Eur Respir J
1992, 5, 53–58

Endocytosis of cigarette-smoke condensate by rabbit alveolar macrophages *in vitro* measured as fluorescence intensity

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ABSTRACT: It has previously been shown that alveolar macrophages (AMs) from smokers have increased fluorescence compared to AMs from nonsmokers. In the present investigation, AMs lavaged from rabbits were exposed to a cigarette-smoke condensate (CSC) in order to study its influence on the fluorescence of AMs. Flow cytofluorometry was used.

Alveolar cells exposed to 0.01% of CSC showed a fluorescence which increased with higher concentrations of the CSC. Incubation with CSC for 5 min was enough to give a significant rise ($p<0.001$) in cell fluorescence compared to controls. After incubating fluorescent cells with untreated non-fluorescent AMs, fluorescent material was distributed to the non-fluorescent AMs within 30 min. Furthermore, fluorescence decreased when exposed cells were cultured in CSC-free medium. When incubating CSC with blood leucocytes, an almost threefold increase in fluorescence was observed among the phagocytic cells at 37°C compared to 4°C, whereas the fluorescence in the non-phagocytic cell population only showed a minor enhancement.

Endocytosis of fluorescent material in CSC can explain the strong fluorescence seen in AMs from smokers. Exocytosis and re-endocytosis of the material by new AMs may explain the slow decline in the fluorescence of AMs observed after cessation of smoking.

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Keywords:
Alveolar macrophages
cigarette-smoke condensate
endocytosis
fluorescence.

Accepted July 1, 1991 after revision.

This study was supported by grants from the Swedish Medical Research Council (grant 16 X-105) and the Swedish Heart-Lung Foundation.

Alveolar macrophages (AMs) are the resident phagocytes in the alveoli. They are located in the epithelial lining fluid and are the only macrophages that live in a body compartment that connects directly with the atmosphere [1–4]. Consequently, in smokers, AMs are exposed to a large variety of substances compared to AMs from nonsmokers. This inhaled cigarette-smoke consists of a gaseous and a particulate phase. Both are known to comprise several thousand components [5]. Many of these substances are known to possess fluorescent properties [6].

The bronchoalveolar lavage (BAL) technique provides excellent opportunities to recruit and study alveolar cells from patients with various diseases, while also providing a relatively benign approach for studying cells from healthy smokers and nonsmokers [7]. The ultrastructure of AMs from smokers differs from that of nonsmokers in that they contain more cytoplasmic organelles and vesicles [8, 9]. In addition, AMs, when examined under ultraviolet light, show a fluorescence which is stronger in those from smokers than from nonsmokers [10, 11]. By the use of flow cytofluorometry we have recently described this difference in fluorescence [12]. The source of the fluorescence in AMs from smokers has not been clarified, and it remains questionable whether it originates from phagocytosed exogenous material or is due to cells that are metabolically active [13–16]. The aim of the present investigation was to elucidate the *in vitro* effect of a cigarette-smoke condensate (CSC) on the fluorescent properties of AMs. This could increase our knowledge about the origin of the strong fluorescence seen in AMs from smokers.

Materials and methods

Animals

White New Zealand rabbits of either sex weighing 2.0–3.5 kg were purchased from a local supplier (ESP-Produkter i Estuna AB, Norrtälje, Sweden). They received food and water *ad libitum*. The study had the approval of the local Ethics Committee.

Bronchoalveolar lavage

The animals were given sedative intramusculary (Hypnorm®, Janssen GmbH, Neuss, FRG) before they were killed by an overdose of pentobarbital (Mebumal Vet®, Nord Vacc, Skärholmen, Sweden). The trachea was identified, and the rabbits were intubated by tracheostomy. Bronchoalveolar lavage was carried out in 10 ml aliquots of Hank's balanced salt solution.

Fluid was recovered by gentle aspiration and it was kept on ice until further processed. The total volume of instilled fluid was 200–300 ml, and at least 80% of the instilled fluid was recovered during the BAL procedure.

