

DNA Synthesis in Alveolar Macrophages and Other Changes in Lavaged Cells Following Exposure of CBA/H Mice to Cigarette Smoke

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Traditional methods to determine the proportion of cells in S-phase use radiolabeled precursors of DNA, such as ³H-thymidine, which become incorporated into DNA during its synthesis and are visualized either in tissue sections or in cell preparations by autoradiography. At the Harwell Laboratory the effects of inhaled α -emitting actinides on the pulmonary alveolar macrophage population of the rodent lung are being studied. For this research the use of an autoradiographic technique to determine the proportion of cells in S-phase is inappropriate, because of the possible presence of competing sources of radioactivity in the cells under investigation. Consequently, an alternative method has been developed. In this method, 5-bromodeoxyuridine (BrdU), an analogue of thymidine, is incorporated into cells undergoing DNA synthesis. Fluorescein-conjugated monoclonal antibodies, highly specific for BrdU substituted DNA, are available commercially and may be used as a probe for BrdU-labeled cells. This technique for identifying cells in S-phase has been described previously for the flow cytometric analysis of cell suspensions and for cells in tissue sections. An adaptation of this technique for use on cytocentrifuge preparations of cells recovered from mouse lung by bronchoalveolar lavage has been developed and its use is described. Some preliminary results of a short-term experiment with CBA/H mice to determine the effects of exposure to cigarette smoke on the DNA synthesis of alveolar macrophages are also included.

Introduction

As yet, the mechanism by which the lung maintains the size of the pulmonary alveolar macrophage (PAM) pool is unknown. It is likely that under normal conditions a homeostatic mechanism exists within the lung, whereby PAM loss is balanced by PAM renewal. PAM are lost from the lungs as a result of cell death and cell clearance via the conducting airways. Masse et al. (1) demonstrated that in rats, the rate of removal of PAM from the lungs via the conducting airways was of the order of 0.75×10^6 cells per day. In the same study it was concluded that half of the total PAM population died within 10 days. Some have suggested that renewal of the PAM population is achieved by the recruitment of monocytes from the bloodstream (1-4), but others have implicated either the proliferation of PAM precursors

within the interstitial tissue of the lung (5), or division of the alveolar macrophages *in situ* (6-10).

The effects of exposure to cigarette smoke on the free cell population of the mouse lung have been studied at this laboratory (11), but no attempt was made to determine the proportions of the PAM population in the different phases of the cell cycle. The aim of the present study was to determine the effects of exposure to cigarette smoke on the DNA synthesis of PAM from CBA/H mice. The method used to identify PAM in DNA synthesis was first described by Gratzner (12), and adapted at Harwell for use on cytocentrifuge preparations. Briefly, BrdU, an analogue of thymidine, was used to label PAM undergoing DNA synthesis. Fluorescent monoclonal antibodies, highly specific for BrdU substituted DNA, were then used to visualize labeled PAM. In addition to estimates of the labeling index (LI) of PAM, measurements were also made of macrophage and other free cell numbers, median PAM diameters, and the frequency of nuclear aberrations in PAM recovered by bronchoalveolar lavage (BAL) from normal mice and those exposed to cigarette smoke. The results of this preliminary experiment, together with comments on the use of the anti-BrdU technique, are presented here.

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Materials and Methods

Animals

Thirty female CBA/H mice from a breeding colony at this laboratory were used in this study and allowed food and water *ad libitum*.

Smoking Regimen

A group of 12 mice (TS) were exposed to mainstream cigarette smoke on a Battelle Geneva Mark III smoking machine (13). The smoking regimen consisted of a 30-min exposure to cigarette smoke followed by a 15-min rest period and a further 30-min exposure to smoke. On the basis of previous experiments (11), the concentration of tar particulate in the smoke to which the mice were exposed was in the range of 1.28 to 1.40 mg/L (mean 1.33 ± 0.04 mg/L) with a CO concentration of approximately 1000 ppm. This regimen started when the mice were approximately 14 weeks old and was repeated each week day until the end of the experiment. An additional 12 mice (SS) were sham-smoked according to the above regimen, but they were exposed only to clean air. The remaining six mice (CC) were used as cage controls and sacrificed before starting the smoking regimen. Three mice from groups TS and SS were killed at 6, 13, 21, and 35 days after commencement of the smoking regimen.

