

# Smoking Cessation Rapidly Reduces Cell Recovery in Bronchoalveolar Lavage Fluid, While Alveolar Macrophage Fluorescence Remains High\*

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Bronchoalveolar lavage (BAL) was performed in smokers ( $22.6 \pm 7.8$  pack-years) before ( $n=18$ ) and 1 ( $n=14$ ), 3 ( $n=13$ ), 6 ( $n=11$ ), 9 ( $n=9$ ), and 15 ( $n=8$ ) months after smoking cessation. The recovery of the BAL fluid increased after smoking cessation ( $p<0.05$ ). The total number of cells and the cell concentration were significantly lower already at one month ( $p<0.05$  and  $p<0.01$ , respectively), and this decline was more pronounced at the following lavages. By using flow cytometry, alveolar macrophage (AM) fluorescence was quantified, since it is known that AMs lavaged from smokers have an increased fluorescence, due to interaction with fluorescent substances in the inhaled smoke. Not until six months after smoking cessation was a significant ( $p<0.05$ ) decrease in AMs fluorescence noted. At 15 months, the fluorescence was still increased, with

great individual variations, compared with AMs from non-smokers. The decline in fluorescence of AMs after smoking cessation was negatively correlated to the previous cigarette consumption. The absence of new, low fluorescent cells in the BAL fluid, despite a slow, but significant decrease in the fluorescence intensity of the whole cell population, suggests that the fluorescent material is redistributed from older AMs to newly recruited cells. These substances can thus remain in the alveolar space for a longer time than the estimated life span of the AMs.

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AMs = alveolar macrophages; FITC = fluorescein isothiocyanate; iq = interquartile; MFI = mean fluorescence intensity; PBS = phosphate-buffered saline solution

Cigarette smoke is one of the most common pollutants that the human lungs are exposed to. The inhaled material is handled either by the mucociliary escalator or by the alveolar macrophages (AMs). These cells, located in the epithelial lining fluid, are the predominant phagocytes in the alveoli. In smokers, they have to take care of a continuous influx of airborne particles originating from tobacco smoke. This results in an accumulation of AMs and in a higher cell recovery in bronchoalveolar lavage (BAL) fluid from smokers compared with nonsmokers.<sup>1</sup> Moreover, the AMs from smokers ultrastructurally differ from those of non-smokers,<sup>2,3</sup> probably as a result of ingestion of or interaction with substances in the inhaled smoke.

In addition, it has been known that AMs, when examined under UV light, have an autofluorescence<sup>4</sup> that is stronger in AMs from smokers than from nonsmokers. By the use of flow cytometry, we have recently described this difference in fluorescence.<sup>5</sup> One possible explanation for the fluorescence is ingestion of fluorescent particles in the tobacco smoke.<sup>4,6,7</sup> Hence, it seems reasonable to assume that

the fluorescence decreases if the smoke exposure is stopped.

The heavy burden of particles that the AMs continuously are exposed to in smokers abruptly discontinues at cessation of smoking, creating a new environment for the AMs in the alveoli. The aim of the present investigation was to elucidate how smoking cessation is reflected by alterations in cell recovery and the composition of cells in BAL fluid. We also wanted to quantify the decrease in fluorescence under such conditions. This fluorescence, originating from fluorescent substances in the smoke, acts as a marker for inhaled particles, thus reflecting alveolar clearance after smoking has ceased. Therefore, a group of heavy smokers underwent BAL before and 1, 3, 6, 9, and 15 months after smoking cessation.

## MATERIAL AND METHODS

### Subjects

Eighteen apparently healthy smokers (mean age, 41.4 years; range, 27 to 61 years; 17 women, 1 man) were included in the study. The majority of the participants were treated with hypnosis at the Stockholm Anti Smoking Clinic during the study. Their mean ( $\pm$  SD) cigarette consumption was  $22.6 \pm 7.8$  pack-years and the consumption exceeded 15 cigarettes per day for the last ten years. Eight subjects participated in all six lavages (Table 1), whereas the others were excluded from the study at different points of time because of relapse in smoking. The exclusion of these subjects did not influence the smoking histories of the remaining groups (Table 1).

