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Smoking-induced changes in epithelial lining fluid volume, cell density and protein

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Smoking-induced changes in epithelial lining fluid volume, cell density and protein. W.M.J. Burke, C.M. Roberts, D.H. Bryant, D. Cairns, M. Yeates, G.W. Morgan, B.J. Martin, H. Blake, R. Penny, J.J. Zaunders, S.N. Breit.

ABSTRACT: Bronchoalveolar lavage has proved a useful research technique for recovering cellular and molecular contents of the lower respiratory tract. Because the recovered fluid is variably diluted, an accurate estimation of molecular and cellular concentrations can only be made if the epithelial lining fluid volume recovered is also known. It has been suggested that smoking may alter epithelial lining fluid volume by reducing clearance or by stimulating production and, thus, affect the interpretation of bronchoalveolar lavage studies.

In this study, urea was used as an endogenous marker of epithelial lining fluid volume in a comparison of 26 smokers and 31 nonsmokers.

The mean epithelial lining fluid volume recovered from smokers was significantly greater than that of nonsmokers (2.4±1.40 ml *vs* 1.2±0.75 ml, $p<0.005$). The total cellular concentration in the bronchoalveolar lavage fluid in smokers was also greater ($94.2\pm46\times10^6$ *vs* $33.9\pm21.5\times10^6$ cells per 300 ml lavage), even when corrected for bronchoalveolar lavage volume recovered ($63.1\pm32.5\times10^6$ *vs* $24.9\pm13.3\times10^6$ cells per 100 ml recovered lavage fluid). This was true for macrophage, lymphocyte and neutrophil cell numbers. However, when corrected for the apparent epithelial lining fluid volume, only the macrophage count remained significantly higher in the smokers compared with nonsmokers ($30.66\pm20.7\times10^6$ *vs* $18.21\pm8.6\times10^6$ macrophages·ml^{-1} ELF). In addition, concentrations of albumin and immunoglobulin M (IgM) were significantly lower in smokers after correction for epithelial lining fluid volume.

These results highlight smoking as a confounding factor in the interpretation of bronchoalveolar lavage data. The increased epithelial lining fluid volume in smokers significantly affected the cellular and protein concentrations in the patients studied. *Eur Respir J., 1992, 5, 780–784.*

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Bronchoalveolar lavage (BAL) has proved to be a safe and effective method of obtaining material from the lower respiratory tract. This technique has allowed accurate estimation of the cellular and molecular components found at the bronchoalveolar surface, giving valuable insights into defence mechanisms and disease processes occurring at that level.

The BAL technique samples epithelial lining fluid (ELF) by the instillation of normal saline into the lower respiratory tract. The recovered fluid is, therefore, a mixture of saline, ELF, and the molecular and cellular components of the ELF. Although the nature of the cellular and molecular content of the ELF can be identified from the recovery fluid, the concentration of these products cannot be known unless the volume of ELF is also known.

Cigarette smoking is known to be associated with at least 20 differences in the content of BAL fluid of smokers compared with that of nonsmokers [1], although these changes have not been widely studied in relation to ELF volume. One recent study [2] has suggested that the ELF volumes may differ between subjects with chronic bronchitis, asymptomatic smokers and nonsmokers, with significant effects upon cellular concentrations within the ELF. This observation, if confirmed, has wide implications for the interpretation of BAL data derived from subjects which include smokers. One suggested method for determining the ELF volume is to use urea as an endogenous marker of ELF dilution [3], thus allowing the calculation of an apparent ELF volume. This then provides a possible framework of reference to measure protein concentrations, as well as a more useful method for expressing cell numbers.

In this study, urea has been used as an endogenous marker of ELF dilution to quantify the apparent ELF volume in a group of smokers and nonsmokers free from symptoms of respiratory disease. Absolute and differential counts of macrophages, lymphocytes and neutrophils, as well as concentrations of albumin,

immunoglobulin G and M (IgG and IgM) were measured in the recovered lavage fluid, and using the calculated apparent ELF volume the cellularity and albumin and immunoglobulin concentrations of the ELF could in turn be calculated.