Preparation of alveolar macrophages

The BAL cells were pelleted by centrifugation at 400 × g, 4°C for 5 min and the supernatants were poured off. Cells were counted in a Bürker chamber and the viability was assessed by trypan blue exclusion. The total cell yield for each rabbit varied from 10–25 × 10^6 cells and the viability always exceeded 90%. Smears for differential counts were prepared by cytocentrifugation at 500 rpm for 3 min (Cytospin 2, Shandon, Southern Products Ltd, Runcorn, UK) and they were stained with May-Grünwald Giemsa. The differential counts revealed that more than 90% of the cells were AMs. Cells were resuspended in a culture medium: RPMI 1640 with 2.00 $g \cdot l^{-1}$ sodium bicarbonate without l-glutamine (Northumbria Biologicals Ltd, Cramlington, UK), supplemented with 10% fetal calf serum and 0.1% gentamicin (Gibco Ltd, Paisley, Scotland, UK).

Preparation of cigarette-smoke condensate

Cigarette-smoke condensate (CSC), a gift from the Swedish Tobacco Company, was prepared as described previously [17]. Briefly, non-filter cigarettes of American blend type (containing 23 mg tar, 11 mg carbon monoxide and 1.8 mg nicotine per cigarette) were smoked in a Borgwaldt automatic smoking machine (Type R 09.01, Heinr. Borgwaldt, Hamburg, FRG) applying standard smoking procedures [18]. The CSC was condensed in an Elmenhorst trap [19], cooled in a dry ice/ethanol mixture. Twenty thousand cigarettes yielded 635 g of CSC, which consisted both of the particulate and volatile fraction. Stock solutions (50 $mg \cdot ml^{-1}$) were made up in dimethyl sulphoxide (DMSO) and stored at -70°C until further used.

Alveolar macrophage culture and exposure to CSC

Culture with different concentrations of CSC. Cell suspensions in culture medium were seeded in tissue culture multiwell plates (Art no 3512, Cluster, Costar, Cambridge, MA, USA) at a concentration of 0.5–1.0 × 10^6 cells per well. They were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 90 min. The supernatants were replaced by fresh medium containing different concentrations of stock CSC (0.0001%, 0.001%, 0.01%, 0.1% and 1% v/v). Cells were then cultured for another 40 h. They were harvested by treating each well with 1 ml cold 0.15 M phosphate buffered saline (PBS), pH 7.4, supplemented with 10 mM edetic acid (EDTA) and 0.02% NaN_3 (PBS-EDTA), and by gently rubbing with a plastic scraper over the bottom of the well. This harvesting procedure did not significantly reduce the cell number. The cells were kept on ice and immediately analysed in the flow cytofluorometer.

Culture with CSC at various periods of time. Cells were seeded as described above and cultured in medium alone for 24 h. The supernatants were then replaced by medium containing 0.1% (v/v) of CSC. The culture was interrupted by harvesting the cells at different intervals between 5 min and 48 h and the cells were kept on ice and immediately analysed in the flow cytofluorometer. Cells cultured in medium alone were used as controls.

Culture of fluorescent cells in CSC-free medium. In this experiment a cell population with maximum fluorescence was cultured in CSC-free medium. The cells were first cultured for 24 h in medium alone. A maximum fluorescence was obtained by exposing them to 0.5% (v/v) of CSC for 120 min. The cells were washed once and pelleted by centrifugation at 400 × g, 4°C for 5 min, resuspended in culture medium and seeded as described above. A parallel experiment was performed in a similar manner but the cells were washed and the culture medium exchanged at 4, 8, 24 and 48 h of culture. No significant cell loss during these treatments was observed. Cells from both experiments were harvested at 8, 24, 48 and 72 h and immediately analysed in the flow cytofluorometer.

Mixture of fluorescent and non-fluorescent cells. Equal amounts (0.5–1.0 × 10^6 cells in 1 ml of culture medium) of non-fluorescent and fluorescent (0.5% of CSC for 120 min) cells were mixed and cultured together (n=4). The cells were harvested and their fluorescence was analysed in the flow cytofluorometer immediately before and after the mixture, as well as at different points of time.