Some of the smokers had a slight morning cough, but none of them had a history of airway obstruction. They were all free of

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**Table 1—Number of Participants, Mean Age, and Previous Cigarette Consumption at the BAL Occasions in the Study\***

| Months | No. of Participants |   | Mean Age, yr (Range) | Pack-Years (mean $\pm$ SD) |
|--------|---------------------|---|----------------------|----------------------------|
|        | F                   | M |                      |                            |
| 0      | 17                  | 1 | 41.4 (27-61)         | 22.6 $\pm$ 7.8             |
| 1      | 14                  | — | 42.5 (29-61)         | 23.4 $\pm$ 7.5             |
| 3      | 13                  | — | 41.1 (29-57)         | 22.1 $\pm$ 6.1             |
| 6      | 10                  | — | 39.4 (29-52)         | 20.4 $\pm$ 5.0             |
| 9      | 9                   | — | 38.8 (29-48)         | 20.2 $\pm$ 5.2             |
| 15     | 8                   | — | 36.8 (29-47)         | 19.5 $\pm$ 5.1             |

\*BAL = bronchoalveolar lavage; F = female; M = male; 0 = before smoking cessation; 1, 3, 6, 9, 15 = months after smoking cessation.

medication. Routine physical examinations and chest roentgenograms prior to the study revealed nothing abnormal. New chest roentgenograms were taken after five lavages, and all were normal. Thirteen nonsmokers served as a reference group.<sup>3</sup> All study subjects, as well as the nonsmoking controls, lived in an urban area. They had not been exposed to any excess amount of pollutants at work or at home. The Ethics Committee at Karolinska Hospital first approved an application for four lavages (0, 1, 3, and 6 months), and then an additional application for three more lavages, due to the findings in the first lavages. Informed consent was obtained from all the participants.

#### Bronchoscopy and BAL

Bronchoscopy was carried out with a flexible fiberoptic bronchoscope (Olympus BF Type 4B2, Olympus Optical Co Ltd, Japan) under local anesthesia. The right bronchial tree was inspected for signs of bronchitis. Edema, erythema, and increased amounts of mucus were considered as such signs. Bronchoalveolar lavage was performed as previously described.<sup>8</sup> The bronchoscope was wedged in a middle lobe bronchus and 37°C sterile saline solution was instilled in five aliquots of 50 ml each. The fluid was gently suctioned back and collected in a siliconized bottle kept on ice that

was transported immediately to the laboratory.

The first investigation was performed as the subjects still smoked. The procedure was then repeated on five occasions: 1, 3, 6, 9, and 15 months after the subjects had stopped smoking. The lavages were performed in the middle lobe but, as far as possible, in different subsegments. The time interval between the lavages prior to smoking cessation and one month after was 1.56  $\pm$  0.20 months (mean  $\pm$  SD). All bronchoscopies with lavages were carried out by one investigator (C.M.S.) and no side effects were noted following the repeated lavages.

#### Preparation of BAL Cells

The BAL fluid was strained through a double layer of Dacron nets (Millipore, Bedford, Ireland). Cells were pelleted by centrifugation at 400  $\times$  g, 4°C for 10 min and the supernatants were poured off. The cell pellet was resuspended in Hanks' balanced salt solution with 1 percent fetal calf serum. The total number of cells was counted in a Bürker chamber and the viability was tested by the exclusion of trypan blue. Smears for differential counts were prepared by cyto centrifugation at 500 rpm for 3 min (Cytospin 2 Shandon, Southern Products Ltd, Runcorn, England). Smears were stained with May-Grünwald Giemsa and 500 cells were counted. Cell samples (3 to 4  $\times$  10<sup>6</sup> cells) were poured in test tubes, and the cells were washed once in cold 0.15 M phosphate-buffered saline solution, at a pH of 7.4, supplemented with 10 mmol/L EDTA and 0.02% NaN<sub>3</sub> (PBS-EDTA). The cells were resuspended in 1 ml PBS-EDTA and were then examined in the flow cytometer.