Several significant differences were observed to occur in smokers: greater overall cellularity; higher absolute counts of all measured cell types in the lavage fluid with a higher differential count being observed for macrophages; an increase in the ELF population of macrophages only; and lower concentrations of albumin and IgM in lavage and ELF. This study describes these changes and addresses the issue of their relevance to smoking-related disease and the practical implications for studies using BAL.

Materials and methods

Patient selection

The population for this study comprised 57 subjects drawn from a group participating in another project [4]. There were 26 smokers and 31 nonsmokers, whose mean ages were 48 and 45 yrs, respectively. All subjects were symptom free, with a normal clinical respiratory examination and normal chest radiograph. The nonsmoking group comprised 25 subjects with connective tissue disorders (scleroderma, Sjogren's syndrome, systemic lupus erythematosis), five with localized breast cancer and one normal subject. The smoking group included 24 subjects with connective tissue disorders (scleroderma, Sjogrens syndrome, systemic lupus erythematosis), one with localized breast cancer and one normal subject. Patients with clinical or radiological respiratory disease were excluded from the study as were any subjects with a recent history of respiratory infection. The parent project from which the data were drawn had been approved by the hospital Ethics Committee and written informed consent was obtained from each patient.

Clinical evaluation

All patients were given a questionnaire designed to elicit a detailed clinical history and respiratory symptoms were evaluated according to Medical Research Council (MRC) criteria [5, 6]. Subjects whose replies suggested respiratory disease were excluded from the study.

Smoking data

Each patient's smoking history was quantitated in terms of duration and daily intake and expressed as pack years, (20 cigarettes·day^{-1} for 1 year=1 pack year). For the purposes of the statistical calculations, those subjects who had not smoked for at least 2 yrs prior to the study were included as nonsmokers.

Respiratory evaluation

Respiratory physiology. Tests of spirometry, vital capacity (VC), alveolar volume (V_A), maximum mid-expiratory flow rate (MMEFR), diffusion capacity of the lungs for carbon monoxide (D_{LCO}) and diffusion capacity corrected for alveolar volume (carbon dioxide transfer coefficient (K_{CO})) were carried out on each subject (P.K. Morgan Transfer Test, Minato AS-600 spirometer). Results were expressed as percentage of the predicted normal mean value using the reference equations suggested by COTES [7].

Bronchoalveolar lavage. This was performed under local anaesthetic as described previously [8]. Briefly, it consisted of the passage, through the mouth, of a flexible fibreoptic bronchoscope, which was wedged in turn into three subsegmental bronchi in three separate lobes. One hundred millilitres of room temperature normal saline was instilled into each lobe in four 25 ml aliquots. Each aliquot was aspirated immediately after inspiration and the material was collected into a chilled siliconized glass container and kept on ice until processed. This procedure was completed in under three minutes for each lobe lavaged.

Evaluation of bronchoalveolar cells. Enumeration of different cell types in BAL fluid was carried out by depositing an aliquot of unprocessed lavage fluid onto a millipore filter. This was then washed, fixed and stained with haematoxylin and eosin and for nonspecific esterase activity in order to accurately differentiate macrophages from lymphocytes. Total cell counts were obtained using unprocessed lavage fluid and a counting chamber. This method gives substantially higher estimates of lymphocyte numbers which are much more accurate than those obtained using cytocentrifuge preparations, which are known to underestimate lymphocyte numbers [8, 9].