Incubation of non-phagocytic and phagocytic cells with CSC

To assess whether the supposed uptake of CSC is due to an endocytic process, an experiment was performed with leucocytes incubated with CSC. Peripheral blood was collected from healthy blood donors (n=6). The erythrocytes were lysed by adding 2 ml NH_4Cl-EDTA to 100 µl of blood. The suspensions were incubated for 2–5 min at 15°C and then washed by centrifugation at 300 × g for 5 min at 4°C. The leucocyte pellets were washed in 2 ml cold PBS-EDTA and incubated with 1 ml of 0.1% of CSC for 30 min at either 4°C or 37°C. Control samples were incubated in RPMI 1640 alone. The incubation was interrupted by washing the cells in cold PBS-EDTA. The fluorescence of CSC-exposed lymphocytes (non-phagocytic cells) and monocytes

(phagocytic cells) was then detected in the flow cytofluorometer after gating the actual cell population.

Measurement of fluorescence intensity

Alveolar macrophages were analysed in an Ortho Spectrum III flow cytofluorometer (Ortho Diagnostic Systems, Westwood, MA, USA) as described recently [12]. In the instrument each cell is assigned two values depending on the light scatter properties as the cell passes through a laser beam: forward scatter and right scatter. These values represent cell size and cell complexity, respectively, and based on these two values each cell is represented by a point in a rectangular co-ordinate system (cytogram). The cell cluster of AMs was identified and a gating frame was set around it. Contaminating blood cells did not interfere with the analysis since they were not within the gating frame in the flow cytofluorometer [20]. In the experiment where peripheral blood was used, a gating frame was set around the lymphocyte and monocyte fields, respectively [20]. The laser power of the instrument was set to 20 mW and the laser wavelength (excitation) was 480 nm. The fluorescence was measured in arbitrary units as the mean fluorescence intensity (MFI) of the cells within the actual field with an emission wavelength range from 515–530 nm. The instrument was calibrated daily with standardized fluorescent (fluorescein isothiocyanate) particles and a linear scale was used.

Statistical analysis

An Anova model (randomized factorial design) was used for the concentration-and time-dependent experiments. In the latter experiment (time-dependent), logarithmic values were used in the calculations since the variances were dependent on the value levels [21]. In the experiment where AMs were cultured in CSC-free medium an Anova model with repeated measures design was used. Dunnett's test was used for testing differences among means [21].

Results

The influence of CSC-exposure on AMs fluorescence

The effects of different concentrations of CSC. Cells exposed to 0.01% (v/v) of CSC showed a significant ($p<0.05$) increase in fluorescence compared to cells cultured in medium alone (fig. 1). At 1% (v/v) of CSC, a maximal fluorescence was obtained in the test system used. When exposed cells were examined with a microscope under ultraviolet light, a granular, cytoplasmic fluorescence was seen.

The effects of various periods of time. Only 5 min of incubation with CSC at a concentration of 0.1% was required to induce a significant ($p<0.001$) rise in cell fluorescence, compared to cells cultured in medium alone (fig. 2). This indicates a rapid uptake of fluorescent material. In addition, the fluorescence intensity was significantly ($p<0.001$) increased at one hour of culture with CSC compared to the fluorescence after 5 min. At 48 h of culture the fluorescence was not increased compared to 24 h, indicating a levelling off effect at longer incubation periods. The fluorescence of AMs cultured in medium alone did not differ significantly during the observation period.

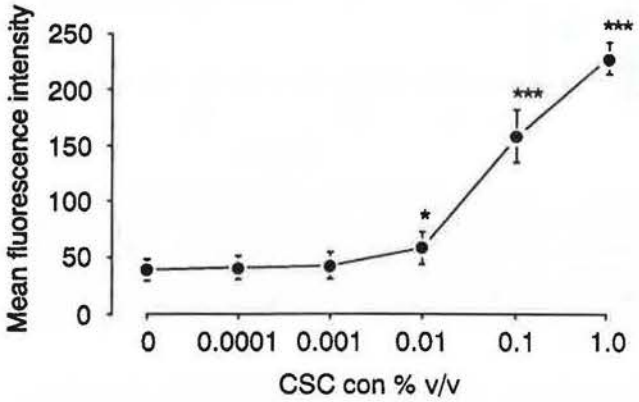

Fig. 1. – Fluorescence of alveolar macrophages (n=5) cultured for 40 h with different concentrations of cigarette-smoke condensate (CSC con), measured as mean fluorescence intensity. Mean values±SD are given. *: $p<0.05$; ***: $p<0.001$.

