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Smoking alters the phenotype of macrophages in induced sputum

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To investigate the effect of chronic smoke exposure on pulmonary macrophages (PM), the expression of seven different surface and intracellular molecules of PM was studied in induced sputum (IS) samples from healthy volunteers – nine smokers and seven non-smokers. Sputum was induced by inhalation of nebulized saline (3·5% NaCl). Cell viability and total cell counts (TCC) were performed immediately. Cell differentials were determined on May–Grünwald Giemsa-stained cytospin preparations. The PM were immunologically characterized by use of the following monoclonal antibodies: RFD1, RFD7, CD11b, CD54, CD68, CD71 and HLA-DR. The stainings were performed with a three-step, indirect immuno-alkaline phosphate method. Viability and TCC did not differ between the groups. Smokers had a higher percentage of macrophages ($P<0·05$) and a lower proportion of neutrophils ($P<0·05$). The percentage of macrophages expressing RFD1, HLA-DR, CD71 ($P<0·01$ for all) and CD54 ($P<0·05$) was significantly lower in smokers, whereas the remaining markers were expressed equally in the two groups. The results indicate that smoking induces a decrease in the expression by PM of surface molecules known to be associated with the antigen-presenting function.

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Introduction

The pulmonary macrophage (PM) population is phenotypically and functionally heterogeneous. Some PM exhibit an effector cell phenotype, possessing a phagocytic and microbicidal capacity (1). Others have immunoregulatory functions, acting as antigen-presenting cells (2) or suppressor macrophages, performing an immunosuppressive function by inhibiting the proliferative capacity of T-cells in the normal lung (3,4). Leukocyte migration, homing, and cell–cell interaction is dependent partly on various adhesion molecules, among which the leukocyte adhesion molecules (LeuCAM; CD11/CD18), which belong to the structural family of integrins and the immunoglobulin-related intercellular adhesion molecules (ICAM), are essential (5).

Previous investigators have studied the effects of cigarette smoke on macrophages in bronchoalveolar lavage (BAL) fluid, showing numerous morphological and functional changes. Smokers have a higher number of alveolar macrophages (AM) in their BAL fluid compared to non-smokers, indicating an increased influx to and/or decreased clearance of AM from the distal airways (6). Smokers' macrophages are larger, show cytoplasmic inclusions and autoimmunofluorescence (6,7) and produce higher levels of oxygen radicals and lysosomal enzymes (7–9). Studies of changes in the immunophenotype of smokers' macrophages have, however, produced somewhat inconsistent results. It has been shown that LeuCAMs are expressed differently in AM from smokers and non-smokers. Some investigators have reported a decreased proportion of CD11/CD18-positive AM in smokers (10,11), while others have reported an increase compared with non-smokers (8).

Contradictory results have also been achieved in studies of the expression of HLA class II surface antigens on AM in BAL. Using different methods, some investigators have shown a reduction of HLA antigen density on AM in smokers compared to non-smokers (11,12), whereas others have found a similar expression of HLA in the groups (6,8).

BAL allows collection of material from the distal airways, but is a laborious method unsuitable for frequent studies in the same subject. An alternative method of collecting material from the airways is induced sputum (IS), a non-invasive procedure that can be performed repeatedly. In this study we aimed to explore the possibility of showing smoking-induced changes in the immunophenotype of pulmonary macrophages in IS material. We investigated macrophage-associated intracellular and surface antigens in IS from smokers and non-smokers in order to identify any difference in expression between the groups.

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Methods

SUBJECTS

Sputum samples were collected from 16 healthy volunteers, nine smokers (one man and eight women, 21–38 years) and seven non-smokers (one man and six women, 21–46 years). The subjects showed no signs of infection. In the non-smoker group, four never had been smokers and three had stopped smoking for at least 5 yr. The cigarette consumption of the smokers was 7·5 (4·9–8·3) pack-years (median, lower and upper quartiles), and the median present consumption at least 15 cigarettes day^{-1} for the last 5 yr. The study had the approval of the local Ethics Committee, and informed consent was obtained.