Analysis of ELF volume and albumin content. The volume of ELF collected in the lavage procedure was estimated as described previously [3]. The urea and albumin levels of the unconcentrated lavage fluid and a simultaneously collected plasma sample were determined by standard methods. Briefly, serum and lavage urea determinations were undertaken using the urease method essentially according to manufacturers instructions (Roche, Basle, Switzerland), by measuring absorbance at 340 nm on a Kone Progress Selective Chemistry Analyser (Finland). Serum albumin was determined on the basis of bromcresol green dye binding (Technicon, USA) using absorbance at 628 nm on the Kone Analyser. Lavage fluid albumin was determined using a turbidometric method employing anti-albumin antibody (Dako, Denmark) and absorbance at 340 nm on a Roche Centrifichem 400 Analyser (Basle, Switzerland). The ELF volume was then calculated using the formula: ELF=(urea concentration of lavage fluid/urea concentration of plasma) (recovered lavage volume). The albumin result was then corrected

for the actual ELF volume recovered. To take into account changes in lavage ELF albumin concentration due to variation in plasma albumin, the result was expressed as the ratio of ELF to plasma albumin (ALBRAT).

Evaluation of ELF IgG and IgM content. The plasma levels of IgG and IgM were measured by standard nephelometric methods using a Beckman Nephelometer. Levels of the immunoglobulins were determined in unconcentrated lavage fluid using a radio-immunoassay as described previously [10, 11]. ELF IgG and IgM were calculated as described above for albumin. Results were also expressed as the ratio of ELF to plasma levels (IgGRAT, IgMRAT) in order to correct for changes in the ELF due to changes in plasma levels.

Statistical analysis

Correlation coefficients (r_s) and significance levels were calculated using Spearman's rank correlation. All r_s values, unless otherwise stated, are significant to at least $p<0.05$. Data are reported as mean±standard deviation.

Results

Respiratory function tests

The only significant difference in lung function between nonsmokers and smokers was in DLCO (values expressed as percentage predicted) (88.4 *vs* 71.9%; $p<0.001$) and KCO (103.9 *vs* 80.4%; $p<0.0005$) (table 1).

Table 1. – Respiratory test profiles of subjects according to smoking status

	Nonsmokers		Smokers		p
Demographics					
Number	31		26		
Age yrs	45		48		
Fluid volumes					
% recovered	46	(14.5)	51	(13.4)	NS
ELF volume ml	1.2	(0.75)	2.4	(1.4)	<0.005
Lavage cells					
Total recovered cells $\times10^6$	33.9	(21.5)	94.2	(46.0)	<0.0001
Cells·ml^{-1} ELF $\times10^6$	27.0	(12.3)	39.4	(21.2)	<0.001
Respiratory function tests					
VC % pred	91	(19.3)	93	(13.0)	NS
VA % pred	87	(14.1)	90	(14.4)	NS
MMEFR % pred	108	(27.6)	95	(28.6)	NS
DLCO % pred	88	(15.6)	72	(18.3)	<0.0005
KCO % pred	103	(22.2)	80	(17.6)	<0.001

Data are presented as mean with SD in parenthesis. ELF: epithelial lining fluid volume; VC: vital capacity; VA: alveolar volume; MMEFR: maximal mid-expiratory flow rate; DLCO: diffusion capacity for carbon monoxide; KCO: carbon monoxide transfer coefficent, DLCO/VA.

Recovered volumes

The mean percentage recovery of instilled lavage fluid was 53.7%. This did not differ significantly between smokers (n=26, 50.5%) and nonsmokers (n=31, 45.9%) (table 1). The mean calculated ELF volume was significantly greater in smokers (n=14) than nonsmokers (n=28) (2.4±1.4 *vs* 1.2±0.75 ml, $p<0.005$) (table 1). A positive correlation was observed in the smoking subjects between smoking history (pack yrs) and ELF volume ($r_s=0.42$).

