

Surface morphology and function of human pulmonary alveolar macrophages from smokers and non-smokers

MASAYUKI ANDO, MINEHARU SUGIMOTO, RYUJI NISHI, MORITAKA SUGA, SUNAO HORIO, HIROTSUGU KOHROGI, KAZUYOSHI SHIMAZU, SHUKURO ARAKI

From the First Department of Internal Medicine, Kumamoto University Medical School, Kumamoto, Japan

ABSTRACT Pulmonary alveolar macrophages were obtained by saline lavage from 23 healthy male volunteers—10 non-smokers and 13 cigarette smokers. Lavage produced three times as many alveolar macrophages in smokers than in non-smokers. When macrophages from smokers and from non-smokers were incubated *in vitro*, more cells from smokers adhered to glass, spread out, and showed enhanced nitroblue tetrazolium (NBT) reduction. The surface morphology of alveolar macrophages from smokers showed more with a plate like appearance and ridge like membrane surface, while the macrophages from non-smokers were predominantly spherical with ruffles. The proportions of cells which stained highly for β galactosidase were 55% in smokers and 11% in non-smokers. Thus, in a resting state *in vitro*, alveolar macrophages from smokers were more active than those from non-smokers. When, however, macrophages from smokers and non-smokers were incubated with immunobeads and with opsonised or non-opsonised BCG, the phagocytic activity and stimulated NBT reduction of alveolar macrophages from smokers were similar to or somewhat less than those of non-smokers.

Alveolar macrophages act as a main cellular defence for the lung against inhaled microorganisms and other particles.¹ Potential damage to these cells by cigarette smoke could lead to microbial infection and parenchymal damage, possibly mediated through excessive release of several reactive products of alveolar macrophages, including both oxidative agents and lysosomal enzymes.^{2,3} Many studies have therefore been directed at the function and biochemistry of alveolar macrophages but data on functional differences between alveolar macrophages from smokers and non-smokers have often been contradictory.⁴ Furthermore, few have described quantitative differences in both cellular morphology and function.

The aims of the present study were (1) to evaluate the functional differences between alveolar macrophages from smokers and non-smokers with respect to adherence and spreading, phagocytosis, nitroblue

tetrazolium (NBT) reduction, and lysosomal enzymes, and (2) to evaluate the relationship between surface morphology and function.

Our results indicate that in the unstimulated condition alveolar macrophages from smokers appear more active than those from non-smokers in both surface morphology and function. Their responsiveness to foreign bodies or bacteria, however, was equivalent or somewhat inferior to that of non-smokers when determined by phagocytosis and by NBT reduction, which reflects superoxide production by the cells.^{5,6}

Methods

SUBJECTS

The 23 healthy male volunteers were medical students or physicians in our university. Ten were non-smokers and 13 were cigarette smokers. All the smokers had a history of more than five pack years, were currently smoking more than one pack per day, and had done so for more than one year before the study. The mean age of the non-smokers was 27.4 (range 23-37) years and that of the smokers was 27.2 (range 23-35) years.

Address for reprint requests: Dr M Ando, First Department of Internal Medicine, Kumamoto University Medical School, 1-1-1 Honjo, Kumamoto 860 Japan.

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PREPARATION OF ALVEOLAR MACROPHAGES

Alveolar macrophages were obtained during fiberoptic bronchoscopy, a total 100 ml of sterile saline being instilled in aliquots into the lateral subsegment of the right anterior basal segmental bronchus and recovered by gentle suction. Lavage fluids were passed through two sheets of gauze to remove mucus and were centrifuged at 400 g and 4°C for 10 minutes. The resulting pellet was washed three times with Hank's balanced salt solution (HBSS) and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum. After the cell yield had been counted a little of the suspension was smeared for differential cell counts and histochemical study of lysosomal enzymes. The remaining cells were cultured. The cells were more than 90% viable by trypan blue exclusion.

PREPARATION OF CELL MONOLAYERS

Alveolar macrophages at a concentration of 4×10^5 /ml were dispersed on eight tissue culture slides (Lab-Tek). After one hour of incubation at 37°C in 5% carbon dioxide and 95% humidified air, chambers were washed four times with culture medium to remove non-adherent cells, and fresh culture medium was added.

