluorescence staining (Janossy *et al.*, 1986). Background staining was identified by comparison with negative control cytospins from which the MoAb was omitted; positive specificity controls were always prepared using sections of human palatine tonsil. In the case of the fluorescent studies a Zeiss microscope equipped with epi-illumination and barrier filters appropriate for FITC and TRITC was used for recording RFD1$^+$RFD7$^+$ cells. Background fluorescence was identified using controls as described above. At least 150 cells were counted in each cytospin, and the percentage of positive cells was recorded.

Acid phosphatase activity

Enzyme studies were performed on unfixed cytospins. Lysozomal enzyme activity was investigated using a standard histochemical reaction for acid phosphatase (Lodja, Vecerek & Pelichova, 1964). All preparations were counter-stained with haematoxylin.

Phagocytosis

Macrophages from each separated subpopulation were incubated with fluorescein-conjugated latex beads of 1 μm diameter (Poly Sciences, Northampton, UK) at a concentration of 100 beads to one cell for 2 h at 37°C in an atmosphere of 5% CO_2. Control plates were set up with the addition of cytochalasin B (to block phagocytosis) at a concentration of 2×10^{-5} M prepared in 0·2% dimethylsulphoxide. Following incubation the suspensions were washed twice to remove excess beads, and cytospin preparations were made. The presence of latex beads within the cells was determined using a Zeiss fluorescence microscope with appropriate barrier filters for FITC. At least 100 cells were counted on each cytospin preparation and the

Table 1. Monoclonal antibodies used in this study

Antibody	Mol. wt (kD)	Specificity in normal tissue	Source	Reference
RFDR1	28–33	Identifies a framework epitope on HLA-DR	RFHSM	Janossy *et al.* (1986)
RFD1	28–33	Identifies inter-digitating cells	RFHSM	Poulter *et al* (1986)
RFD7	77	Identifies mature phagocytic macrophages	RFHSM	Poulter *et al.* (1986)
RFD90	—	Identifies epithelioid cells and tingible body macrophages	RFHSM	Munro *et al.* (1987)
EMB11 (CD68)	110	Identifies all cells of the macrophage series	Dakopatts, Glostrup, Denmark	Kelly *et al.* (1988)
UCHMI (CD14)	52	Identifies antigen present on monocytes	P. L. C. Beverley*	Hogg *et al.* (1984)
Anti-C3b	205	Reacts with the receptor for the third component of human complement	Dakopatts	Gerdes *et al.* (1982)
Anti-fibronectin	—	Reacts with fibronectin in human cells	Dakopatts	Kradin *et al.* (1988)
10.1		Fc receptor for IgG	N. Hogg†	Dougherty *et al.* (1987)

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presence of five or more latex beads in a cell was considered to constitute phagocytosis.

Allogeneic MLR

Aliquots of 1×10^4 unfractionated BAL cells and similar aliquots of the separated RFD1$^+$RFD7$^+$ subsets were co-cultured in triplicate wells of 96-well microtitre plates with a standard population of 1×10^5 allogeneic normal peripheral blood mononuclear cells (PBMC) from a single donor.

In other experiments, varying concentrations of RFD1$^+$RFD7$^-$ and RFD1$^+$RFD7$^+$ subsets of alveolar macrophages were incubated again with a constant number of allogeneic PBMC. In some experiments the proportions of one macrophage subset remained constant while a second varied from ratios of 1:10 to 10:1. In other experiments, the proportions of both cell populations varied to maintain a constant number of stimulating cells within each culture well. To ensure 'one-way' reactions, inducer populations were pre-incubated in mitomycin C (25 μl/ml) for 45 min at 37°C, and then washed three times in RPMI 1640 before adding to the MLR cultures.