#### Analysis of AMs Fluorescence

The fluorescence of AMs was quantified in an flow cytometer (Ortho Spectrum III, Ortho Diagnostic Systems, Westwood, Mass). In the instrument, each cell is represented by a point in a coordinate system, based on the light scatter properties as the cell passes a laser beam. The fluorescence of the AMs was measured as the mean fluorescence intensity (MFI), and this was obtained by gating the AM's cell cluster.<sup>9</sup> The excitation wavelength was 480 nm and the fluorescence was measured with a wavelength range from 515 to 530 nm (fluorescein isothiocyanate [FITC]). The instrument was calibrated daily with standardized fluorescent particles, and a linear scale was used.

**Table 2—General Characteristics of BAL Fluid Recovered from Healthy Smokers before (0) and 1, 3, 6, 9, and 15 Months after Smoking Cessation\***

| Months  | 0<br>(n = 18)       | 1<br>(n = 14)        | 3<br>(n = 13)       | 6<br>(n = 10)        | 9<br>(n = 9)         | 15<br>(n = 8)       |
|---|---------------------|----------------------|---------------------|----------------------|----------------------|---------------------|
| Recovery, %   | 60<br>(56-61)       | 66<br>(60-69)        | 66†<br>(63-70)      | 62<br>(60-68)        | 65†<br>(62-72)       | 64<br>(62-78)       |
| Viability, %  | 90<br>(87-93)       | 89<br>(85-94)        | 91<br>(89-93)       | 88<br>(84-90)        | 89<br>(86-94)        | 95<br>(92-95)       |
| Total cell yield, $\times$ 10 <sup>6</sup>                      | 54.5<br>(34.0-70.0) | 32.4†<br>(27.0-38.9) | 28.5<br>(19.6-37.2) | 16.3†<br>(12.8-25.0) | 17.0†<br>(12.5-20.2) | 14.2<br>(12.0-19.9) |
| Cell concentration,<br>$\times$ 10 <sup>6</sup> cells per liter | 317<br>(237-499)    | 195‡<br>(150-227)    | 187†<br>(114-207)   | 103†<br>(85.3-149)   | 106†<br>(71.2-122)   | 91.2<br>(68.4-121)  |
| Differential cell count,<br>% of total cells                    |                     |                      |                     |                      |                      |                     |
| AM  | 93<br>(90-95)       | 92<br>(90-94)        | 88<br>(86-93)       | 85<br>(75-90)        | 88<br>(86-89)        | 90<br>(88-94)       |
| Ly  | 5<br>(3-8)          | 7<br>(4-7)           | 7<br>(5-13)         | 13<br>(9-19)         | 9<br>(8-12)          | 8<br>(5-10)         |
| Neu   | 1.0<br>(0.4-1.6)    | 1.0<br>(0.4-1.2)     | 1.4<br>(0.8-1.8)    | 1.7<br>(1.0-2.6)     | 2.2<br>(1.0-2.2)     | 1.0<br>(0.4-1.4)    |

\*Results are given as medians with interquartile ranges (25th and 75th percentiles). AM = alveolar macrophages; Ly = lymphocytes; Neu = neutrophil granulocytes; BAL = bronchoalveolar lavage. Significance levels are calculated vs 0.

†p < 0.05.

‡p < 0.01.

### Statistical Methods

The results are given as medians with 25th and 75th percentiles, unless otherwise stated. The paired Wilcoxon rank sum test was used to evaluate systematic changes within the group. Generally, due to a number of statistical tests, and to avoid a mass significance effect, a Bonferroni adjustment was used.<sup>9</sup> Thus, a  $p$  value  $<0.01$  was considered significant in order to approximately maintain an overall 5 percent level. Correlations were calculated according to Spearman.