CELLULAR ADHERENCE AND MORPHOLOGICAL CLASSIFICATION

The alveolar macrophages remaining adherent around the centre of the culture slide in the unit area (0.55 mm²) were counted, and the cells were grouped as rounded or spreading cells by morphological criteria. The former were designated non-activated macrophages and the latter activated macrophages.⁷

QUALITATIVE NBT REDUCTION BY STIMULATED AND NON-STIMULATED ALVEOLAR MACROPHAGES

Spontaneous reduction of nitroblue tetrazolium (NBT) by non-stimulated alveolar macrophages was assessed qualitatively in all subjects after one hour's incubation of macrophages monolayers by the supravitral method of Park *et al.*,⁸ as previously reported.⁹

In the stimulated NBT reduction test,

immunobeads (rabbit antihuman IgG, IgA, and IgM; BIO-RAD laboratories) and BCG, which were preincubated with or without fresh serum from a patient with pulmonary tuberculosis, were used as stimulants. The test with immunobeads was performed in six of 10 non-smokers and in 10 of 13 smokers, and the test with BCG in three non-smokers and five smokers. Alveolar macrophages and the immunobeads or the BCG were mixed in a Falcon tube at a ratio of 1:10 in the presence of 0.3% NBT solution. The tubes were incubated in a shaking bath at 37°C for 30 minutes. After incubation the tubes were centrifuged at 400 g at 4°C and the cells were smeared. Alveolar macrophages containing clumped formazan were designated positive cells.

HISTOCHEMICAL STAINING OF LYSOSOMAL ENZYMES

The cells were fixed with cold, buffered 1.25% glutaraldehyde for four minutes and rinsed three times in saline.

Acid phosphatase was demonstrated by the method of Dannenberg *et al.*¹⁰ Five milligrams of naphthol AS-BI phosphate (Sigma) were dissolved in 0.5 ml of dimethylformamide. Fifty millilitres of 0.1 mol/l acetate buffer (pH 5.2) and 10 mg of fast red violet LB were added, along with two drops of 10% manganese dichloride solution as a positive activator. The somewhat opalescent solution was then filtered and the smeared cells were incubated on its surface at room temperature for 20 minutes.

Beta galactosidase was demonstrated by the method of Yarborough *et al.*¹¹ Two milligrams of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Sigma) were dissolved in 0.5 ml of dimethylformamide. Thirty one ml of 0.1 mol/l acetate buffer (pH 5.4), 0.5 ml of saline, and 8 mg of spermidine were added. The smeared cells were incubated in the solution for five hours at 37°C.

Macrophages which stained 2+ to 4+ for the lysosomal enzyme were considered active alveolar macrophages.^{12,13}

SCANNING ELECTRON MICROSCOPY

Alveolar macrophages monolayers for scanning electron microscopy were obtained from six non-

Table 1 Results of bronchoalveolar lavage

	Cell recovery ratio (%)	Total cell count ($\times 10^6$)	Differential cell count (%)		
			AM (%)	Lym (%)	PMN (%)
Non-smokers (n = 10)	36	2.7	83	15	2
Smokers (n = 13)	42	8.4	92	6	2
p	NS	<0.001	<0.01	<0.025	NS

AM—alveolar macrophages; Lym—lymphocytes; PMN—polymorphonuclear leucocytes; NS—not significant.

Table 2 Adherence to glass, morphological alterations, and response to spontaneous nitroblue tetrazolium (NBT) reduction test of alveolar macrophages (AM) from smokers and non-smokers

	No of AM per unit area (0.55 mm ²)	Spreading cells	NBT positive cells
		No of cells per unit area (%*)	No of cells (%*)
Non-smokers (n = 10)	152	49 (32)	51 (32)
Smokers (n = 13)	374	185 (49)	157 (42)
p	<0.001	<0.001	NS

*Percentages refer to proportion of adherent cells.
NS—not significant.

smokers and five smokers. The monolayers were prepared on the coverslips in Lab-Tek chambers. After incubation for one hour the coverslips were removed with forceps, rinsed in 0.2 mol/l cacodylate buffer, and fixed in Karnovskí's fixatives at 4°C for 60 minutes. The samples were post fixed in 1% osmium tetroxide at 4°C for 30 minutes and then passed through graded solutions of ethanol and isoamyl acetate. The specimens were critical point dried, sputter coated with gold palladium, and examined in a JEOL JSM-50A scanning electron microscope at 15 KV and at a tilt angle of 45°.14

STATISTICAL METHODS

Differences between values were analysed by the Wilcoxon rank sum test. Values are given as means. A test result was considered significant if the p value was less than 0.05.

Results

ADHERENCE TO GLASS

Table 1 lists the results of the recovery ratio of bronchoalveolar lavage fluids and total and differential cell counts. The total cell number recovered from smokers was on average three times that from non-smokers ($p < 0.001$), although the recovered volumes of fluid showed no significant differences. In the differential cell counts a significantly greater percentage of alveolar macrophages was found in the fluid of the smokers.