Recovery of Free Cells by Bronchoalveolar Lavage

Mice were injected (IP) prior to sacrifice with 50 mg/kg body weight of BrdU (Sigma) prepared as a 10 mg/mL stock solution in sterile saline, and stored frozen in 5-mL aliquots. One hour later, the mice were killed by an IP injection of sodium pentobarbitone (Sagatal, May and Baker, Ltd., Dagenham, UK) and exsanguinated via the brachial artery. Free cells were recovered from the lungs by BAL *in situ* as described by Moores et al. (14). Briefly, this involves 10 washes with 0.55 mL of sterile physiological saline (Polyfusor, Boots, Nottingham, UK) at room temperature. The combined washes were collected in preweighed plastic universal containers (Sterilin, Middlesex, UK) and the volume of fluid recovered determined by weight.

Cell Counts and Measurements of PAM Diameter

To determine the concentration of PAM in lavage fluid, a 0.5-mL aliquot was dispensed into 19.5 mL of Isoton II (Coulter Electronics, Luton, UK). The size distribution of recovered cells was determined over a 60-sec period with a Coulter counter (Model ZM, Coulter Electronics) linked to a 1024-channel pulse height analyzer installed in a microcomputer (Apple IIe plus). Thresholds were set to exclude fine debris and the number of cells/mL in the lavage fluid determined (mean of three counts). A cell-free blank was prepared for each

sample by centrifuging the lavage fluid at approximately 500g for 5 min. The size distribution of debris in the blank was determined as above and subtracted from the size distribution of PAM. Median macrophage diameter, geometric standard deviation, and the percentage of abnormally large cells (greater than 14 μ m) were calculated from the resulting difference spectrum.

Preparation of Cytospins

Six 250- μ L aliquots of lavage fluid per animal were used to prepare cytopins using a cyto centrifuge (Shandon) operated at 1000 rpm for 5 min. The cytopins were air dried and fixed in 70% ethanol for 30 min at room temperature. Slides not processed immediately were stored frozen for subsequent investigation.

Differential Cell Counts

Two cytopins from each animal were stained using the technique of May-Grünwald/Giesma for differential cell counts. At least 1000 cells for each animal were examined and classified as either lymphocytes, macrophages, monocytes, or neutrophils. The frequency of binucleate (BiPAM) and micronucleate (MiPAM) macrophages was also assessed and expressed as a percentage of all PAM.

Determination of DNA Synthesis

Two cytopins from each mouse were processed to determine the proportion of PAM in DNA synthesis. Denaturation of double-stranded DNA was achieved by immersing the slides in a fresh solution containing ethanol and 0.1 M sodium hydroxide (2:5 v/v) for 4 min at room temperature (15). The slides were then rinsed in 97% ethanol for 2 min and air dried. BrdU incorporated into DNA was then visualized by incubation of the cell deposit with 20 μ L of undiluted fluorescein-conjugated anti-BrdU monoclonal antibody (Beckton Dickinson, mouse IgG, clone B44) for 1 hr at room temperature in a dark, humidified box. After incubation, the slides were rinsed three times in distilled water and allowed to air dry. Prior to examination, the slides were coverslipped with one drop of fluorescence mounting medium (Sigma). At least 1000 cells were examined on each of two cytopins per animal. Transmitted white light was used to count the total number of cells per field and a small correction was made to this figure to exclude granulocytes and lymphocytes from the analysis. Subsequently epi-fluorescent illumination was used to determine the number of PAM in the same field that were labeled with anti-BrdU.

Cells recovered by the disaggregation of spleens from mice injected with BrdU were used as a positive control. A negative control was provided by PAM recovered from mice not injected with BrdU.

The student's *t*-test was used to compare the means of smoke-exposed and sham-exposed groups for all parameters measured. The null-hypothesis (i.e., that

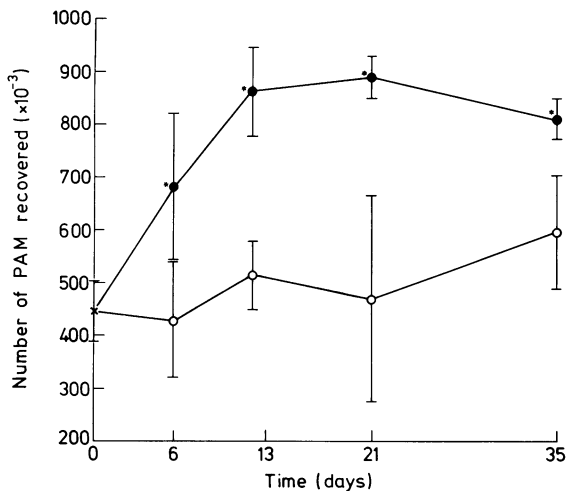


FIGURE 1. The total numbers of pulmonary alveolar macrophages (PAM) recovered from control (x), sham-exposed (O) and smoke-exposed (●) mice, with time after starting the smoking regimen. Mean ± SD. Asterisk (*) significantly different at $p < 0.05$ or greater.

there were no differences between the two groups) was rejected at a significance level of $p = < 0.05$. The variation in the labeling index was analyzed by the Fisher test for the analysis of variance.

