Br. J. exp. Path. (1986) 67, 407–413

Human pulmonary alveolar macrophages with smokers' inclusions: their relation to the cessation of cigarette smoking

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Received for publication 14 October 1985
Accepted for publication 4 December 1985

Summary. Bronchoalveolar lavage (BAL) was performed in 47 patients (24 smokers, 18 ex-smokers and 5 who never smoked). Cytocentrifuged preparations were stained with May-Grunwald-Giemsa (MGG) and differential counts of pulmonary alveolar macrophages (PAM) with smokers' inclusions were performed. The data supported an exponential reduction in this percentage and indicated that about 3 years elapsed before this percentage approximated to the values in patients who had never smoked. Electron microscopy was performed on eight BALs and the results supported those obtained by light microscopy.

Keywords: bronchoalveolar lavage, cigarette smoking, pulmonary alveolar macrophages, smokers' inclusions

The handling of inhaled particulate material in the respiratory tract is undertaken by the mucociliary transport mechanism and by the pulmonary alveolar macrophage (PAM). In cigarette smokers PAM have been shown to contain yellow brown inclusions (Pratt *et al.* 1969; Martin 1973) with basophilic features when stained with Romanowsky stains. These smokers' inclusions also have distinctive electron micrographic features which may represent silicates originating from the earth in which the tobacco was grown (Brody & Craighead 1975). Under the conditions of bone marrow transplantation in man, the lifespan of the PAM is approximately 81 days (Thomas *et al.* 1976). However, the *in vivo* kinetics of material phagocytosed by PAM and the morphological changes induced by it is largely speculative although this knowledge may be relevant to the relationship between smoking history and pulmonary disease. Within the limits of ethical constraints we directed our study to determining the *in vivo* kinetics of human PAM involved in phagocytozing exogenous material by studying these cells in BAL of patients with different cigarette smoking status.

Materials and methods

We obtained the informed consent of, and studied only, patients who had a clinical indication for fibroeoptic bronchoscopy, generally for the investigation of possible pulmonary malignancy. Before BAL, one of the authors who was not their clinical attendant questioned them about their

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smoking habits, ie. whether they had ever smoked or not, their daily cigarette consumption and, if they had stopped smoking, the time since stopping. Patients either with clinical, radiographic or histological features of cryptogenic fibrosing alveolitis which is known to be associated with a reduction in PAM with smokers' inclusions (Haslam *et al.* 1980), or with other diseases known to be associated with alveolitis (notably sarcoidosis), or with occupational exposure to dusts which might cause inclusions in PAM, were excluded.

No patient was subjected to BAL more than once. All smokers and exsmokers had smoked for at least 4 years. Thus a total of 47 BAL were included in the study (24 from current smokers, 18 from ex-smokers and 5 from patients who had never smoked). The age range was 9 to 76 years (mean 60 years) and all but six were males. The ex-smokers had stopped smoking for between 5 days and 34 years (mean 1433 days; median 174 days).

Bronchoalveolar lavage was performed as previously described (Cole *et al.* 1980). BAL fluid was collected in siliconized glass bottles at 4°C and filtered through surgical gauze to remove mucus. The cells were washed with Hanks Balanced Salt Solution (Flow Laboratories, UK). Cytocentrifuge (Shandon Cytospin) preparations were made in duplicate within 90 min of BAL, stained with May-Grunwald-Giemsa, mounted and randomly coded. At the end of the study the slides were examined in a random sequence. One thousand macrophages were counted on each slide and the macrophages with inclusions identified according to their morphological features. Cells with five or more discrete inclusions and diffuse cytoplasmic basophilia were expressed as a percentage of the total PAM.

Statistical comparisons between groups were made using the unpaired *t*-test to assess the significance of differences between means. Associations between the % PAM with inclusions and other variables were studied by least squares regression analysis. In addition certain transformations were tested to explore possible kinetics such as an exponential change in % PAM with inclusions or the effect of correcting this for cigarette consumption.

EM studies were performed on cells from eight patients (two never smoked, two current smokers, four ex-smokers). The BAL cells were fixed as a pellet in 2.5% glutaraldehyde in sodium cacodylate buffer, post-fixed in 1% buffered osmium tetroxide, dehydrated and embedded in Araldite. Ultra-thin sections, stained with uranyl acetate followed by lead citrate, were randomized, blind coded and examined by transmission electron microscopy. Approximately 100 macrophages were examined in each case.