When the cells were incubated at the same concentration of alveolar macrophages, twice as many adherent macrophages were observed in the monolayers of smokers as of non-smokers ($p < 0.001$, table 2). The increased number of adherent cells in the monolayers of smokers was accompanied by a greater proportion of spreading cells. There were also more alveolar macrophages positive for the spontaneous NBT reduction test in the monolayers of smokers. Since a greater proportion of alveolar macrophages in the monolayers of non-smokers were non-adherent and since detached cells are usually negative for the spontaneous NBT reduction

tests, there was a significantly lower overall proportion of NBT positive cells in non-smokers. These results indicate that alveolar macrophages in cigarette smokers are very different from those in non-smokers not only in cell number but also in morphology and function.

SURFACE MORPHOLOGY

To investigate morphological differences more precisely, the surface morphology of the alveolar macrophages was observed by scanning electron microscopy. As shown in table 3, more than half of the macrophages in the monolayers of non-smokers were spherical, their cell surface was covered with ruffles, and they attached to glass with a thin undulating membrane (fig 1). Only 9% were plate like. On the other hand, 33% of the macrophages in the monolayers of smokers showed a plate like appearance ($p < 0.005$). They adhered to glass polygonally with a ridge like cell surface ($p < 0.005$) (fig 2). These alterations of surface morphology of the alveolar macrophages from cigarette smokers parallel the enhancements in adherence, spreading, and spontaneous NBT reduction.

SPONTANEOUS AND STIMULATED NBT REDUCTION TEST

When spontaneous NBT reduction by non-

Table 3 Surface morphology of alveolar macrophages from smokers and non-smokers

	% of non-smokers (n = 6)	% of smokers (n = 5)
Shape		
Plate	9	33*
Hemisphere	28	34
Round	63**	33
Surface		
Ridge	12	36*
Ruffie	88*	64
Adherence		
Polygonal	11	35*
Lamellipodia	14	15
Filopodia	7	6
Undulation	68***	44

Significance of difference between smokers and non-smokers: * $p < 0.005$; ** $p < 0.01$; *** $p < 0.025$.

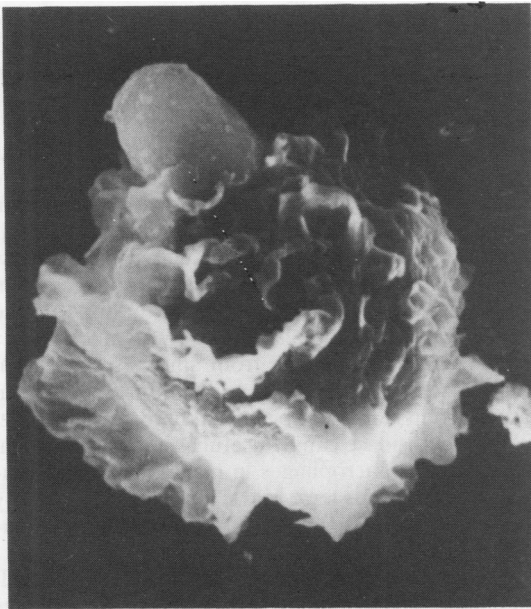


Fig 1 Pulmonary alveolar macrophage from a non-smoker showing the rounded appearance and the cell surface covered with ruffles ($\times 4000$.)

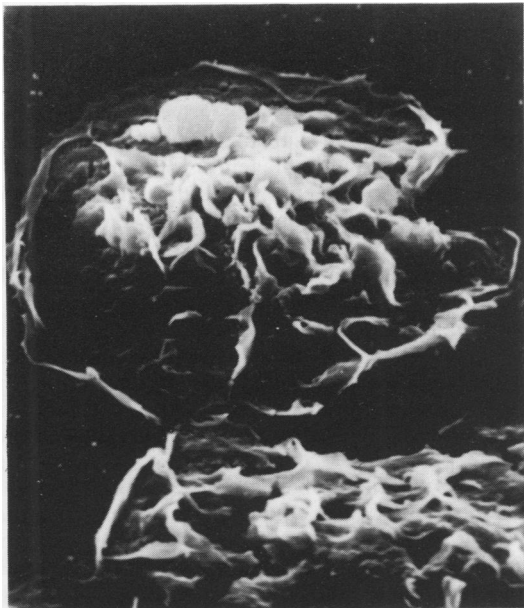


Fig 2 Pulmonary alveolar macrophage from a smoker showing the plate like appearance and the ridged cell surface ($\times 2700$.)

