

Short-term *in vivo* exposure to cigarette-smoke increases the fluorescence in rat alveolar macrophages

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Short-term in vivo exposure to cigarette-smoke increases the fluorescence in rat alveolar macrophages. C.M. Sköld, K. Andersson, J. Hed, A. Eklund. ©ERS Journals Ltd 1993.

ABSTRACT: Alveolar macrophages (AMs) from human smokers exhibit a strong intracellular fluorescence. In order to study the mechanisms behind the fluorescence, we wanted to determine whether it could be induced by short term exposure to smoke.

We therefore exposed rats for either one or five days to cigarette smoke. AMs were recovered by bronchoalveolar lavage (BAL), and their fluorescence was quantified by flow cytometry.

AMs from rats exposed to two cigarettes, on a single occasion, showed an increased fluorescence compared to controls. The fluorescence was further enhanced by exposing rats to two cigarettes, for five consecutive days. Larger and more granular/complex AMs were more fluorescent than smaller and less granular/complex cells. Smoke-exposed rats (five consecutive days) lavaged immediately after the exposure, had less cells in their BAL fluid than control animals. However, when rats were lavaged three smoke-free days after the exposure, the cell recovery increased, probably due to less airway obstruction.

In conclusion, we found that a short-term *in vivo* exposure to cigarette smoke results in an increased AM fluorescence. The findings can be one explanation for the strong fluorescence seen in AMs from human smokers, possibly due to endocytosis of fluorescent material.

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Alveolar macrophages (AMs) are the main phagocytic cells in the alveoli. Human smokers have more AMs in their alveoli than nonsmokers, as reflected by an elevated cell yield in bronchoalveolar lavage (BAL) fluid [1]. AMs from human smokers exhibit a strong intracellular fluorescence [2], which can persist for more than 2 yrs after cessation of smoking [3, 4]. We have recently shown [5], in an *in vitro* model, that this fluorescence is due to endocytosis of fluorescent particles from tobacco smoke. Thus, the fluorescence is rapidly inducible *in vitro* by incubating AMs with a cigarette-smoke condensate. The aim of the present study was to assess whether a short-term *in vivo* exposure to cigarette-smoke alters the fluorescent properties of rat AMs.

Material and methods

Animals

Male, pathogen-free, Sprague-Dawley rats (250 g) were used in the study. They were given food pellets and wa-

ter *ad libitum*. The study had the approval of the local Ethics Committee.

Smoke exposure

The animals were exposed to cigarette smoke in a Walton smoke exposure machine [6] (Process & Instruments Corp., Brooklyn, NY, USA). In this apparatus, six rats can simultaneously be exposed to smoke from one cigarette. The smoke is transferred into smoke exposure chambers, in which the animals are kept. Each puff of smoke leads to a 30 s smoke exposure period, which is followed by a purge period of 30 s, to sweep out the smoke. Smoke dilution of about 1/10 is attained in the chamber. The total exposure time for one cigarette is 10 min.

In the present study, two groups of six rats each were exposed to two cigarettes, for one or five days, respectively. Kentucky reference cigarette IR-1 (Tobacco and Health Institute, University of Kentucky, Lexington, KY, USA) were used, generating 40 mg tar, 2.6 mg nicotine and 17 cm³ carbon monoxide per cigarette. A control group (n=6) was sham-exposed with air, for four days.

Bronchoalveolar lavage

Rats were sacrificed by exposure to carbon dioxide, immediately after the last smoke exposure. Bronchoalveolar lavage was then performed as described previously [7]. Briefly, the trachea was cannulated, and the lungs were lavaged with Hank's balanced salt solution (HBSS). The lavage medium was introduced into the lungs by gravity in aliquots of 5 ml. The total volume instilled was 50 ml. The lavage fluid was drained into a tube placed on ice. Massage was given over the thorax to increase the recovery. The BAL fluid was strained through a double layer of Dacron nets, and then washed by centrifugation at $400 \times g$ for 5 min at 4°C . Cells were resuspended in Hank's balanced salt solution. Total cell count was performed in a Bürker chamber. Smears for differential counts were performed by cytocentrifugation at 500 rpm for 10 min (Cytospin 2, Shandon, UK). Smears were stained with May-Grünwald Giemsa, and 500 cells were counted.