SPUTUM INDUCTION AND PROCESSING

After administration of 200 μg salbutamol (Ventoline®, Glaxo, U.K.), sputum was induced by inhalation of 3·5% sterile solution (Apoteksbolaget, Sweden). The saline was nebulized with an ultrasonic nebulizer (De Vilbiss Ultraneb 2000, U.S.A.) and inhaled for up to 20 min. After 5 min, and at subsequent intervals of 3–5 min, subjects were asked to rinse their mouths and try to cough sputum into clean plastic tubes. The first and third expectorates were pooled and stored for future analysis, and the second expectorate was collected in a separate tube for use in cell analysis. The volume of the sputum sample was determined, and 5 ml of the material was diluted with 0·1 ml 10% dithiothreitol (DTT, Sigma, MO) and 1·9 ml Hank's balanced salt solution (HBSS, Sigma), gently mixed and incubated until homogenized. The sample was centrifuged at 800 rpm for 10 min and the cell pellet was resuspended in 1–2 ml HBSS. Fifty microlitres of the cell suspension was used to assess viability by trypan blue cell exclusion and to determine the total cell count in a Bürker chamber. Cytospins were prepared using aliquots of cell suspension equivalent to 60 000 cells per slide. The material was centrifuged at 800 rpm for 6 min in a Shandon cytocentrifuge (Cytospin 2, Shandon, U.K.). Two slides were stained using May–Grünwald Giemsa for differential cell counts and the rest stored at − 70°C, until further processed.

IMMUNOCYTOLOGICAL STAININGS

The stainings were performed using a three-step, indirect immuno-alkaline phosphatase (ALP) method. Frozen slides were warmed to room temperature before processing. Slides were fixed in − 20°C acetone for 10 min and rehydrated in tris-buffered saline (TBS), pH 7·6 for 5 min. The slides were subsequently incubated with 0·07 ml of the appropriate dilutions of primary monoclonal antibodies (Table 1), ALP-conjugated rabbit anti-mouse antibody (DAKO, Denmark) and ALP-conjugated swine anti-rabbit antibody (DAKO), in humid chambers for 30 min each, and carefully washed with TBS between the steps. The immunological reaction was visualized using freshly prepared ALP substrate (Phosphatase Fast Red Sigma solution, Sigma) containing 1 mmol l^{-1} of levamisole (Sigma) to inhibit endogenous ALP activity. The enzyme–substrate reaction was interrupted with tap water and the slides were counterstained with haematoxylin for 30 s, blued in tap water and air-dried. For mounting, a glycerine mountant (Merck, Germany) was used. The slides were viewed with a light microscope (Nikon, Japan). Macrophages were identified on the basis of morphological features, and positive cells were recognized by red staining (Plate 1). A minimum of 600 cells were counted by two independent observers, and the mean value of the two observations was used. An irrelevant monoclonal antibody (MNF116, Anti-human cytokeratin, DAKO) was used as negative control).

STATISTICAL ANALYSIS

The data are presented as medians with upper and lower quartile values. Statistical comparisons were made using the non-parametric Mann–Whitney U test. A P value $<0·05$ was considered as significant.

Results

TOTAL AND DIFFERENTIAL CELL COUNTS

The results are expressed as medians with upper and lower quartiles. The cell viability and total cell count did not differ significantly between smokers and non-smokers (Table 2). Smokers had a higher percentage of macrophages, 69·4 (51·4–75·9)%, compared with non-smokers, 36·4 (26·7–48·4)%, ($P<0·05$), and a lower percentage of neutrophils, 29·6 (14·7–36·4)%, compared with 63·4 (49·7–66·4)%, ($P<0·05$).

The percentage of lymphocytes and eosinophils did not differ significantly between the groups (Table 2).

IMMUNOCYTOLOGICAL STAININGS OF MACROPHAGES

The results are expressed as the percentage of positive cells showing the morphological characteristics of PM (Table 3). Plate 1 shows a picture of PM recovered with IS, stained for HLA II. The percentage of PM expressing the RFD1 and HLA class II molecules was significantly lower in smokers, 57·0 (42·0–62·5)% and 93·0 (84·0–95·0)% respectively, compared with non-smokers, 86·0 (76·0–89·0)% and 100 (100)%, ($P<0·01$ both, Fig. 1). The expression of CD54 by smokers' PM, 33·0 (14·0–43·0)%, was lower than the expression of the same molecule by non-smokers' PM, 57·5 (35·0–62·0)%, ($P<0·05$, Fig. 1).

The expression of CD71 was lower in smokers, 68·0 (64·0–71·5)%, compared with non-smokers, 91·5 (80·0–93·5)%, ($P<0·01$), whereas the expression of CD11b, CD68 and RFD7 did not differ between the groups.