## RESULTS

### Endobronchial Findings

Signs of bronchitis (edema, erythema, and increased amounts of mucus) were observed macroscopically at the first investigation. After smoking cessation of three months, however, there was a less pronounced bronchitis, and after six months, the bronchial mucosa looked normal.

### General Lavage Data

The median recovery of the instilled fluid was more than 60 percent at all lavage occasions (Table 2). There was a significantly higher recovery after smoking had ceased compared with the recovery at lavages done when the subjects still smoked ( $p<0.05$  at 3 and 9 months). The viability of the cells did not differ between the lavages. A significant decrease both in the total number of cells and in the cell concentration

was found one month after smoking cessation ( $p<0.05$  and  $p<0.01$ , respectively). As seen in Table 2 (and Fig 1), the decline in the total number of cells and in the cell concentration was more accentuated at the later lavages. However, the number of subjects that participated at 15 months ( $n=8$ ) was not enough to give a statistically significant difference. At 6, 9, and 15 months, the total number of cells and the cell concentration did not differ statistically (Mann-Whitney  $U$  test) from our reference group of nonsmokers. (These values, based on 13 nonsmoking subjects<sup>5</sup> are as follows: total cell yield  $\times 10^6$ : median 18.0, interquartile [iq] range 16.4 to 18.8. The corresponding values for the cell concentration  $\times 10^6/L$  are as follows: median, 94.9; iq range, 83.8 to 113.1). The proportion of cells in the differential counts showed no significant changes between the BAL occasions.

### Alveolar Macrophage Fluorescence

There was a significant ( $p<0.05$ ) decrease in the MFI six months following smoking cessation compared with before the smoking stop. At 9 and 15 months, this decline was further accentuated (Fig 2). However, at 15 months the number of subjects that participated ( $n=8$ ) was not enough to give a significant decrease with the statistical methods used. The MFI value of the AMs before smoking cessation was a median of