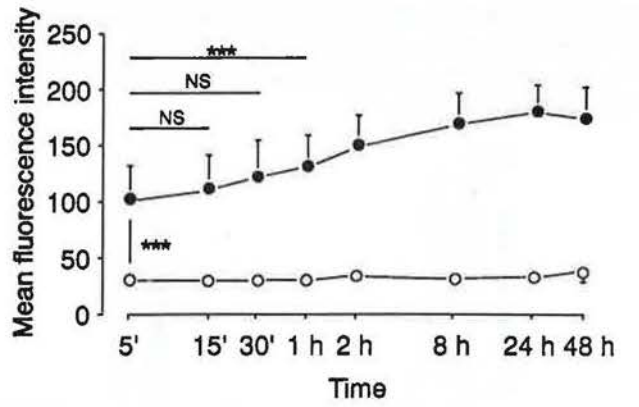

Fig. 2. – Fluorescence of alveolar macrophages (n=5), measured as mean fluorescence intensity (MFI), cultured in the presence of 0.1% CSC (●) at various periods of time (logarithmic scale). Cells cultured in medium only (○) were used as controls. There was a significant ($p<0.001$) difference between CSC-exposed cells and controls at all points of time. Mean values±SD are given. ***: $p<0.001$. CSC: cigarette-smoke condensate; NS: nonsignificant; ': min.

The effects of culturing exposed cells in CSC-free medium. In this experiment, CSC-exposed cells, with maximum fluorescence were cultured in medium alone. In one parallel experiment exchange of culture medium was performed at 4, 8, 24 and 48 h. A significant ($p<0.001$) decrease in fluorescence was seen in both groups after 8 h (fig. 3). However, the most pronounced decline at this point of time was noticed in the group where the culture medium was exchanged at 4 h, which made a significant ($p<0.05$) difference between the groups. When the medium was

exchanged at 8 h, a further decline was observed at 24 h, which made the difference between the two groups even larger ($p<0.001$).

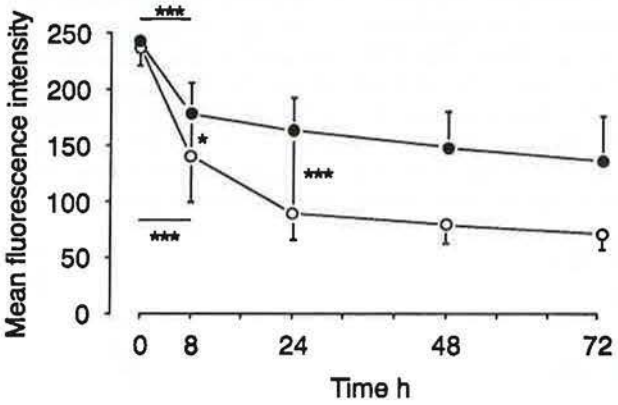

Fig. 3. – Fluorescence of alveolar macrophages (n=6), measured as mean fluorescence intensity (MFI), exposed to cigarette-smoke condensate (CSC) and cultured in CSC-free medium. The diagrams represent the fluorescence without exchange of culture medium (●) and with cell-washing and exchange of culture medium (○) at 4, 8, 24, and 48 h. Mean values±SD are given. *:$p<0.05$; ***: $p<0.001$.

The effects of mixture of fluorescent and non-fluorescent cells. When unexposed, low fluorescent AMs were admixed with exposed, fluorescent AMs, the unexposed cells engulfed fluorescent material which originated from the fluorescent cell population. The transference of fluorescent material from exposed to unexposed AMs occurred within 30 min. After 8 h, the two former cell populations appeared to be a homogeneous one (fig. 4).

The effects of incubating non-phagocytic and phagocytic cells with CSC

The CSC-exposed phagocytic cells (monocytes) showed a substantial increase in fluorescence intensity at 37°C (53.8±3.8, mean±SD) compared to 4°C (19.3±2.4). The fluorescence of the exposed non-phagocytic cell population (lymphocytes) increased to a much lesser extent at 37°C (table 1). Notably, at 4°C there was a higher fluorescence in both cell types after CSC-exposure compared to controls incubated in medium alone.