Results

The mean and (SE) percentage return of instilled buffered saline was 38% (1.8%). The mean volume of buffered saline returned from the BAL was 98 ml (3.4 ml). The mean number of nucleated cells yielded by the BAL was 30×10^6 (6×10^6).

Table 1 shows the three categories of patients, ie. non-smokers, smokers and ex-smokers, together with diagnoses and percentage counts of macrophages with inclusions.

The mean cigarette consumption of the smokers was 18.9 (SD 8.87) per day, range 5–40. The mean cigarette consumption of the ex-smokers had been 22.9 (SD 10.2) per day, range 10–50. There was a significant difference ($P<0.001$) between the mean % macrophages with inclusions in the non-smoker group and the % in either of the other groups but the difference in mean % was not significant between the smoker and ex-smoker groups.

When data from the 18 smokers was analysed separately and the % PAM with inclusions regressed against the daily cigarette consumption no significant linear correlation was present ($r=0.30$) although a statistically significant correlation ($r=0.41$, $P<.05$) was present when both variables

Table 1.

		Current smokers	Ex-smokers	Never smokers
Males		21	16	4
Females		3	2	1
Diagnosis:	Malignancy	10	14	1
	Others	14	4	4
Age:	Mean (SD)	61.0 (11.2)	62.8 (9.4)	44.4 (18.3)
	Range	19–75	42–76	19–66
Cigarettes/day:	Mean (SD)	18.9 (8.8)	22.9 (10.2)	—
	Range	5–40	10–50	—
% PAM with inclusions:				
	Mean (SD)	19.8 (12.6)	13.2 (13.0)	1.14 (0.61)
	Range	2.6–49.2	0.1–40.4	0.3–1.9

were expressed on natural logarithmic coordinates.

Figure 1 shows the scatter of the data points for the % PAM with inclusions plotted against the time since smoking was stopped in days. The latter is expressed logarithmically because of the skewed distribution of the available data. The current smokers are portrayed as last having smoked 1 day before BAL. To the extreme right (off the scale) the scatter of points for the non-smokers is included for comparison. This data suggests that it takes about 3 years after stopping smoking before the % PAM with inclusions approximates the values for non-smokers. A matrix of correlations for the various relationships studied between % PAM with inclusions (or its transformations) against time (or its logarithmic transformation) is shown in Table 2. This indicates a reduction in variance (increase in the value for the correlation coefficient) when the natural logarithm of time is the independent variable, showing that the longer the time since stopping smoking the slower the reduction in % PAM with inclusions. Exponential plotting of the % PAM with inclusions and correction for the daily cigarette consumption also tend to reduce the variance.

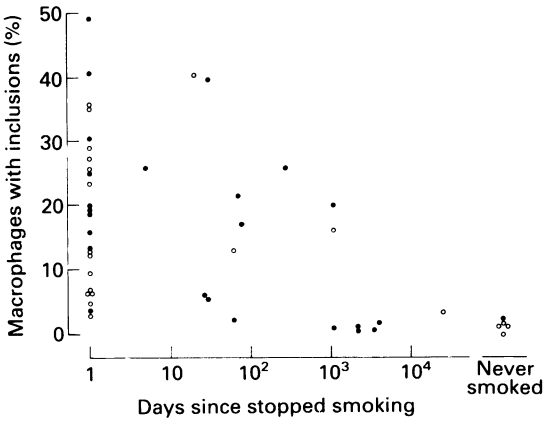

Figure 1. Relationship between % macrophages with inclusions and days since stopping smoking. Current smokers plotted as a '1 day'. ●, proven pulmonary malignancy; ○, others.

Since the number of BALs studied by electron microscopy was small and only limited numbers of cells could be assessed it was not feasible to analyse the results quantitively in the same way as the light microscopy data. Instead, the following empirical ranking was employed:

Rank Criteria

0: No smokers' inclusions observed.

1: Few phagolysosomes containing inclusions and then only one or two inclusions.

Table 2. Matrix of correlation coefficients (r).

		Independent variable (x axis)	
		Time (days)	$\log_e$ time
Dependent variable (y axis)	% PAM with inclusions	−0.313* (−0.372)	−0.395† (−0.583*)
	$\log_e$ % PAM with inclusions	−0.407† (−0.350)	−0.589‡ (−0.589*)
	$\log_e$ % PAM with inclusions $\log_e$ cigarettes/day	−0.429† (−0.369)	−0.625‡ (−0.665†)

Values are for pooled ex-smokers and current smokers (assumed to have last smoked 1 day before BAL). Values in brackets are for ex-smokers only.