In initial experiments, we observed that the cell yield in the BAL fluid decreased when the rats were lavaged immediately after smoke exposure. Therefore, additional groups of rats were exposed, as described above, for one ($n=6$) and five ($n=6$) days, and lavaged after three smoke-free days.

Measurement of fluorescence intensity

AM fluorescence was measured in an Epics Profile flow cytometer (Coulter Electronics, Hialeah, FL, USA). In the instrument, each cell is represented by a point in a co-ordinate system, depending on the light scattering properties, as the cell passes a laser beam. Forward scatter represents cell size. Side scatter yields information on surface topography and internal structures of cells. This parameter is designated complexity/granularity. In addition to size and complexity/granularity, cell fluorescence can be measured. The fluorescence intensity was quantified in arbitrary units, as the mean fluorescence intensity (MFI) of the AMs within the gating frame (figs 1 and 2). The whole AM population was analysed by gating the AM cell cluster [2, 8]. In addition, gating frames were set around two subpopulations of AMs: a cluster of less complex cells, and a population of larger and more complex AMs (fig. 1). This was done to determine how size and complexity may influence the fluorescent properties of the AMs.

The excitation wavelength was 488 nm, and emission was measured with a wavelength range of 525–550 nm. The instrument was calibrated daily with standardized fluorescent particles and a logarithmic scale was used.

Statistical methods

The data are presented as median and range, unless otherwise stated. Analysis of the significance levels were made with the non-parametric Mann-Whitney test.

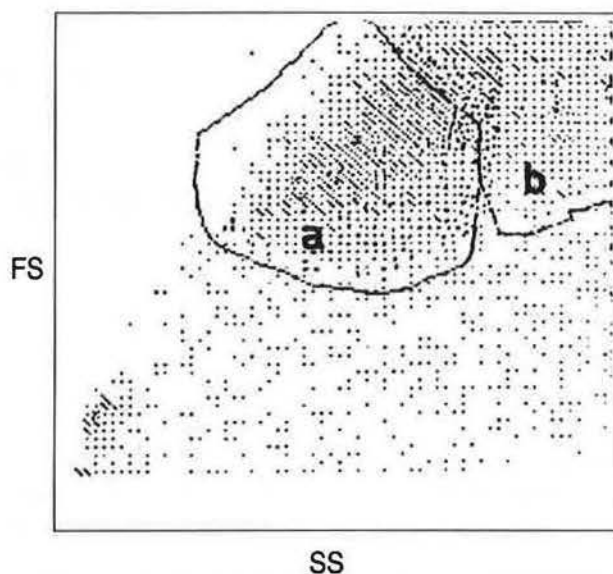


Fig. 1. - Cytogram, indicating rat alveolar macrophages (AMs) cell cluster. The X-axis (SS, side scatter) and Y-axis (FS, forward scatter) represent cell-complexity/granularity and cell-size, respectively. Each cell is, in principle, represented by a point in a co-ordinate system. The gating frames were set around the whole AM population (a+b), smaller and less complex/granular AMs (a) and larger, more complex/granular cells (b).