Table 1. – Fluorescence of CSC-exposed leucocytes

	Lymphocytes		Monocytes	
	Controls	CSC	Controls	CSC
4°C	0.6±0.1	12.3±2.0	1.7±0.3	19.3±2.4
37°C	0.6±0.1	17.6±0.7	1.9±0.2	53.8±3.8

The mean fluorescence intensity of human peripheral blood leucocytes (lymphocytes and monocytes), n=6, incubated with 0.1% CSC for 30 min at 4°C and 37°C, respectively. The control samples were incubated in RPMI 1640 alone. Results are given as mean±SD. CSC: cigarette-smoke condensate.

Discussion

The origin of the previously reported [12] strong fluorescence in AMs from human smokers compared to nonsmokers has not been clarified. This fluorescence is associated with an increased intracellular

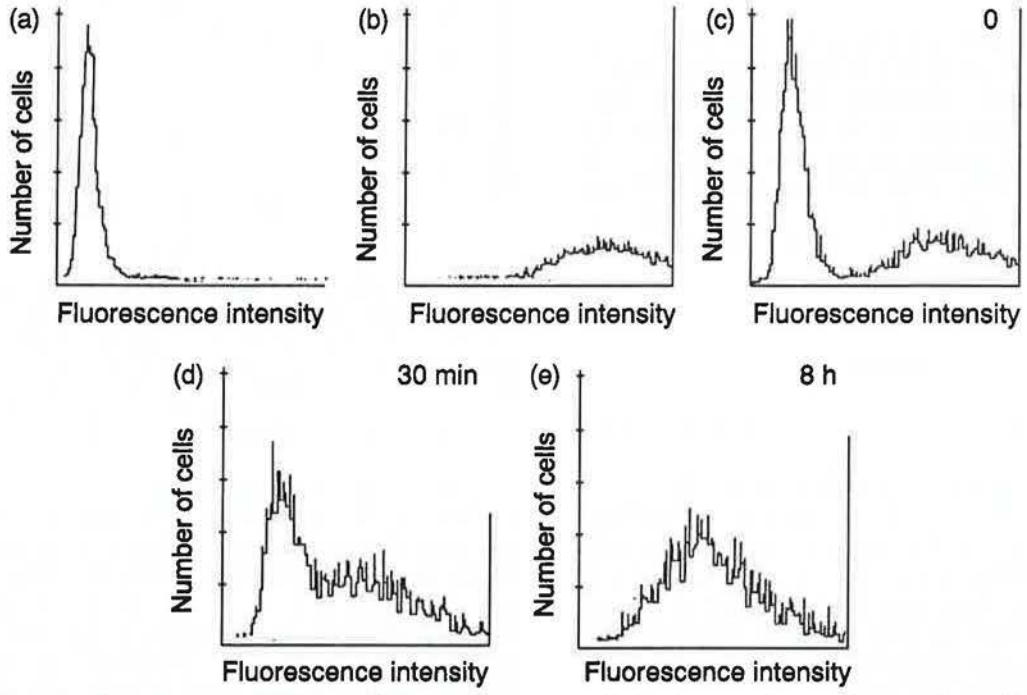

Fig. 4. – Histograms from one representative experiment where non-exposed alveolar macrophages (a) were mixed with equal numbers of cells exposed to smoke condensate (b). The flow-fluorescent (a) and high fluorescent (b) cell populations are seen in a common histogram immediately (time=0) after mixture of the cells (c). After 30 mins (d) the two curves fuse and 8 h after the mixture (e) the cells seem to form a homogeneous population.

complexity or granularity of the cells, suggesting an increased number of vacuoles, granules and lysosomes [12]. Whether the fluorescence of AMs from smokers originates from phagocytosed fluorescent material in tobacco smoke or is due to a metabolic activation of the cells is unclear. In the context of the latter possibility flavoproteins, lipofuscin and other storage pigments have been proposed to cause the fluorescence [13, 15, 16].