Results

The numbers of PAM recovered by lavage from control, sham-exposed, and smoke-exposed mice are shown in Figure 1. At all time points during the smoking regimen, the number of PAM recovered was significantly greater for smoke-exposed than for sham-exposed mice. The recovery of PAM from mice exposed to cigarette smoke rose steeply over the first 2 weeks of exposure to reach a peak value at 21 days; at this time the recovery was approximately twice that of sham-exposed mice.

After 21 days the numbers of PAM recovered from smoke-exposed mice decreased slightly but remained significantly ($p < 0.05$) above the corresponding values for sham-exposed mice. During the course of the experiment a small increase was seen in the mean values of PAM recovered from the lungs of sham-exposed mice, but this was not significant.

Differential counts of the cells obtained by BAL are given in Table 1. In all cases, PAM accounted for more than 94% of the cells recovered, both from the lungs of sham- and smoke-exposed mice. The incidence of lymphocytes was variable, ranging between 1 and 3%; no significant differences were apparent between sham- and smoke-exposed animals. Monocytes represented less than 1% of all the cells, and there was no significant increase in their incidence after exposure to smoke. For sham-exposed mice, neutrophils accounted for less than 0.4% of all the cells recovered. Higher incidences were observed after exposure to smoke but the increase was not significant.

Measurements of median PAM diameters are presented in Table 2. At all time points there was a significant increase in the diameter of PAM recovered from mice exposed to smoke relative to sham-exposed animals. The incidence of large PAM (greater than 14 μm in diameter; Table 2) from sham-exposed animals was on average 5.6% of the total PAM population. For smoke-exposed mice the percentage of PAM greater than 14 μm in diameter increased to a mean value of 11.2%, twice the value observed for sham-exposed mice. This increase, however, was only significant at 6 and 35 days after starting the smoking regimen.

Also given in Table 1 is the incidence of PAM with nuclear aberrations. In all cases, binucleate PAM (BiPAM) account for less than 1% of all PAM recovered from sham-exposed mice. After 13 days the incidence of BiPAM was higher for smoke-exposed than sham-exposed mice, reaching a peak incidence at 21 days (3.5 times that of the mean incidence of BiPAM from sham-exposed mice). However, this difference was only significant at 21 days.

Table 1. The percentage of pulmonary alveolar macrophages (PAM), lymphocytes (Lym), monocytes (Mono), and neutrophils (Neut) in the free cell population recovered by lavage and the percentage binucleate (BiPAM) and micronucleate (MiPAM) alveolar macrophages from control, sham-exposed, and smoke-exposed mice at times after starting the smoking regimen.

Time, days	Differential cell count, % ^a				Frequency of nuclear aberrations, % of all PAM ^a	
	PAM	Lym	Mono	Neut	BiPAM	MiPAM
Cage-controls						
0	99.5 ± 0.3	0.4 ± 0.1	<0.1 ± 0.1	<0.1 ± 0.1	0.3 ± 0.4	0.2 ± 0.2
Sham-exposed						
6	96.5 ± 2.6	2.1 ± 2.4	0.6 ± 0.2	0.3 ± 0.3	0.6 ± 0.4	0.1 ± 0.1
13	96.9 ± 2.1	2.1 ± 1.9	<0.1 ± 0.2*	1.0 ± 0.8	0.1 ± 0.1	
21	95.8 ± 1.4	3.0 ± 1.6	0.6 ± 0.1	0.2 ± 0.3	0.3 ± 0.3	0.2 ± 0.1
35	97.5 ± 0.7	1.0 ± 0.7	0.3 ± 0.1*	0.3 ± 0.1*	0.6 ± 0.7	0.2 ± 0.2
Smoke-exposed						
6	94.4 ± 0.3	2.8 ± 1.7	0.9 ± 0.6	1.9 ± 1.4	0.6 ± 0.2	0.3 ± 0.3
13	96.5 ± 3.2	2.7 ± 2.8	0.6 ± 0.2	0.2 ± 0.3	1.5 ± 1.4	0.1 ± 0.1
21	97.1 ± 0.8	1.1 ± 0.5	0.3 ± 0.2	1.2 ± 0.9	2.1 ± 0.9*	0.5 ± 0.1*
35	98.2 ± 1.3	0.9 ± 0.7	0.5 ± 0.4	0.1 ± 0.1	1.3 ± 0.8	0.2 ± 0.1

^aMean ± one standard deviation.