Discussion

In this study we wanted to investigate smoking-induced changes in the immunophenotype of macrophages in

TABLE 1. Panel of primary monoclonal antibodies

Antibody	Antigen cluster designation	MW antigen (kDa)	Clone	Source	Specificity	Reactivity
Anti-human dendritic cells, RFD1	RFD1		RFD1	SEROTE C	Reacts with an epitope within the HLA class II complex	Dendritic cells; non-reactive with monocytes, granulocytes
Anti-human mature macrophages RFD7	RFD7		RFD7	SEROTE C	Reacts with an antigen expressed predominantly in mature tissue phagocytes	Macrophages
Anti-human C3bi receptor, CD11b	CD11b	165	2LPM19c	DAKO	Reacts with the cell surface receptor for the C3bi complement fragment	Granulocytes, monocytes, tissue macrophages
Anti-CD54	CD54, ICAM-1		6.5B5	DAKO	Reacts with the human intercellular adhesion molecule-1 (ICAM-1)	Broad; leukocytes, endothelial and epithelial cells
Anti-human macrophage, CD68	CD68	110	PG-M1. (1)	DAKO	Reacts with an intracytoplasmatic antigen associated with lysosomal granules	Macrophages; non-reactive with antigen-presenting cells
Anti-human transferrin receptor, CD71	CD71	180	Ber-T9	DAKO	Reacts with the transferrin receptor	Proliferating cells; macrophages
Anti-human HLA-DR	HLA class II	28	CR3/43	DAKO	Reacts with the beta-chain of all products of the DP, DQ and DR subregions	Leukocytes
Anti-human cytokeratin	Cytokeratin	40–60	MNF116	DAKO	Reacts with an epitope present in a wide range of cytokeratins	Broad; human epithelial cells (negative control)

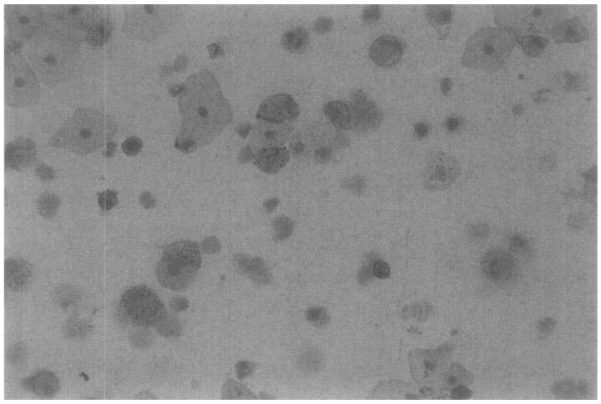

PLATE 1. Three-step immunocytochemical stain for HLA II on macrophages recovered with induced sputum. Positivity shows as strong dark staining of the cytoplasm of the macrophages.

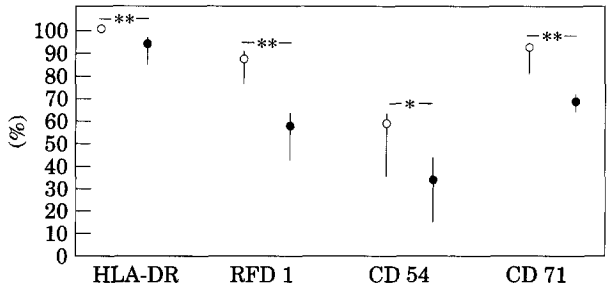

FIG. 1. Percentage of PMs expressing HLA-DR, RFD1, CD54 and CD71 in non-smokers (○; $n=7$) and smokers (●; $n=9$). $^{*}P<0{\cdot}05$; $^{**}P<0{\cdot}01$.

induced sputum. The cell viability and total cell count did not differ between smokers and non-smokers. Smokers had a significantly higher percentage of macrophages in their sputum samples compared to non-smokers. This corresponds well to results shown earlier in BAL fluids (6,12). The increased number of macrophages in smokers' BAL and sputum material could hypothetically depend on a decreased clearance of macrophages from the airways as well as an increased recruitment of blood monocytes to the alveoli. In contrast to previous findings in sputum from normal subjects (13–15), our non-smokers had somewhat high neutrophil counts. The reason for this is at present not obvious to us.

The expression of different surface antigens on pulmonary macrophages is fundamental to their function as a vital part of the lung's immune defence. Our immunocytological staining results showed a significantly decreased percentage of macrophages expressing the HLA II and HLA II-associated RFD 1 molecules in smokers. Using different immunofluorescence methods, Mancini *et al.* (11) and Pankow *et al.* (12) have reached similar results, showing a decrease in HLA-DR expression on smokers' AM in BAL.