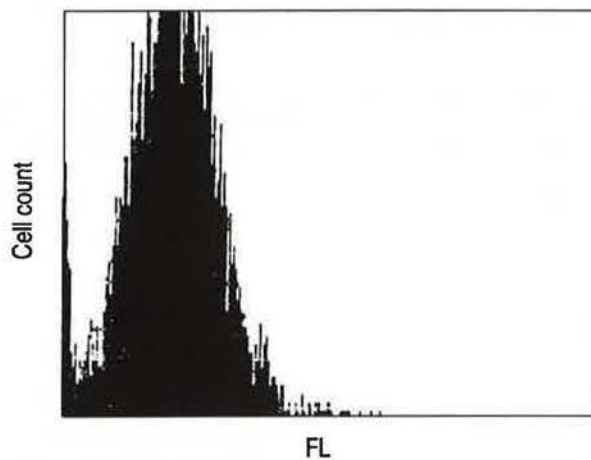


Fig. 2. - Representative histogram indicating the fluorescence (FL) of the whole non-exposed alveolar macrophage population. The number of cells are indicated on the Y-axis.

Results

General lavage data

The recovery of the instilled fluid was $>90\%$ in all rats. Viability, as tested by trypan blue exclusion, was $>95\%$ in all samples. The total number of cells and the cell concentration were lower in the groups lavaged immediately after smoke exposure compared to controls ($p < 0.01$ for rats exposed 1 day, and $p < 0.05$ for rats exposed 5 days) (table 1). In contrast, rats exposed for one day, and then lavaged after three smoke-free days, had no significant alterations in their total number of cells, or in their cell concentration, compared to controls.

Table 1. - General characteristics of BAL fluid recovered from rats exposed to cigarette smoke *in vivo*

Group	Total cell yield $\times 10^6$	Cell concentration $\times 10^6 \cdot l^{-1}$	Differential count % of total cells			
			AM	LY	PMN	EOS
Controls n=12	5.0 (3.7-6.1)	96.9 (83.3-130.0)	96.8 (84.0-99.2)	2.0 (0.6-3.5)	1.3 (0.2-12.7)	0 (0-4.8)
Rats lavaged immediately after smoke exposure						
1 day exposure n=6	2.6** (1.0-4.4)	56.7** (23.3-96.7)	89.0 (74.0-98.0)	5.8 (1.0-22.0)	3.0 (1.0-6.0)	0 (0-1)
5 days exposure n=6	3.4* (1.4-4.5)	75.8* (31.6-100.0)	97.0 (74.0-97.0)	2.0 (1.0-16.0)	1.0 (1.0-9.0)	0 (0-1.4)
Rats lavaged three days after smoke exposure						
1 day exposure n=6	4.1 (3.3-6.3)	92.2 (73.3-140.0)	94.2 (75.3-98.3)	2.8 (1.4-10.3)	2.0 (0.3-2.7)	0.5 (0-12.9)
5 days exposure n=6	7.1** (5.6-9.3)	155.0** (121.7-206.7)	94.0 (70.0-98.0)	2.8 (1.3-20.0)	1.5 (0.5-9.0)	0.6 (0-1.2)

Data are given as median and range in parenthesis. BAL: bronchoalveolar lavage; AM: alveolar macrophages; LY: lymphocytes; PMN: neutrophils; EOS: eosinophils. Significance levels are calculated vs controls. *: $p < 0.05$; **: $p < 0.01$.