All cultures were incubated for 5 days at 37°C in a humidified atmosphere with 5% CO_2. The cultures were then pulsed with ^{3}H-thymidine for 18 h and harvested using a semi-automatic cell harvester (Titer-Flow, McLean, VA). The amount of incorporated thymidine was measured in a liquid scintillation counter and expressed as average ct/min of triplicate cultures.

RESULTS

Bronchoscopic findings

No evidence of bronchial infection or inflammation was found in any subject. The mean percentage of lavage fluid was 72 ± 11% of the instilled volume.

Differential cell count

The mean (s.e.m.) cell yield was 9·7 (1·9) × 10^6 cells. The mean number of alveolar macrophages was 8·8 (1·9) × 10^6 cells. In the 15 volunteers studied, the mean proportion of lymphocytes and polymorphonuclear cells was less than 9% and 2%, respectively. Viability of unfractionated cells and separated subsets was always greater than 90%, as determined by trypan blue exclusion. All the cells in the separated populations exhibited the morphological characteristics of macrophages.

Table 1. Phenotypic characteristics of isolated macrophage subsets from normal bonchoalveolar lavage (BAL) (counts performed on three sets of represented cells)

Phenotype tested	Whole BAL*	RFD1$^+$RFD7$^-$ cells	RFD1$^+$RFD7$^+$ cells
RFD1$^+$RFD7$^-$	12·3 ± 4·0	97 ± 2·4	< 1% +
RFD1$^-$RFD7$^+$	48·2 ± 10·6	< 1%	< 1% +
RFD1$^+$RFD7$^+$	7·3 ± 2·0	< 1%	97·4 ± 1·6
CD68	98·6 ± 1·4	95·4 ± 0·61	96·3 ± 0·21
CD14	5·0	< 1%	< 1%
HLA-DR	82 ± 4·6	98·3 ± 1·2	95·4 ± 3·5
RFD9	11·1 ± 6·3	< 1%	5·4 ± 1·12
Fc IgG	NT	20·3 ± 1·21	85·3 ± 1·24
C3br	NT	< 1%	35·7 ± 0·45
Fibronectin	NT	30·3 ± 0·21	95·3 ± 3·41
Phagocytosis (%)	NT	39·3 ± 4·1	73 ± 8·1
Acid phosphotase$^+$	NT	25·0 ± 2·9	74 ± 3·6

*Mean ± s.e.m. (%) of morphologically identifiable macrophages positive for phenotype marker.
< 1%, No positive cells recorded in 150 counted; NT, not tested.

Immunocytological analysis

The isolation procedure produced more than 90% homogeneity in the two phenotypically distinct macrophage populations (Table 2). No significant difference was observed in the proportions of each subset exhibiting HLA-DR, CD68, or CD14 antigens. The RFD7$^+$RFD1$^+$ population differed from the RFD1$^+$RFD7$^-$ cells in terms of Fc and C3b receptor expression and the presence of fibronectin in that in each case RFD1$^+$RFD7$^+$ populations contained a significantly greater proportion of positive cells (Table 2). A small number of RFD9$^+$ cells (epithelioid cell marker) were also found in the RFD$^+$RFD7$^+$ population, while no RFD1$^+$RFD7$^-$ cells exhi-

Table 3. Allogeneic mixed lymphocyte reactions with separated subsets

Experiment no.	PBMC	PBMC/M	PBMC/RFD1$^+$RFD7$^-$	PBMC/RFD1$^+$RFD7$^+$	RFD1$^+$RFD7$^+$	RFD1$^+$RFD7$^+$
1	8429 (714)	147 (13·7)	40 520 (3156)	13 118 (642)	121 (19·8)	84 (13·2)
2	4464 (555)	30 (4)	14 360 (1644)	9357 (589)	96·8 (28)	182 (20)
3	8604 (1451)	157 (19·8)	20 404 (775)	9825 (1124)	81 (8·7)	87 (7·1)
4	1132 (71)	108 (20·6)	4181 (1685)	198 (8)	94 (31·5)	140 (44·1)

PBMC, Peripheral blood mononuclear cells (normal donor); PBMC/M, PBMC treated with mitomycin.
Results are mean ct/min (±s.d.) of triplicate wells.