Statistical significance, * $P<0.05$, † $P<0.01$, ‡ $P<0.001$.

Table 3.

Patient	Age	Sex	Diagnosis	Smoking status	EM ranking of inclusions
JM	59	M	Carcinoma	Still 35 cigs/day	4
MB	49	M	Bronchitis	Still 20 cigs/day	3
SE	60	M	Carcinoma	Ex 5 days 25 cigs/day	1
CP	42	M	No abnormality detected	Ex 21 days 20 cigs/day	4
JG	58	F	Carcinoma	Ex 30 days 30 cigs/day	2
HP	75	M	Carcinoma	Ex 270 days 20 cigs/day	2
VM	59	F	Bronchiectasis	Never smoked	0
HB	61	M	Bronchitis	Never smoked	0

2: Phagolysosomes containing more than one inclusion, usually two or three.

3: Most phagolysosomes containing inclusions, some with three or four or more.

4: As in 3: but with most with more than three or four inclusions.

The data is shown in tabular form in Table 3. This shows that even 270 days after stopping smoking (the longest interval in the

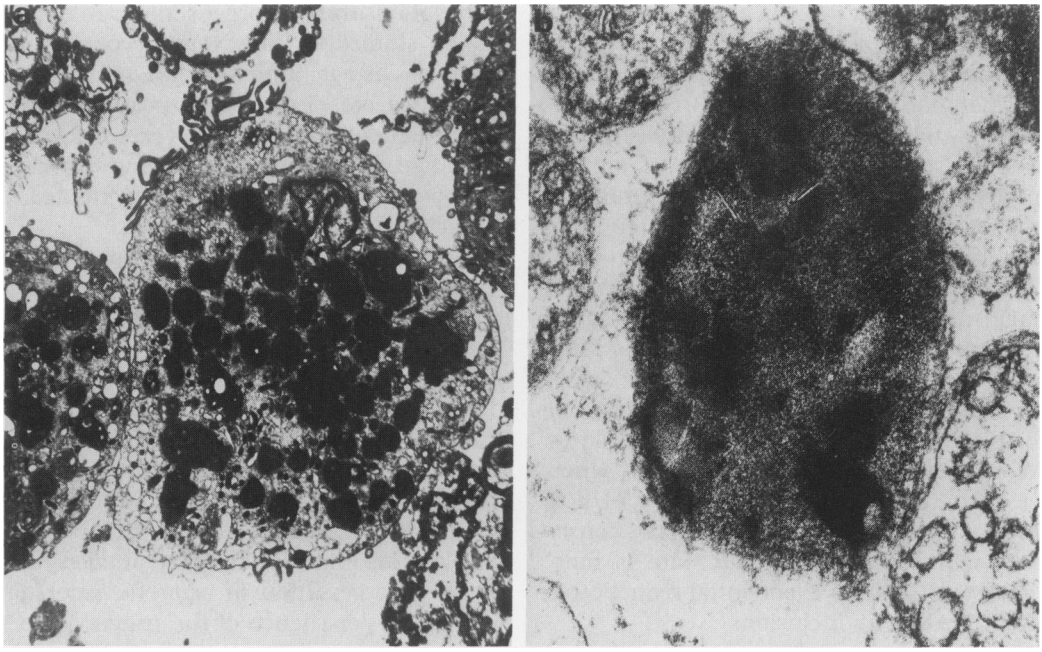

Figure 2. a, electron micrograph (×4200) showing a pulmonary alveolar macrophage from a patient who stopped smoking 270 days before BAL. The phagolysosomes (corresponding to the smokers' inclusions seen under the light microscope) are clearly evident. b, electron micrograph (×56000) showing one phagolysosome within a pulmonary alveolar macrophage, containing plate-like inorganic structures (as described by Brody & Craighead 1975) which are seen as linear electron translucencies.

electron microscopic study) smokers' inclusions were clearly evident. Figure 2 illustrates the ultrastructural appearance of PAM in subject HP who gave up smoking 270 days before BAL.