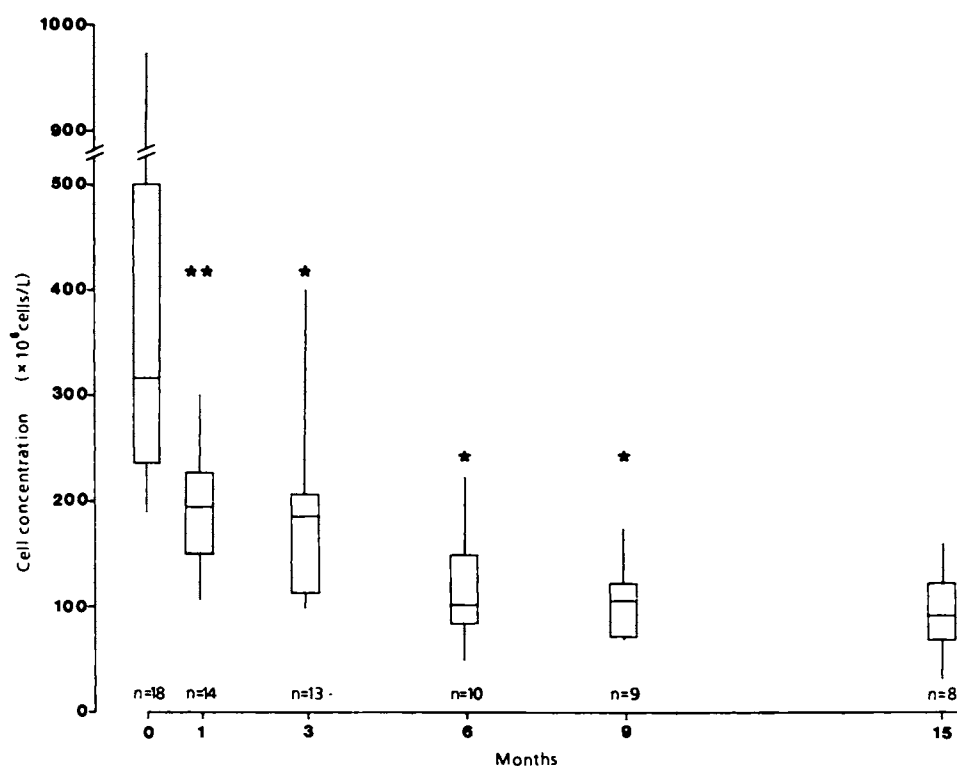


FIGURE 1. The cell concentration in bronchoalveolar lavage fluid recovered from smokers before (0) and 1, 3, 6, 9, and 15 months after smoking cessation. The boxes cover values within the interquartile ranges, the central line is at the median, and the minimum and maximum values are also given. The number (n) of subjects undergoing lavage at each occasion is presented. One asterisk =  $p<0.05$ ; two asterisks =  $p<0.01$ .

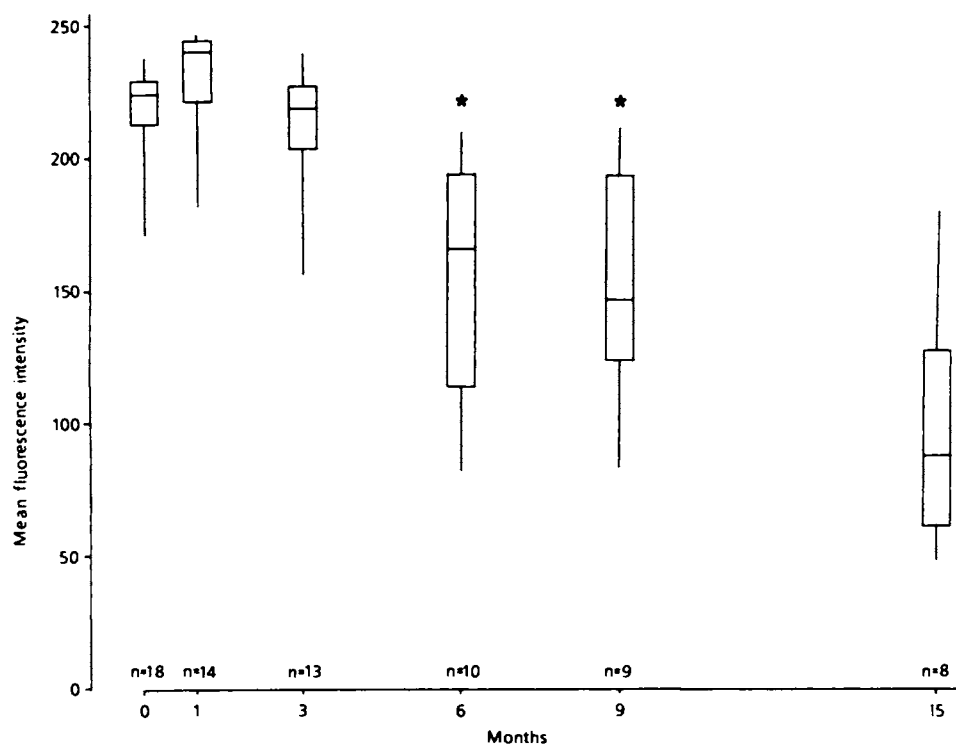


FIGURE 2. The fluorescence of human alveolar macrophages lavaged from smokers before (0) and 1, 3, 6, 9, and 15 months after cessation of smoking. The mean fluorescence intensity (MFI) is given in arbitrary units. The boxes cover values within the interquartile ranges, the central line is at the median, and the minimum and maximum values are also given. The number (n) of subjects undergoing lavage at each occasion is presented. Asterisk =  $p < 0.05$ .