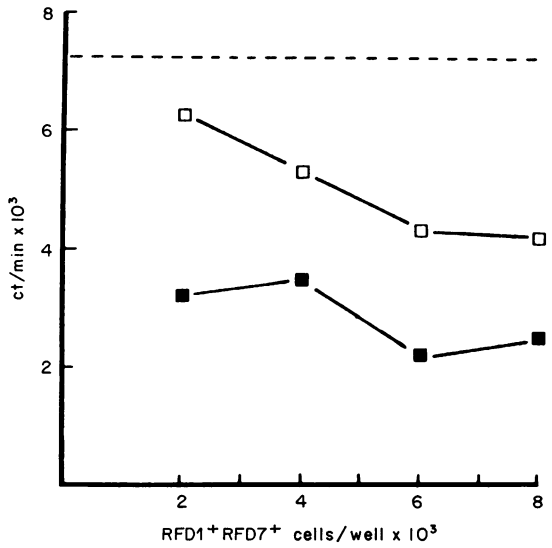

Fig. 1. The effect of varying concentrations of RFD1$^+$RFD7$^+$ lung macrophages on the ability of RFD1$^+$RFD7$^-$ cells to stimulate allogenic peripheral blood mononuclear cells (PBMC). Hatched line represents thymidine uptake achieved with 4×10^3 RFD1$^+$RFD7$^-$ admixed with 10^5 PBMC; ■, uptake by lymphocytes when mixed with varying concentrations of RFD1$^+$RFD7$^+$ cells; □, resulting lymphocyte stimulation when the varying concentrations of RFD1$^+$RFD7$^+$ cells are added to the fixed number of RFD1$^+$RFD7$^-$ cells. Results are mean ct/ min from triplicate wells of a typical experiment. The study was repeated on five samples.

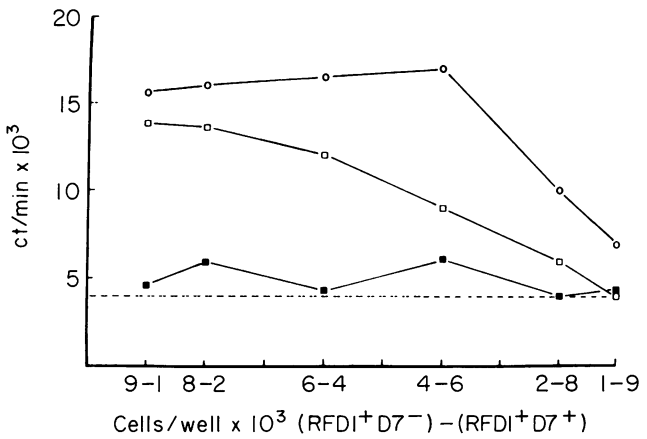

Fig. 2. Dose-dependent suppression of allogenic mixed lymphocyte reaction (MLR) by the RFD1$^+$RFD7$^+$ macrophage subset obtained from normal bronchoalveolar lavage. A constant number (10^5) allogeneic peripheral blood mononuclear cells (PBMC) is present in all cultures. Hatched line represents thymidine uptake by PBMC above (autologous reaction); ○, MLR when varying numbers of RFD1$^+$RFD7$^-$ cells alone are added; ■, MLR when varying numbers of RFD1$^+$RFD7$^+$ cells are added; □, MLR reactivity when varying proportions of RFD1$^+$RFD7$^-$ and RFD1$^+$RFD7$^+$ cells are used together as the stimulator population. Results are mean ct/min from tiplicate wells in one study. The experiment was repeated on five samples.

bited RFD9 positivity. The presence of acid phosphatase activity was recorded in 74% of RFD1$^+$RFD7$^+$ cells; only 26% of RFD1$^+$RFD7$^-$ cells exhibited this reactivity.