Discussion

Despite several studies (Pratt *et al.* 1969; Martin 1973; Warr & Martin 1978) of the light microscopic morphology of PAM exposed to tobacco smoke *in vivo*, there is as yet no consensus on the criteria for identifying, and hence counting, such cells differentially. The classification adopted by Warr & Martin (1978) is probably the most comprehensive but was considered too detailed for this study for which therefore a simpler scheme was adopted. On descriptive grounds our PAM with inclusions correspond to the Warr and Martin types 3 and 4 cells. Our counts tended to be lower than theirs, possibly attributable to the use of a different Romanowsky stain. The random 'blind' counting of all slides by the same observer at the end of the study favoured consistency in our results.

In current smokers the proportion of PAM with smokers' inclusions is probably multifactorial, depending on the extent of tobacco smoke exposure in terms of pack years, daily consumption, depth of inhalation, etc., as well as host factors. The latter include age, possible inherited factors and also concomitant disease which may involve macrophage function (Haslam *et al.* 1980).

The kinetics of the human PAM under normal steady state conditions is not known but after bone marrow transplantation, host macrophages disappear in a linear fashion and the data indicates a life span of approximately 81 days (Thomas *et al.* 1976). How-

ever the human PAM is capable of replication and to some extent the macrophage compartment can be sustained by local cell proliferation (Golde *et al.* 1974). Our findings suggest that the time interval between cessation of smoking and recovery of a PAM population similar in morphology to that of the non-smoker is considerably longer than the life span of the PAM. This is even more likely when one considers that our PAM with inclusions are expressed as a percentage of the total PAM—in absolute terms their numbers would be even higher since smokers have more PAM returned in the BAL than non-smokers (Pratt *et al.* 1969; Warr & Martin 1978). Furthermore, whereas the change in the percentage of host PAM after marrow transplantation is consistent with a linear fall, our data is more consistent with an exponential reduction in the % PAM with inclusions.

These observations can be used to propose several interesting hypotheses. If smokers' inclusions consist to a significant degree of an inorganic core (Brody & Craighead 1975) then, once PAM reach the end of their life span and die (apoptosis), their contents may be taken up, to a varying extent, by other PAM. There is some experimental evidence to support this concept (McLemore *et al.* 1977). Our limited EM study has shown significant inclusions with morphology suggestive of an inorganic silicate content (as described by Brody & Craighead, 1975) in patients up to 270 days after stopping smoking (no ex-smokers having been studied for longer).

Reduced mucociliary clearance in smokers may prolong the slow reduction of % PAM with inclusions after stopping smoking. The better correlations, observed when the original daily cigarette consumption and a natural logarithmic relationship are taken into account, suggest that reduction in % PAM with inclusions does not occur at a constant rate but may be related to cigarette consumption and probably to other factors including clearance. The wide scatter of points indirectly attests to such multifactorial relationships but larger series would have to be studied to take into account such variables as age, lung function, mucociliary clearance, etc. Moreover it is possible that in some patients who had never smoked, or who had stopped smoking, passive exposure to tobacco smoke could have contributed to the small % PAM with inclusions.

Changes in surface antigen expression in cigarette smokers (Lawrence *et al.* 1983) may be a function of alveolar macrophage heterogeneity. Exposure to tobacco smoke may induce the proliferation of a subgroup of PAM, conditioned with respect to their phagocytic, enzymatic or other functions, to handle such an insult. Such a PAM population either might originate from a subtype of circulating monocytes or might maintain itself by local intrapulmonary proliferation. Even after cessation of cigarette smoking, either the persistence of the trigger factors (eg. small amounts of inorganic silicate) or some other endogenous mechanism could perpetuate the population for a long period. The observation of lower % PAM with inclusions in patients with interstitial lung disease (Haslam *et al.* 1980) could be a consequence of changes in PAM populations and/or function in such disease.

After stopping smoking, at least 10 years has to elapse (depending on the original cigarette consumption) before the risks of lung cancer and other smoking-relate pulmonary disease is reduced to that of subjects who never smoked (The Royal College of Physicians of London 1971). This slowly reducing risk may be mainly the consequence of damage inflicted while the subjects were still smoking. However, it is possible that the continued existence of exogenous agents within PAM and/or persisting macrophage activation also may contribute to continuing morbidity and excess mortality in ex-smokers.

Acknowledgements

RMA was supported by the Wellcome Trust to whom we are grateful for support of this

work. We gratefully acknowledge the EM expertise of Miss Teresa Cox and Miss Ann Dewar, and the statistical assistance of Dr M. Campbell.

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