Cell density

The total white cell count (WCC) in the lavage fluid was significantly greater in smokers (n=26) than nonsmokers (n=31) (94.2×10^6 *vs* 33.9×10^6, $p<0.0001$) as was the total cell count·ml^{-1} of ELF (39.4×10^6 *vs* 27×10^6, $p<0.001$) (table 1). The difference also remained highly significant when expressed as cells·100 ml^{-1} of recovered lavage fluid ($63.1\pm32.5\times10^6$ *vs* $24.9\pm13.3\times10^6$, $p<0.0001$).

There were significant increases in the lavage fluid of smokers in the absolute number of recovered macrophages ($49.8\pm30\times10^6$ *vs* $16.9\pm9.5\times10^6$, $p<0.0001$); lymphocytes ($12.2\pm6.7\times10^6$ *vs* $7.4\pm5.4\times10^6$, $p<0.005$) and neutrophils ($1.1\pm0.2\times10^6$ *vs* $0.6\pm0.07\times10^6$, $p<0.05$). When the cellularity of the lavage fluid was corrected for the calculated ELF volume (*i.e.* the cellularity of the ELF), the only cell type where the difference reached significance was macrophages ($30.7\pm20.7\times10^6$ *vs* $18.2\pm8.6\times10^6$, $p<0.01$) (fig. 1).

The only significant increase in the differential cell count in smokers was seen for macrophages (77.2 *vs* 67.1%, $p<0.005$) with a reciprocal decrease in the percentage of lymphocytes (21.2 *vs* 31.5%, $p<0.005$) (fig. 2).

Significant positive correlations were noted in smokers between smoking history (pack yrs) and ELF cellularity ($r_s=0.45$), ELF macrophage content ($r_s=0.52$), WCC in the recovered fluid ($r_s=0.6$) and the absolute ($r_s=0.66$) and differential ($r_s=0.41$) macrophage count.

Protein concentration

There were significantly lower ELF concentrations in smokers of IgM (1.24±1.1 *vs* 3.65±2.8 g·dl^{-1}, $p<0.05$) and albumin (0.32±0.19 *vs* 0.58±0.21 g·dl^{-1}, $p<0.005$). The ELF concentration of IgG was not significantly different in smokers and nonsmokers (data not shown).

An ELF:plasma ratio for each substance was calculated (IgGRAT, IgMRAT, ALBRAT). The ratio was significantly lower in smokers (n=9) compared with nonsmokers (n=11) for IgMRAT (0.008±0.01 *vs* 0.031±0.03, $p<0.0005$) and ALBRAT (0.009±0.01 *vs* 0.015±0.01, $p<0.0005$) but not IgGRAT (0.11±0.06 *vs* 0.09±0.06 (fig. 3). Negative correlations were observed between smoking history (pack-yrs) and IgMRAT ($r_s=-0.65$ and ALBRAT ($r_s=-0.4$) in the cigarette smokers.

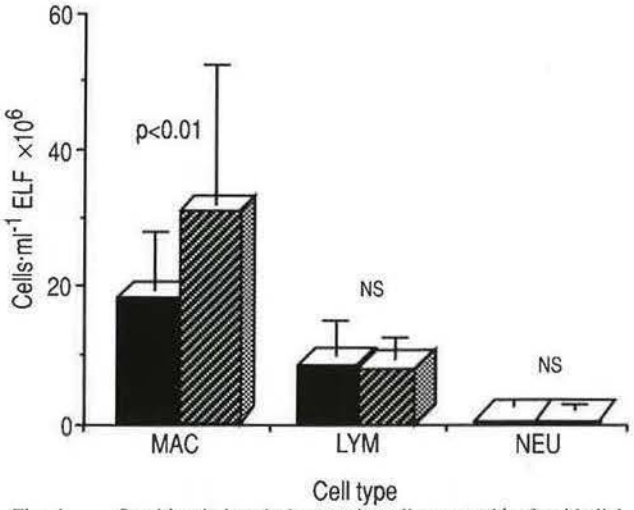

Fig. 1. – Smoking-induced changes in cell count·ml^{-1} of epithelial lining fluid (ELF). ■: nonsmokers; ▨: smokers; MAC: macrophages; LYM: lymphocytes; NEU: neutrophils; NS: nonsignficant.