Using fluorescent latex beads as a marker, phagocytosis activity was determined in the separated alveolar macrophage subsets. With the RFD1$^+$RFD7$^+$ population, 73% of cells contained phagocytosed beads after a 2-h incubation. In contrast, less than 40% of RFD1$^+$RFD7$^-$ cells exhibited phagocytic capacity (Table 2).

Lymphocyte stimulation

In four experiments (Table 3) the admixture of 1×10^4 RFD1$^+$RFD7$^-$ cells with 1×10^5 allogeneic PBMC resulted in a 2·5–5-fold increase in ^{3}H-thymidine incorporation compared with the autologous reactivity of the responder population. In two of these experiments, stimulation with RFD1$^+$RFD7$^+$ was radically reduced, in one no stimulation was seen, and in the fourth a suppression of autologous reactivity was recorded. In a subsequent experiment the numbers of RFD1$^+$RFD7$^-$ cells were standardized at 4×10^3 cells/well mixed with 10^5 allogeneic lymphocytes and then varying numbers of RFD1$^+$RFD7$^+$ cells were added. It was found that 4×10^3 RFD1$^+$RFD7$^-$ cells caused marked allogeneic stimulation, and RFD1$^+$RFD7$^+$ cells failed to cause any significant stimulation irrespective of the numbers used (Fig. 1). When the RFD1$^+$RFD7$^+$ cells were admixed with the RFD1$^+$RFD7$^-$ cells, a progressive suppression of allogeneic lymphocyte transformation was seen.

The latter result could have been due to the increasing total numbers of alveolar macrophages in these cultures. To test this possibility, further experiments were performed in which the numbers of both RFD1$^+$RFD7$^-$ and RFD1$^+$RFD7$^+$ cells were varied to maintain a constant number of stimulator cells. This study again revealed that the RFD1$^+$RFD7$^+$ populations failed to stimulate the lymphocytes at any concentration.

The RFD1$^+$RFD7$^-$ cells exhibited stimulatory capacity at a concentration range of $9–2 \times 10^3$ cells/well. However, the admix-

ture of RFD1$^+$RFD7$^+$ cells to the stimulating population was seen to suppress progressively the lymphocyte transformation induced by RFD1$^+$RFD7$^-$ cells, and this suppression was most marked when the concentration of RFD1$^+$RFD7$^+$ used was promoting optimum stimulation (i.e. 4×10^3 RFD1$^+$RFD7$^-$ cells and 6×10^3 RFD1$^+$RFD7$^+$ cells; Fig. 2).

DISCUSSION

We have shown that phenotypically distinct alveolar macrophage subpopulations can be identified within BAL of normal subjects. These discrete cell types share a similar morphology, yet exhibit phenotypic and functional characteristics implying spearate capabilities. One isolated macrophage subset appears to express phenotypic characteristic of both dendritic cells and 'classic' macrophages (RFD1$^+$RFD7$^+$). Functional studies with these cells show for the first time that a specific subset of human alveolar macrophages can act to suppress the induction of T cell responses.

The question of macrophage heterogeneity has assumed some importance in recent years, with greater appreciation of the multiplicity of roles of these cells in the immune response (Gordon, Keshav & Chung, 1988; Johnston, 1988). Macrophages have been fractionated on density gradients into subpopulations that exhibit different morphological and cytochemical features (Sandron *et al.*, 1986a; Nakstad *et al.*, 1989), as well as phagocytic (Sharma & Remington, 1981), migratory (Brannen & Chandler, 1988) and tumouricidal capacities (Evans, 1973). It has been postulated that these macrophage density fractions represent different stages of maturation (Shellito & Kaitreider, 1984). In this present study all cell subpopulations were CD68$^+$ and none expressed CD14 antigen, implying that these macrophages had matured and not recently been recruited from the circulating pool of blood monocytes. This being the case, the diversity in terms of phenotype and function revealed here suggests that local factors within the lung may control macrophage phenotype after maturation.