The aims of the present investigation were to examine the effects of a soluble cigarette-smoke condensate on the fluorescent properties of AMs. We found a rapid increase of the fluorescence intensity in a dose-dependent manner when AMs were exposed to CSC. To determine whether this uptake is due to an endocytic process, leucocytes from peripheral blood were incubated with the CSC. When the phagocytic cell population (monocytes) were examined in the flow cytofluorometer an almost threefold increase in fluorescence was observed at 37°C compared to 4°C. This was in contrast to the non-phagocytic cells (lymphocytes) which only showed a slightly enhanced fluorescence at 37°C. These findings suggest that the CSC-uptake is mainly an endocytic process, since low temperature effectively inhibits such mechanisms [22]. On the other hand, an increased fluorescence was observed in both phagocytic and non-phagocytic cells at 4°C compared to control samples. Therefore, in the present study, we cannot exclude that some of the fluorescence originates either from fluorescent materials that have diffused through the cell membrane, or from substances adhered to the cell surfaces.

In general, the endocytic process in macrophages is accompanied by exocytosis, *e.g.* there is a continuous considerable recycling of plasma membranes [22–24]. This endo-exocytic process is rapid and membranes can in minutes move from one cellular compartment to another [25]. A similar uptake and release of material was observed in the present experiments. Thus, when AMs were cultured in CSC-free medium the fluorescence intensity of the cells decreased, indicating an exocytosis of fluorescent material, and a corresponding endocytosis of culture medium. These findings are in agreement with SWANSON [26], who found an initial, high accumulation of the soluble fluorescent dye Lucifer Yellow in mouse alveolar macrophages, and an efflux of the dye when the cells were reincubated in medium alone. In the present investigation, the decrease in fluorescence seems to be a continuing process until it levels off. Furthermore, in the experiment where non-fluorescent, non-exposed AMs were admixed with fluorescent cells, the non-fluorescent AMs were able to ingest fluorescent material which originated from the fluorescent cell population.

The present findings provide one possible explanation for the high fluorescence observed in AMs from smokers, *i.e.* the ingestion of fluorescent material from tobacco smoke. It seems unlikely that the strong and rapidly induced fluorescence observed *in vitro* in the present investigation could originate from fluorescent substances synthesized within the cells. Besides, a study by VASSAR *et al.* [10] showed that the fluorescence originating from a smoke condensate was identical with that observed from macrophages in smokers sputum. However, our findings do not explain the low-intensity fluorescence observed in AMs from nonsmokers. This fluorescence could be related to an activation of the cells since the AMs from nonsmokers show a state of heightened activity, compared to corresponding blood monocytes [20].

The clearance of many types of particles from the alveolar space is a slow process. Thus, in an experimental model in humans it was calculated that particles were eliminated from the alveolar space with a half time exceeding 6 yrs [27]. In a recent study, we have found that fluorescent material from tobacco smoke can remain for over a year in the alveoli after cessation of smoking (Sköld, Chest, in press). Based on the results in this study it appears possible that the fluorescent material is continuously released from older AMs and subsequently endocytosed by newly recruited cells. This model of recycling and redistribution of particles between AMs in the alveolar space has been discussed previously by other investigators [28–30]. As a consequence, inhaled particles may remain in the alveoli for a considerably longer period than the estimated life span of the AMs [31]. The exchange of material between older and newly recruited macrophages in the alveolar space may also be of importance in certain diseases such as hypersensitivity pneumonitis. In this disease inhaled organic particles cause a pronounced accumulation of lymphocytes in the alveoli, probably as a result of antigen-presentation by macrophages [32]. Our findings can explain why such an alveolitis may remain even a long time after withdrawal of exposure [33].

In conclusion, AMs rapidly engulf fluorescent material from cigarette-smoke condensate. The mechanisms at least partly explain the strong fluorescence observed in AMs from smokers and the slow decline observed after cessation of smoking.

Acknowledgements: The authors thank B. Dahlberg at the Department of Thoracic Medicine, Karolinska Hospital, for excellent technical assistance. M. Curvall and E. Kazemi from the Swedish Tobacco Company are gratefully acknowledged for providing cigarette-smoke condensate. Thanks also to E. Berg at the Department of Medical Information Processing, Karolinska Institute, for valuable help with the statistics.

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