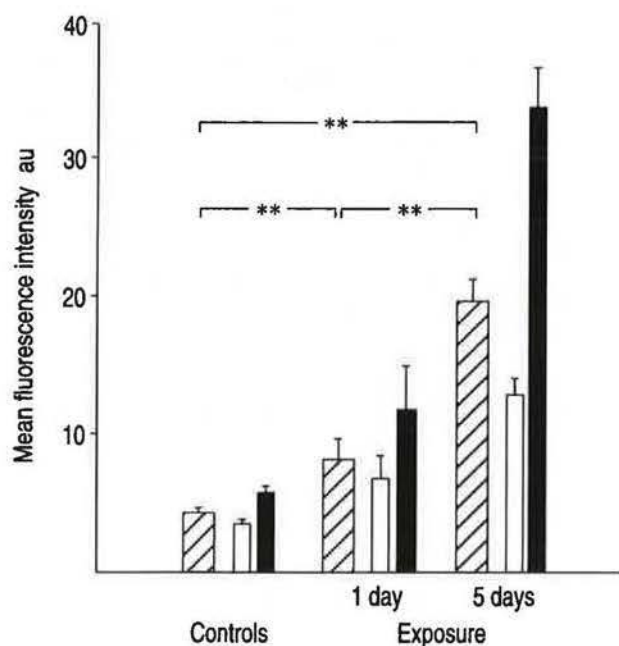


Fig. 3. - Mean fluorescence intensity (arbitrary units) of alveolar macrophages (AMs) lavaged from rats exposed to two cigarettes *in vivo* for one (n=6), or five (n=6) consecutive days. \square : whole AM populations; \square : smaller, less complex AMs; \blacksquare : larger, more complex AMs, (see text). Mean values with standard deviations are given. **: $p < 0.01$. au: arbitrary units.

The five days exposed animals, which were lavaged three days after the smoke exposure, had, however, a significantly ($p < 0.01$) increased total cell yield and cell concentration, compared to the control animals (table 1). There were no differences between the groups in the proportions of AMs, lymphocytes, neutrophils or eosinophils in the BAL fluid.

Alveolar macrophage fluorescence

The fluorescence of the AMs, lavaged immediately after smoke exposure, increased ($p < 0.01$) after exposure to two cigarettes on a single occasion. After exposure to two cigarettes for five consecutive days, the AM fluorescence was further enhanced ($p < 0.01$) (figure 3). The larger and more complex AMs had higher fluorescence than the smaller and less complex cells. This was found in all three groups of animals, including the non-exposed (control) group.

Discussion

The fluorescence in AMs from human smokers is known to be higher than in AMs from nonsmokers [2]. It is a matter of controversy whether the fluorescence originates from storage pigments, is due to altered metabolic status of the cell, or to endocytosed particles from tobacco smoke [9-11]. We have previously shown [5] that *in vitro* exposure of AMs to a soluble cigarette-smoke condensate, rapidly increases the fluorescence, suggesting an endocytosis of fluorescent material. Furthermore, the fluorescent material could be transferred from one cell population to another.

In this study, we found an enhanced fluorescence in a dose-dependent manner in rat AMs after exposure to cigarette smoke *in vivo*.

AMs from smokers contain cytoplasmic inclusions ("smokers' inclusions") which fluoresce [12-14]. Previous studies [2, 3, 15] have also shown that AM fluorescence is associated with the cell size and the cell complexity/granularity, and that the distribution of fluorescent material in the AMs is granular. The present study supports these findings. Thus, we found that the AMs with the highest fluorescence were also the largest and most complex/granular. This suggests an uptake of fluorescent

material into lysosomes and phagolysosomes. However, it cannot be completely ruled out, that an activation of the cells with more endocytic vesicles could also contribute to the enhanced fluorescence in the lavaged cells.

The number of AMs lavaged from human smokers compared to nonsmokers is considerably increased [1]. A corresponding increase has also been shown in animals lavaged after smoke exposure [16]. Notably, in the present study, the total number of cells, as well as the cell concentration in the smoke-exposed rats decreased significantly when the animals were lavaged immediately after the smoke exposure. Since human smokers may have an obstructive lung function impairment, [17, 18], one possible explanation for the decreased number of cells could be that the exposure *per se* might have induced airway obstruction. This may have caused an impaired penetration of the instilled fluid into the alveolar space, resulting in a decreased cell recovery. This was not observed when the rats were lavaged after three smoke-free days, indicating an "acute" effect of the smoke-exposure.

In conclusion, we found a significantly increased fluorescence in rat AMs after short-term *in vivo* exposure to cigarette smoke. The findings can explain the strong fluorescence observed in AMs from human smokers. It also supports the hypothesis that endocytosis of fluorescent material is a possible cause of the high fluorescence in AMs from smokers.