*Significantly different at $p < 0.05$

Table 2. Median pulmonary alveolar macrophage (PAM) diameters and the percentage of PAM greater than 14 μm for control, sham-exposed, and smoke-exposed mice at times after starting the smoking regimen.

Time, days	Median PAM diameter, μm^a		PAM > 14 μm in diameter, % ^b	
	Sham-exposed	Cigarette-exposed	Sham-exposed	Cigarette-exposed
6	11.02 \pm 0.10	12.34 \pm 0.14*	4.6 \pm 1.00	13.1 \pm 4.06*
13	11.24 \pm 0.14	11.74 \pm 0.27	6.4 \pm 0.45	8.9 \pm 2.97
21	11.10 \pm 0.18	11.99 \pm 0.19*	6.3 \pm 4.96	11.7 \pm 2.29
35	10.95 \pm 0.12	11.89 \pm 0.22*	5.21 \pm 1.21	10.9 \pm 1.72*

^aValue for cage controls at day 0 = 11.25 \pm 0.05 μm , mean \pm SD.

^bValue for cage controls at day 0 = 3.4 \pm 1.59%, mean \pm SD.

*Significantly different at $p < 0.05$ or greater.

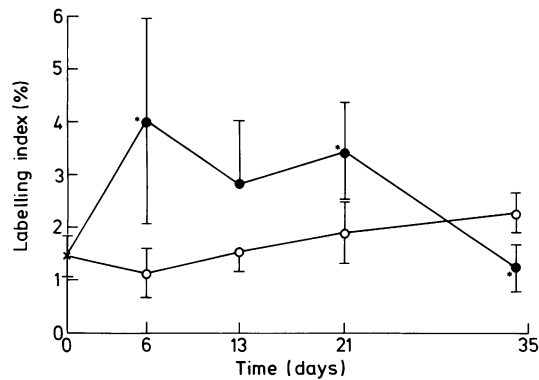


FIGURE 2. The labeling index of alveolar macrophages recovered from control (x), sham-exposed (O) and smoke-exposed (●) mice, with time after starting the smoking regimen. Mean \pm SD. Asterisk (*) significantly different at $p < 0.05$.

Very few PAM with micronuclei (MiPAM) were recovered from sham-exposed mice, the mean value being only 0.15%. In general, MiPAM were also rare in smoke-exposed mice; however, at 21 days the incidence of MiPAM from smoke-exposed mice was three times that of the mean value for the sham-exposed group ($p < 0.05$).

The percentage of recovered PAM in DNA synthesis (labeling index, LI) was determined by the anti-BrdU method, and the results are shown in Figure 2. After six days exposure the LI was 3.5 times higher for mice exposed to smoke relative to sham-exposed animals (significant at $p < 0.05$). This difference decreased at days 13 and 21, and by 35 days the LI of PAM from smoke-exposed mice was significantly below that of the sham-exposed group. The labeling index of PAM from sham-exposed mice ranged between 1.1% and 2.3% of all PAM, and the mean values were seen to increase slightly during the course of the experiment. The variation in LI between replicate measurements on different cytopspins for the same mouse was low, but the variation between individual mice, killed at the same time and from the same group, was much greater. In general the variation in LI between animals was greater for smoke-exposed than sham-exposed animals, particularly at the earlier time points.

Discussion

The ability of cigarette smoke and other particulate material to stimulate an increase in the number of PAM

is well documented (11,16,17). The majority of these studies however have been concerned with changes in the lung after long-term exposure; in this study the effects of short-term exposure to cigarette smoke on the free cell population of the mouse lung were studied. In previous work at this laboratory, an increase was observed in the number of PAM recovered from the lungs of mice after 3 months exposure to cigarette smoke (11). The present study demonstrates that an increase in the number of PAM can be detected in mice after only 6 days of exposure to cigarette smoke.