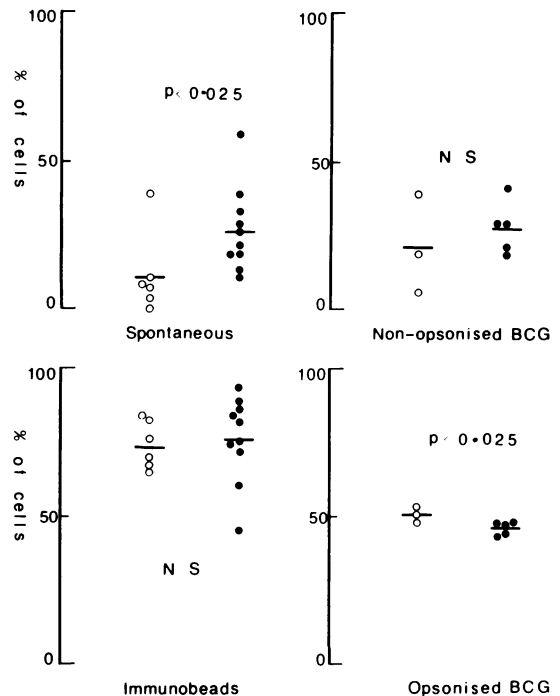


Fig 3 Spontaneous and stimulated nitroblue tetrazolium reduction by macrophages from non-smokers (○) and smokers (●). NS—not significant.

stimulated alveolar macrophages was examined in a tube by shaking incubation, more macrophages positive for the NBT test came from the smokers (fig 3). This is consistent with the results of the alveolar macrophages monolayers cultured on chamber slides. These results show that, compared with non-smokers, macrophages from smokers show enhanced activity of spontaneous NBT reduction in any state of free or adherent cells.

On the other hand, when alveolar macrophages from smokers and non-smokers were incubated with immunobeads 90% of the macrophages phagocytosed the immunobeads, and most of the phagocytosed cells showed greatly enhanced NBT reduction (fig 3). There were, however, no significant differences between macrophages from smokers and non-smokers in terms of the percentage of phagocytosed cells or of the percentage of cells which were positive for the stimulated NBT reduction test. Similar results were obtained when opsonised or non-opsonised BCG were used. Alveolar macrophages from either smokers or non-smokers phagocytosed opsonised BCG more than non-opsonised BCG ($p < 0.05$), and showed enhanced

Table 4 Lysosomal enzyme activities of alveolar macrophages from smokers and non-smokers

	% of cells 2+—4+	
	β galactosidase	Acid phosphatase
Non-smokers (n = 10)	11	89
Smokers (n = 13)	55	97
p	<0.001	<0.01

NBT reduction when the macrophages phagocytosed opsonised BCG but not non-opsonised BCG ($p < 0.05$). But, except for decreased NBT reduction of macrophages from smokers which phagocytosed opsonised BCG no differences were observed between macrophages from smokers and non-smokers.

These results indicate that, in a resting state, alveolar macrophages from smokers are more active than alveolar macrophages from non-smokers but that their responsiveness to foreign matter or bacteria is the same or less.

LYSOSOMAL ENZYMES

Histochemical methods showed apparent differences in activity between acid phosphatase and β galactosidase in human alveolar macrophages. Generally, the former was highly active and the latter relatively inactive. The proportion of cells strongly positive (2+ to 4+) for acid phosphatase among the macrophages was 97% in smokers and 89% in non-smokers ($p < 0.01$) (table 4). In the case of cells highly positive for β galactosidase, however, the proportions were 55% in smokers and 11% in non-smokers ($p < 0.001$). Monocytes from either smokers or non-smokers stained no more than 1+ for both enzymes. These results show that a histochemical study for β galactosidase is useful as a marker of local activation in human alveolar macrophages, and that the lysosomal enzyme activities of macrophages in smokers are increased.

Discussion

The results in this report clearly show that in a resting state *in vitro* alveolar macrophages from smokers differ from those of non-smokers in surface morphology and function. The characteristic features of alveolar macrophages from smokers are similar to those of activated macrophages as judged by our criteria of macrophage activation. In our experimental system, macrophages activated *in vitro* or *in vivo* by various stimulants show enhancement of adherence, spreading, and NBT reduction, accompanied by increases in superoxide production and antibacterial activity.^{6,7} The plate like appearance and adherence to glass polygonally with ridge

like cell surface are also consistent with the surface morphology of activated macrophages.¹⁴ As our previous report showed, perturbation of cell membranes of alveolar macrophages correlated well with enhanced production of superoxide by the cells.¹⁵