223.5 (iq range, 212.8 to 229.1), and after one smoke-free month, the median was 239.9 (iq range, 221.4 to 244.6). The fluorescence of the AMs at the following lavages were as follows: three-month median, 219.0 (iq range, 204.5 to 227.4); six-month median, 166.4 (iq range, 113.9 to 194.3); nine-month median, 147.5 (iq range, 124.8 to 193.8); and 15-month median, 88.4 (iq range, 62.4 to 128.3). At 15 months, there was, however, still a significant ( $p = 0.017$ , Mann-Whitney *U* test) difference compared with the fluorescence in AMs from our reference group of nonsmokers. (The MFI values of the AMs in our reference group,<sup>5</sup> based on 11 nonsmokers, are median of 53.1 and iq range of 47.7 to 62.7.) The higher value at one month was not significant compared with the baseline value prior smoking cessation, but in 12 of the 14 subjects, the MFI value had increased at this point of time. Without the correction for mass significance (see "Statistical Methods"), this difference would be statistically significant ( $p < 0.05$ ).

We could not detect any accumulation of newly recruited cells with low fluorescence during the lavages, *ie*, there were no signs of two different cell populations in the same histogram with low and high fluorescence intensity, respectively. In contrast, only one homogenous cell population with declining MFI values was observed (Fig 3).

Before and at the first lavages after cessation of smoking, the AMs have the maximal fluorescence possible to detect in the test system used, which explains the small differences in fluorescence among the subjects at these occasions (Fig 2). Furthermore, large individual variations in the decline in the fluorescence was noted. The decline at 6, 9, and 15 months ( $\Delta$  MFI values) were plotted against the pack-years, to assess whether any correlation occurred between these parameters. A negative correlation was found (Fig 4), *eg*, the subjects having the highest consumption of cigarettes showed the slowest decline in fluorescence. This correlation was strongest at nine months ( $p < 0.01$ ), but occurred also at 6 and 15 months ( $p < 0.05$  for both).

## DISCUSSION

In this study, we investigated the effect of smoking cessation on the cellular components in the BAL fluid by performing consecutive BALs on healthy smokers before and at different points of time after smoking had stopped. Furthermore, the decline of fluorescence in the AMs was followed with a flow cytometric technique.

As the interval between the first and the second lavage was short, the findings in the second lavage could have been influenced by the previous lavage. To