The RFD1$^+$RFD7$^-$ cells isolated exhibit the characteristics of 'dendritic' antigen-presenting cells in having low lysozymal enzyme content, limited expression of Fc and C3b receptors and being strong stimulators of MLR. Such results confirm our previous observations (Poulter & Duke, 1983; Poulter *et al.*, 1986). The RFD1$^+$RFD7$^+$ cells, on the other hand, have the phenotypic characteristics of mature phagocytes and seem capable of suppressing T cell responses. Such cells are not identified in lymphoid tissue and do not 'naturally' develop from monocytes in culture (Poulter *et al.*, 1986). The only other anatomical location where significant numbers of these cells have been reported is in the lamina propria of the gut (Allison *et al.*, 1989). This distribution raises the fascinating possibility that such cells are uniquely associated with mucosal immunity and perhaps represent part of the immunoregulatory mechanisms active at sites where antigenic bombardment is constant. Similar observations have been made in animal studies. Holt *et al.* (1988) described the presence of non-adherent, Ia-positive, FcR-negative, ultra-low-density mononuclear cells in rat lung parenchyma capable of antigen presentation *in vitro*, and called these cells 'putative dendritic cells'. Their function was seen to be inhibited by the presence of endogenous adherent FcR$^+$ cells, described as 'putative macrophages'.

It has been suggested in previous studies of unfractionated alveolar macrophage that the enhancing and suppressing effects seen with regard to T cell reactivity could depend on the varying proportion of 'stimulatory' macrophages *in situ* (Holt *et al.*, 1985). While this may be true, it is also evident from our present results that allogeneic MLR suppression can also be modulated by macrophages. Irrespective of the varying cell concentration, allogeneic PBMC reactivity remained low or abolished with RFD1$^+$RFD7$^+$ macrophages, but stimulated to varying degrees with RFD1$^+$RFD7$^-$ cells. These observations hint at a paradox, as the majority of our 'suppressor' RFD1$^+$RFD7$^+$ macrophages express HLA-DR, a requirement for antigen presentation. However, Unanue *et al.* (1984) suggested that although the minimum requirement for an antigen-presenting cell is the expression of Ia or HLA-DR, a given Ia (or HLA-DR) bearing cell may not necessarily present all or any antigen, or indeed be capable of stimulating all T cells. In fact, differences in functional DR-antigen expression, with shielding of the reactive epitopes in the DR-complex by N-linked carbohydrate moieties has been postulated (Ferro *et al.*, 1987), and could explain the 'suppressor' activity of RFD1$^+$RFD7$^+$ cells. Two other possibilities are the secretion of 'suppressive' factors from these cells and/or the absence of other surface antigens not tested for here, that may be required in stimulating MLR, e.g. HLA-DQ (Bach, 1985; Ettensohn, Duncan & Jankowski, 1989). In addition, some reports have stressed that membrane-bound accessory molecules such as LFA-1 and LFA-3 are crucial for antigen-presenting cell–T cell interaction (Krensky *et al.*, 1983). With specific regard to the expression of antigens seen by MoAbs RFD1 and RFD7 (the feature that separates the functionally distinct alveolar macrophages studied here), it is relevant that this expression can be altered by contact with soluble mediators. For example, interferon-gamma has been shown to up-regulate RFD1 expression (Poulter *et al.*, 1987); and corticosteroids can up-regulate RFD7 expression (manuscript in preparation). Whether these induced phenotypic changes result in functional change is currently being investigated. Nevertheless, the results presented here support the hypothesis that under normal circumstances in humans, the alveolar macrophage populations represent a dynamic system comprising phenotypically and functionally distinct subpopulations, the delicate balance of which may contribute to immunoregulation.

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