It is likely that the size of the PAM population within the lung is maintained by a balance between PAM renewal and PAM loss. Three possible mechanisms to explain the observed increase in the number of PAM following the exposure of mice to cigarette smoke can therefore be put forward: *a*) an increased input of PAM to the lungs, whether by local division of macrophages on the alveolar surface or by the recruitment and maturation of PAM precursors; *b*) impaired clearance of PAM from the lung; and *c*) increased efficiency of the lavage procedure in smoke-exposed mice. Clearance of PAM from the lungs may be decreased following exposure to cigarette smoke because of saturation of the mucociliary escalator with particulate material or to the impairment of ciliary action by nicotine. Adolkofer et al. (18) demonstrated partial deciliation of tracheal epithelial cells in rats after 90 days of exposure to a smoke particulate concentration of only 0.04 mg/L and concluded that this might lead to some changes in mucociliary function. It is estimated that in the present study the concentration of tar particulate in the smoke to which the mice were exposed was in the range of 1.3 to 1.4 mg/L so that impairment of clearance cannot therefore be overlooked. Other studies have provided evidence of clustering of PAM near terminal airways following the exposure of mice to cigarette smoke (11). It is possible that such macrophages may be washed out of the lung more easily than cells in more distal regions, resulting in an increase in the recovery of PAM after exposure to smoke.

An increase in the size of PAM following the exposure of mice to cigarette smoke for both 3 months (11) and 9 months (19) has been reported. Similar increases in the median diameter of PAM and also in the frequency of large PAM were observed in this experiment after as little as 6 days of exposure to smoke. In accordance with

other studies, it appears that PAM accounted for more than 94% of all the free cells recovered by BAL. No evidence of lymphocyte or monocyte infiltration in the alveoli was apparent. After 3 months exposure to cigarette smoke under similar conditions, a mild inflammatory response was observed, characterized by an increase in the number of neutrophils recovered (11,20). In this study a small but insignificant increase in the number of neutrophils was observed after 6 and 21 days of exposure to smoke.

Micronuclei are thought to arise from chromosomal fragments and chromosomes that are not included in the daughter nuclei at cell division. The frequency of this type of aberration depends both upon the rate of chromosomal breakage and the rate of cell division (21,22) and may serve as a useful indicator of damage to DNA. With the exception of a small increase at 21 days, the incidence of PAM with micronuclei in smoke-exposed mice was not increased relative to the sham-exposed group. This confirms the results of an earlier study (11) in which the frequency of MiPAM was not found to increase after 3 months exposure to cigarette smoke.

An increase in the number of BiPAM in mice exposed to cigarette smoke has been reported (11,23), but in these studies the animals were exposed to smoke for much longer periods. The results of the present study demonstrate that changes in the number of BiPAM can be seen after much shorter periods of exposure. It is possible that, unlike micronuclei, BiPAM are produced by errors in cytokinesis, and the frequency of this aberration may increase during periods of increased PAM turnover.

The proportion of PAM in DNA synthesis in sham-exposed mice was in the range of 1 to 2%. In earlier studies (24) with the same strain of mice, a value of 1.3% was obtained for control mice using ^3H -thymidine. The recovery of PAM in DNA synthesis from the mouse lung supports the view that macrophages are capable of entering into DNA synthesis on the alveolar surface of the lung (6-10); moreover, the recovery of such cells suggests that the macrophage is not a terminally differentiated cell incapable of further replication. It is possible that DNA synthesis may take place without cell division; however, the recovery of PAM in metaphase which has been observed in other studies (6,11) suggests that the alveolar macrophage is able to undergo mitosis.

An increase in the DNA content of lungs has been recorded in mice after exposure to cigarette smoke (25). This increase was observed after exposure to smoke for 7 days and persisted for more than 17 weeks. Holt et al. (26) demonstrated an increase in DNA synthesis following the exposure of peritoneal macrophages to cigarette smoke *in vitro*. In the present study the proportion of PAM in DNA synthesis recovered from mice exposed to cigarette smoke was increased, relative to sham-exposed mice during the first 21 days of exposure. A large variation was observed in the LI of PAM, particularly for smoke-exposed animals at the earlier time points. This variation can be attributed to differences in the response

of individual animals to cigarette smoke rather than day to day variation in the method; in general the variance between replicate slides was small (mean variance 0.44, $p < 0.0001$).

The mechanism by which the number of PAM increases following exposure to cigarette smoke cannot be determined from the results of this study. It appears, however, that a relationship exists between the LI and the number of PAM recovered. It is unlikely that an influx of monocytes into the alveoli contributed significantly to the increase in the number of PAM because no increase was observed in the number of monocytes recovered by BAL. This view cannot be ignored if, as has been suggested (27), the monocytes undergo a maturation stage before a final division on the alveolar surface.

The anti-BrdU technique presented here provides a sensitive method for detecting DNA replication in the PAM population recovered by BAL. The method is both cheaper and quicker to perform than alternative techniques and avoids the use of radiolabeled materials; moreover, the method will be of particular use at this laboratory where the effects of inhaled α -emitting actinides on the rodent lung are being studied.

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