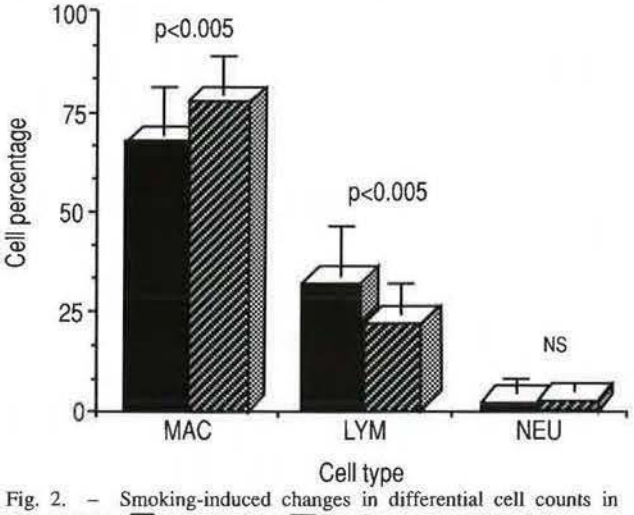

Fig. 2. – Smoking-induced changes in differential cell counts in lavage fluid. ■: nonsmokers; ▨: smokers. For abbreviations see legend to figure 1.

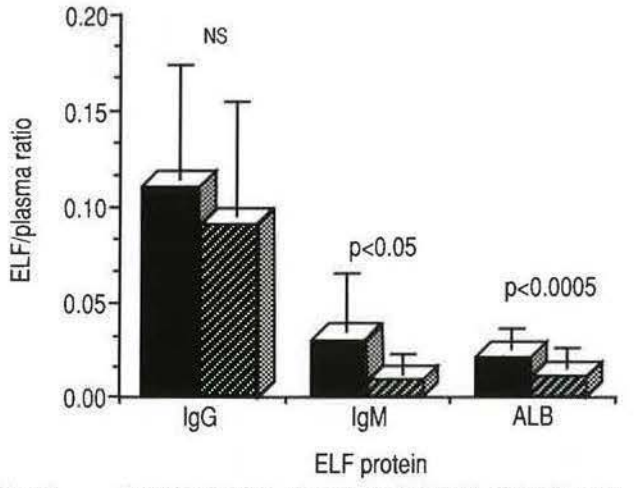

Fig. 3. – Smoking-induced changes in IgGRAT, IgMRAT and ALBRAT. ■: nonsmokers; ▨: smokers; ELF: epithelial lining fluid; IgG: immunoglobulin G; IgM: immunoglobulin M; ALB: albumin; IgGRAT, IgMRAT, ALBRAT: ratio of ELF to plasma IgG, IgM and albumin, respectively.

Discussion

The results of this study suggest that the apparent ELF volume retrieved from smokers is significantly greater than that from nonsmokers (table 1). This observation affects the interpretation of the concentration of BAL samples from subject groups containing smokers and highlights smoking as a confounding factor in previous studies not controlled for smoking.

This study demonstrates the importance of separating smokers and nonsmokers in defining normal subjects to avoid misleading results. The smokers in our study did not differ significantly from the nonsmokers in either clinical evaluation of their respiratory status or laboratory measurement of lung function, with the exception of gas diffusion (table 1), which has been shown to be significantly reduced in a proportion of subjects after relatively brief periods of smoking [12]. Although, this defect in diffusion may also be exhibited by subjects with pulmonary involvement from connective tissue diseases. Strict criteria were applied to our subjects to ensure the absence of clinical pulmonary disease in both smoking and nonsmoking groups.