Furthermore, we found a decreased percentage of PM expressing the adhesion molecule CD54 (ICAM-1) in smokers. Since the HLA II complex and the leukocyte adhesion molecules are vital in antigen presentation by macrophages, a decrease in expression of these molecules could,

TABLE 2. Sputum cell count data in smokers and non-smokers (medians with upper and lower quartiles)

	n	Viability (%)	TCC ($\times 10^6$)	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)
Non-smokers	7	95·0 (89·0–97·0)	1·9 (1·3–3·2)	36·4 (26·7–48·4)*	1·7 (0·7–2·7)	63·4 (49·7–66·4)*	0·7 (0·4–0·7)
Smokers	9	90·0 (81·0–92·0)	1·9 (1·3–2·3)	69·4 (51·4–75·9)	1·0 (0·8–1·0)	29·6 (14·7–36·4)	0·7 (0·2–3·0)

$^{*}P<0{\cdot}05$.

TABLE 3. Percentage of sputum macrophages expressing antigens as detected by monoclonal antibodies, using a three-step alkaline phosphatase immunocytochemical method (data are expressed as medians and interquartile ranges)

Monoclonal antibodies	Smokers ($n=9$)	Non-smokers ($n=7$)	Mann–Whitney U test
RFD1	57·0 (42·0–62·5)	86·0 (76·0–89·0)	$P<0{\cdot}01$
RFD7	58·0 (50·0–65·0)	68·0 (56·0–78·0)	n.s.
CD11b	52·0 (38·0–54·5)	64·0 (42·0–67·0)	n.s.
CD54	33·0 (14·0–43·0)	57·5 (35·0–62·0)	$P<0{\cdot}05$
CD68	42·0 (35·0–46·0)	32·0 (28·0–36·0)	n.s.
CD71	68·0 (64·0–71·5)	91·5 (80·0–93·5)	$P<0{\cdot}01$
HLA DR	93·0 (84·0–95·0)	100	$P<0{\cdot}01$

n.s. = not significant.

hypothetically, give rise to a reduced antigen-presenting capacity.

The expression of CD11b did not differ between smokers and non-smokers. This is consistent with earlier studies of smoke exposure effects on AM in BAL fluid (16).

We detected a lower expression of the transferrin receptor (CD71) in the smoking population. The expression of CD54, CD71 and RFD1 has been shown to correlate directly with the CD4/CD8 ratio and percentage of BAL lymphocytes in sarcoidosis (17). This disease is characterized by an accumulation of $CD4^+$ T lymphocytes in the alveoli and up-regulation of CD54 (18,19), CD71 (17,20) and RFD1 (17) on BAL macrophages. It has been suggested that alterations in the balance of signals from lung macrophages may be an important factor in enhanced $CD4^+$ T-cell activity in sarcoidosis (21). The fact that smokers' macrophages show a decreased expression of markers specifically up-regulated on macrophages in sarcoid patients could, hypothetically, offer one explanation for the lower incidence of sarcoidosis in the smoking population.

When processing sputum according to the method used in this study, sputum plugs are not separated from saliva. The method of processing the whole expectorated sample guarantees that no material loss takes place during the processing, but it gives a varying percentage of squamous epithelial contamination in the sample. A large number of squamous cells can sometimes disturb the assessment of macrophages. This problem can be solved in the majority of cases by diluting the sample before producing cytospins. The ALP immunostain gives clearly detectable red stain in positive cells and allows, together with HTX counterstain, a good detection of morphological characteristics. When assessing the positivity, the observer graded the colouring as weak, moderate and strong stain and then established a limit below which cells were assessed as negative. For some markers the limit between positive and negative cells was difficult to determine. The samples were therefore assessed by two independent observers and a large number of cells were counted to avoid errors.

In this study we have shown in sputum a smoking-induced change in cell proportions and expression of macrophage-specific antigens that has previously been demonstrated by several investigators in material recovered by BAL. BAL and IS do, however, provide the investigator with material deriving from different compartments in the lung. The origin of cells collected with BAL is mainly the alveoli. Cells recovered by sputum are more likely to derive from the proximal airways. Using airway lining fluid (ALF), which gives an opportunity to assess cells deriving from major airways selectively, Rankin *et al.* (22) have shown that viable macrophages reside in the airways and that they express similar surface and cytoplasmic antigens compared to macrophages recruited by BAL.

Hypothetically, the phenotype of cells in induced sputum could correspond to the phenotypic patterns of cells recovered with BAL. Induced sputum could then, together with immunological methods, provide a possibility for repeatedly sampling the pulmonary milieu and following dynamic, immunological processes in different pathological conditions. Further studies, comparing macrophages in sputum and BAL, are indicated.

In conclusion, we found that chronic exposure to smoke changes the phenotype of macrophages retrieved by induced sputum. Smokers' macrophages show a decreased expression of antigens that are up-regulated on macrophages in sarcoidosis and important in the antigen-presenting process. The changed phenotype of pulmonary macrophages induced by chronic exposure to smoke could hypothetically explain the lower tendency to develop sarcoidosis in the smoking population.

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