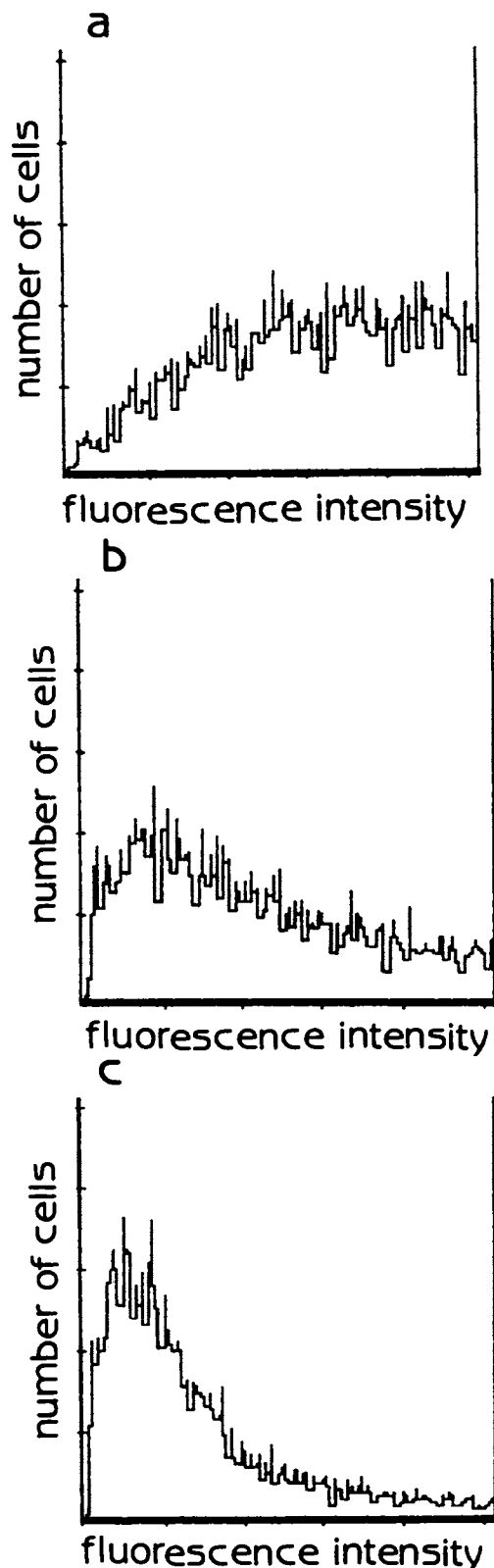


FIGURE 3. Histograms indicating fluorescence intensity in alveolar macrophages from one of the participants in the study before (a), 6 months (b), and 15 months (c) after smoking cessation. The mean fluorescence intensity was reduced from 214.6 to 137.5 at 6 months and to 75.1 at 15 months.

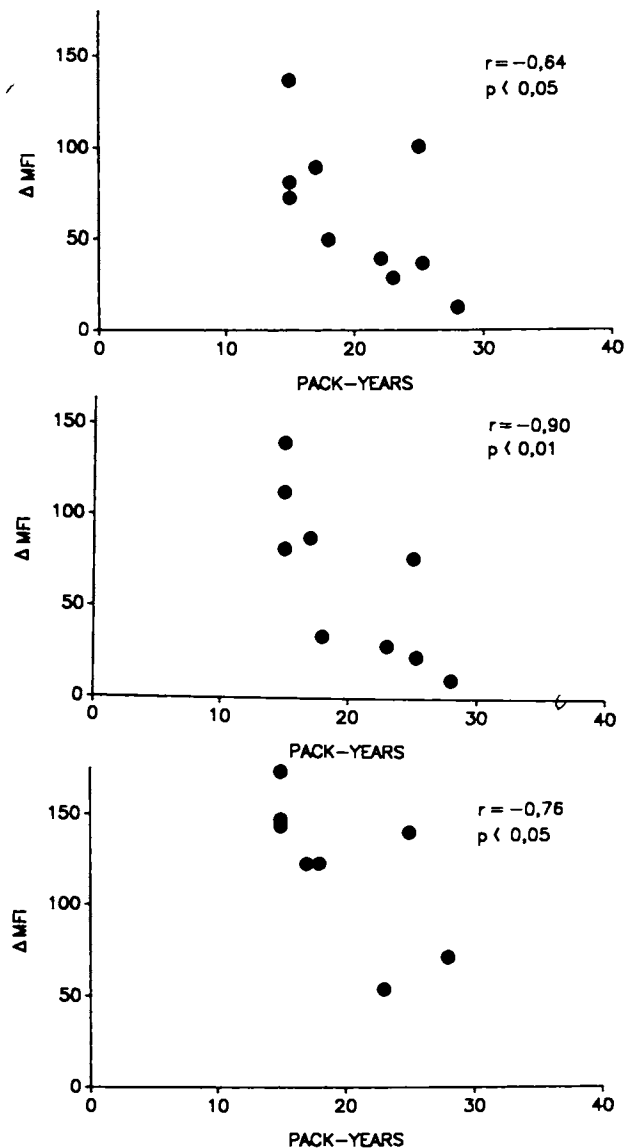


FIGURE 4. The decline in alveolar macrophage fluorescence ( $\Delta$  mean fluorescence intensity [MFI]) for each individual subject plotted against the pack years at 6 (a), 9 (b), and 15 months (c) after smoking cessation.

minimize such an influence, we performed the lavages, whenever possible, in different subsegments.