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References

1. Cherniack RM (co-ordinator). – Bronchoalveolar lavage constituents in healthy individuals, idiopathic pulmonary fibrosis, and selected comparison groups. *Am Rev Respir Dis* 1990; 141: S169–S202.
2. Sköld CM, Eklund A, Halldén G, Hed J. – Autofluorescence in human alveolar macrophages from smokers; relation to cell surface markers and phagocytosis. *Exp Lung Res* 1989; 15: 823–835.
3. Sköld CM. – Mechanisms of smoke-induced fluorescence in alveolar macrophages. Thesis, Karolinska Institute, Stockholm, Sweden, 1991.
4. Sköld CM, Hed J, Eklund A. – Smoking cessation rapidly reduces cell recovery in bronchoalveolar lavage fluid, while alveolar macrophage fluorescence remains high. *Chest* 1992; 101: 989–995.
5. Sköld CM, Eklund A, Hed J, Hernbrand R. – Endocytosis of cigarette-smoke condensate by rabbit alveolar macrophages *in vitro* measured as fluorescence intensity. *Eur Respir J* 1992; 5: 53–58.
6. Guerin M, Stockley J, Higgins C, Moneyhun J, Holmberg G. – Inhalation bioassay chemistry: Walton Horizontal Smoking Machine for inhalation exposure of rodents to cigarette-smoke. *J Nat Cancer Inst* 1979; 63: 441–448.
7. Tornling G, Eklund A, Engström-Laurent A, Hällgren R, Unge G, Westman B. – Hyaluronic acid in bronchoalveolar lavage in rats exposed to quartz. *Br J Ind Med* 1987; 44: 443–445.
8. Sköld CM, Eklund A, Halldén G, Hed J. – Different cell surface and phagocytic properties in mononuclear phagocytes from blood and alveoli. *Acta Pathol Microbiol Scand* 1990; 98: 415–422.
9. Aubin JE. – Autofluorescence of viable cultured mammalian cells. *J Histochem Cytochem* 1979; 27: 36–43.
10. Benson RC, Meyer RA, Zaruba ME, McKhann GM. – Cellular autofluorescence: is it due to flavins? *J Histochem Cytochem* 1979; 27: 44–48.
11. Edelson JD, MacFadden DK, Klein M, Rebuck AS. – Autofluorescence of alveolar macrophages: problems and potential solutions. *Med Hypotheses* 1985; 17: 403–408.
12. Martin RR. – Altered morphology and increased acid hydrolase content of pulmonary macrophages from cigarette smokers. *Am Rev Respir Dis* 1973; 107: 596–601.
13. Roque AL, Pickren JW. – Enzymatic changes in fluorescent alveolar macrophages of the lungs of cigarette smokers. *Acta Cytol* 1968; 12: 420–429.
14. Vassar PS, Culling C, Saunders AM. – Fluorescent histiocytes in sputum related to smoking. *Arch Pathol* 1960; 70: 649–652.
15. Kradin RL, McCarthy KM, Preffer FI, Schneeberger EE. – Flow cytometric and ultrastructural analysis of alveolar macrophage maturation. *J Leuk Biol* 1986; 40: 407–417.
16. Hornby SB, Kellington JP. – DNA synthesis in alveolar macrophages and other changes in lavaged cells following exposure of CBA/H mice to cigarette smoke. *Environ Health Perspect* 1990; 85: 107–112.
17. Woolcock AJ, Peat JK, Salome CM, *et al.* – Prevalence of bronchial hyperresponsiveness and asthma in a rural adult population. *Thorax* 1987; 42: 361–368.
18. Wright JL, Lawson LM, Paré PD, Wiggs BJ, Kennedy S, Hogg JC. – Morphology of peripheral airways in current smokers and ex-smokers. *Am Rev Respir Dis* 1983; 127: 474–477.