Recovered volumes did not differ significantly between smokers and nonsmokers but an important finding, which may lead to a reappraisal of other observed changes, was that of a 100% increase in the ELF volume of smokers, in the absence of any difference in the total amount of lavage fluid recovered (table 1). The reason for this is not clear but could conceivably relate to either increased ELF production, or be a consequence of impaired clearance. This, however, appears to be a true increase in volume and does not merely reflect a nonspecific increase in vascular permeability. Had this been so, a parallel increase in albumin in the lavage and in the ELF of smokers would have been expected, whereas the reverse was observed. Assessment of the inflammatory response at alveolar level in any condition necessitates a reproducible quantitation of the volume of fluid lining the alveolar spaces, on which can be based calculations of the local concentrations of inflammatory cells and mediators. In this study, urea has been used to calculate the apparent ELF volume and, thence, to measure concentrations of cells and proteins in the ELF.

Urea is a normal plasma component, the molecular weight of which allows it to diffuse readily across various membranes and the concentration of which is easily measured. The urea method of ELF volume estimation has been widely studied in the estimation of ELF volume and has been shown to have several advantages over other available methods [13, 14]. However, the urea method itself is handicapped by the tendency of urea to readily diffuse into the BAL fluid from the lung interstitium within one minute of infusion. Minimizing the dwell time and volume of the lavage fluid reduces this artefact. Overall, this effect tends to result in an over-estimation of the true ELF volume [3, 11, 15] and the term "apparent ELF volume" should be used to describe the fluid volume determined by the urea method. In this study, the dwell

time was minimal as the lavage aliquots were aspirated immediately upon instillation. The small aliquot volumes used facilitated this, further controlling the inherent error of the technique. In addition, the same methodology was applied to both subject groups. It is important that the findings of this study be confirmed using other methods of ELF determination because of these technical difficulties in measurement of ELF volumes.

When the cellular contents of the BAL fluid recovered are considered, there was a highly significant increase in total WCC of the fluid in the smoking subjects (table 1), which was unchanged when corrected either for the volume of fluid instilled or recovered. This increase appears to have two components, *i.e.* an increase in all cell types secondary to the increased ELF volume and, in the case of macrophages an additional increase greatly in excess of that due to ELF changes. The predominant cell in this observed response was the alveolar macrophage. There was a highly significant increase in the absolute and differential count for alveolar macrophages (fig. 1) and also a significant increase in the number of macrophages per volume of ELF (fig. 2). Whilst absolute numbers of both polymorphs and lymphocytes increased this was only in proportion to the increase in ELF volume.

Macrophages have a primary role in host defence against foreign particles, such as exist in cigarette smoke, so that the increase of this cell type in smokers is not surprising. It has, however, been difficult in asymptomatic smokers without bronchitis to explain the observed increase in neutrophils [1]. Although it appears from these data that a true increase occurs in macrophage numbers, the increase in neutrophils may simply reflect the increase in ELF volume.

It was observed that there were significantly lower IgMRAT, ALBRAT and ELF concentrations for IgM and albumin in smokers (fig. 3). There were no significant changes observed in the corresponding IgG values. It is likely that the decrease in IgM and albumin in smokers is a dilutional effect due to the increase in ELF volume. This relative preservation of IgG in the lavage fluid and ELF is obviously not due to greater diffusion from the circulation as IgG (MW about 150,000 Da), although a considerably smaller molecule than IgM (MW about 900,000 Da in its usual pentameric form) is considerably larger than albumin (MW about 67,000 Da). This suggests the existence of processes to enhance its concentration at the bronchoalveolar surface, such as local synthesis and secretion [16], and may involve selective transport similar to that observed for IgG_1 and IgG_2 across the placenta [17]. IgG secreting cells have been recovered in normal lavage fluid [18], so that a mechanism does exist for local input directly into airway secretions.

The results of this study emphasize the need to consider cigarette smoking as an independent factor affecting the cellular and molecular concentrations of recovered bronchoalveolar lavage fluid.

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