Activation of the alveolar macrophages of smokers in the resting state may be induced locally in the lung by stimulation by particulate matter present in smoke. Warr and Martin¹⁶ reported a strong correlation between lysosomal enzyme activity and yellow brown pigmentation in alveolar macrophages from smokers. This is consistent with our finding that the lysosomal enzyme β galactosidase was much higher in alveolar macrophages from smokers than from non-smokers, whereas human monocytes from either group stained at best weakly for this enzyme. Beta galactosidase can be useful as a marker of local activation of macrophages in man as well as in animals because this enzyme is increased locally by immunologically specific and non-specific stimulation.¹⁷

Because the number of lavaged cells was small, qualitative NBT reduction by alveolar macrophages was performed to observe the superoxide production indirectly. In our experimental system more than half of the NBT reduction by alveolar macrophages was due to superoxide.⁶ Thus the observation that macrophages from smokers showed enhanced NBT reduction indicates that the cells produce superoxide excessively and release it extracellularly. Superoxide and its derivatives, highly toxic oxidants, could cause injury to the lung parenchyma or to the macrophages themselves. This might in turn lead to release of lysosomal enzymes, including proteolytic substances, which are particularly related to the pathogenesis of emphysema.^{2,3}

The enhanced activity of the alveolar macrophages of smokers in the resting state might be favourable to the handling of foreign materials and bacteria, though the functional enhancement might lead to tissue injury in other ways. Superoxide is a major component of the bactericidal agents of macrophages as well as of polymorphonuclear leucocytes, and lysosomal enzymes participate in the digestion of ingested particles or killed bacteria in phagosomes.^{17,18} Our results, however, showed that the responsiveness of alveolar macrophages from smokers to immunobeads and opsonised or non-opsonised BCG was equivalent to or less than that of non-smokers with respect to phagocytosis and stimulated NBT reduction. In other words, macrophages from non-smokers are fully capable of reacting against foreign materials and bacteria, with or without opsonic requirements. Thus chronic stimulation of alveolar macrophages by particulate matter present in cigarette smoke may be harmful

rather than beneficial to the lung defence by macrophages.

Finch *et al*⁴ recently summarised many of the general observations concerning differences between alveolar macrophages from smokers and non-smokers, and they reported that the data on functional differences were often inconsistent. For example, with regard to adherence of macrophages from smokers, Mann *et al*¹⁹ reported greater adherence to glass, while Rasp *et al*²⁰ reported decreased adherence to nylon fibres and Fisher *et al*²¹ reported macrophages from smokers and non-smokers to be equivalent. Fisher *et al*²¹ and Martin²² reported diminished phagocytic activity of alveolar macrophages from smokers, while Harris *et al*,²³ Cohen *et al*,²⁴ Reynolds *et al*,²⁵ and Territo *et al*²⁶ reported no differences from non-smokers. Similar discrepancies have been observed in the results of chemotactic activity and oxidative metabolism.^{23 27-29} This may be due to differences between the subjects, including their smoking history, as well as between experimental methods.

Undoubtedly, the effects of cigarette smoke on the morphology and function of alveolar macrophages are related to regular daily cigarette consumption and they are aggravated by the amount and duration of smoking. Hoidal *et al*³⁰ reported that macrophages from older smokers (mean 40 pack years) had a more intense, generalised enhancement of oxidative metabolism than macrophages from younger smokers (mean seven pack years). Martin²² reported that the increases of acid phosphatase and β glucuronidase in macrophages from smokers correlated with the daily cigarette consumption and our results are consistent with this observation.

In relation to the appearances on scanning electron microscopy, Finch *et al*³¹ reported that alveolar macrophages from smokers had a greater incidence of rounded cells, surface ruffles, filopodia, and multiple surface features, while macrophages from non-smokers had greater incidences of spreading cells and featureless cells. The cells from smokers also showed depressed phagocytosis.²¹ The observations of this group are virtually opposite to ours. The discrepancy may be due to the differences in the experimental conditions. They held the cells over wet ice at 4°C in a centrifuge tube for about six hours during shipping from hospital to laboratory, whereas we cultured immediately after harvesting of the cells. Conceivable the preservation could impair the morphology and function of highly activated cells more than of non-activated, resting cells. In our present study no attempt was made to separate the alveolar macrophages from other cells to avoid procedures after lavage that could affect the results. Almost all of the adherent cells were macrophages,

however, and contamination by lymphocytes and polymorphonuclear leucocytes was negligible. Other results^{23 26 30} support our findings that the cell yield in smokers increased threefold to fourfold, and that over 90% of the cells were macrophages.

In summary, our results suggest that chronic stimulation of cigarette smoke is harmful for lung defence as assessed by both morphology and function of alveolar macrophages.

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