We found an initial rapid phase in the normalization process after smoking cessation. Thus, the total number of cells as well as the cell concentration in the BAL fluid decreased significantly already after one smoke-free month, and the values were comparable to a nonsmoker after six months. It is well known that BAL fluid recovered from smokers contain four to five times as many cells as fluid from nonsmokers,<sup>1</sup> primarily due to an increased number of AMs. One possible explanation could be that the number of phagocytic cells in the alveoli is increased in order to take care of the great influx of particles in smokers. In the present study, the fluorescence of the AMs had not decreased after one month, indicating the persist-

ence of fluorescent tobacco material in the alveolar space. Thus, there may also be mechanisms other than the presence of these factors for the elevated number of AMs among smokers. During the first rapid phase, we also found an increased recovery of the BAL fluid and a disappearance of the macroscopic signs of bronchitis. Since the BAL recovery usually is lowered in patients with airway obstruction, one possible explanation for this increased recovery may be the fact that smokers have a higher degree of bronchial reactivity compared with nonsmokers.<sup>10</sup>

The results indicate that smoking *per se* induces an increase in the number of AMs, and that this increase is not just due to the presence of fluorescent smoke components. At the later lavages, the total cell yield and the cell concentration continued to be at a nonsmoker level indicating the participants maintained to be smoke free.

The median life length of the AMs in the alveoli has been estimated to be 80 days.<sup>11</sup> The fluorescence of AMs was still increased compared with the fluorescence of AMs from nonsmokers more than 80 days after smoke cessation. Hence, the decline of fluorescence seems to represent a slower phase in the normalization process after smoking cessation. It further indicates that fluorescent material has not been fully cleared from the alveoli despite the fact that new AMs, primarily derived from the peripheral blood,<sup>12,13</sup> are recruited to the alveoli. One explanation for this phenomenon could be that fluorescent material is redistributed from older AMs to newly recruited cells.<sup>7,14,15</sup> Since AMs continuously are recruited from the bloodstream to the alveoli, these new AMs are supposed to have a lower fluorescence intensity compared with AMs from smokers,<sup>5</sup> but higher fluorescence than the corresponding blood monocytes.<sup>16</sup> However, we could not detect any increased proportion of low fluorescence intensity cells, *eg*, there were no histograms showing two different cell populations, which would suggest one newly recruited cell population with low fluorescence intensity and one older population (recruited prior smoking cessation) with higher fluorescence intensity. In contrast, only one homogenous cell population with declining MFI values was found during the lavages. This may also explain the fluorescence intensity at one month compared with before smoking cessation. As the total number of cells at this point of time had decreased significantly, the fluorescent material will be distributed to a substantially lower number of AMs, giving a higher fluorescence intensity per cell.

In our study, the fluorescence intensity in AMs from some of the subjects had decreased to a nonsmoker level at 15 months, whereas in AMs from other subjects it had not. Thus, there were great individual variations in the decline in fluorescence. The subjects who had

the most elevated fluorescence intensity at 6, 9, and 15 months also had the highest cigarette consumption history. A correlation was found between the smoking history as reflected in pack-years and the decline in fluorescence, *eg*, the more cigarettes, the slower decline in fluorescence. It seems that the more organic material that the alveoli have been exposed to, the longer it takes to clear them.

Our findings are in agreement with Agius et al<sup>17</sup> who demonstrated smoking-related inclusions in AMs up to 270 days after smoking had ceased, and calculated that three years must pass before they are comparable to nonsmokers' AMs. Other authors<sup>18</sup> found, at autopsy, fluorescent material in AMs from smokers up to two years after smoking had stopped. In contrast, fluorescent cells in sputum could not be detected in subjects who had abstained from smoking for longer than three months.<sup>4</sup> However, Vassar et al<sup>4</sup> investigated expectorated cells, which probably made the recruitment of AMs more hazardous than recruitment with the BAL technique. In relation to the results in our study, the data of Vassar et al would primarily reflect the first rapid phase in the normalization process after smoking cessation.

In contrast to our findings after cessation of smoking, inhalation of organic material in humans may give rise to an increased cell recovery, even long after exposure has ceased.<sup>19</sup> The reason for these conflicting results is unclear, but it indicates different cell responses after uptake of different particles and is probably due to various inflammatory properties of the inhaled material.

In conclusion, we found a rapid normalized cell count in the BAL fluid after smoking cessation, despite a persistence of fluorescent smoke particles in the alveoli. The slow decline in fluorescence after smoking cessation showed great individual variations that were correlated to the previous smoking history.

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