

## Tobacco smoke exposure suppresses radiation-induced inflammation in the lung: a study of bronchoalveolar lavage and ultrastructural morphology in the rat

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*Tobacco smoke exposure suppresses radiation-induced inflammation in the lung: a study of bronchoalveolar lavage and ultrastructural morphology in the rat. L. Björner, Y. Cai, K. Nilsson, S. Hellström, R. Henriksson. ©ERS Journals Ltd 1993.*

**ABSTRACT:** Previous studies on patients with breast cancer, who received postsurgical irradiation, displayed a markedly suppressed inflammatory response in the lung of smoking patients compared to nonsmokers. The aim of the present study was to investigate further the effect of exposure to tobacco smoke on the development of irradiation-induced pneumonitis in the rat. Four groups of animals were used: controls (C); those exposed to tobacco smoke (S); those irradiated but not exposed to smoke (RNS); and those irradiated and exposed to tobacco smoke (RS). The rats were exposed to a diluted main stream of cigarette smoke, at a concentration of about 0.4 mg·l<sup>-1</sup>, in a nose-only exposure system for 1 h·day<sup>-1</sup>, 5 days·week<sup>-1</sup> for 10 weeks. Exposure to tobacco smoke started 3 weeks before irradiation. The basal one third of both lungs was exposed to a single radiation dose of 28 Gy (6 MeV photons). All animals were killed 7 weeks after irradiation. We compared findings in bronchoalveolar lavage (BAL) and tissue morphology.

The alveolar tissue showed less inflammation in the RS-group than in the RNS-group. Most strikingly, mast cells were increased one hundredfold in the lung interstitium and thirty fold in the peribronchial area in the RNS-group, whereas no increase was found in the RS-group or in the controls. The alveolar septa of the RNS-group were thickened, with occurrence of inflammatory cells and mast cells, whereas the RS-group displayed no difference as compared to the non-irradiated, nonsmoking group (C). There was a marked discrepancy between the findings in BAL and tissue of the alveolar space or lung interstitium. In BAL, neutrophils, and to a lesser extent lymphocytes, were increased both in the RS- and RNS-group; however, with significantly higher numbers in the RNS-group. In contrast, the cells in the alveolar space and interstitium were dominated by mononuclear cells, mainly macrophages. Moreover, a more than twenty fold increase in total cells in the alveolar space was observed, whereas the numbers of cells in BAL only increased about five times.

These findings probably reflect lower recovery of alveolar macrophages in the irradiated animals due to an increased cell adherence. Thus, this experimental study further supported the suppressive effect of smoking on radiation-induced pneumonitis.

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Cigarette smoking is a well-known risk factor, not only for the development of malignant diseases of the lung, but also for other respiratory diseases, such as chronic bronchitis and emphysema. Paradoxically, sarcoidosis and extrinsic alveolitis occur less frequently in smokers than in nonsmokers [1, 2]. Components in the smoke have been shown to have an immune suppressive function, making the individual more susceptible to respiratory tract infections [3]. Bronchoalveolar lavage (BAL) makes it possible to sample material from the lower respiratory tract. In smokers, as well as in nonsmokers, the normal BAL is dominated by macrophages, usually constituting more than 80% of the total cell numbers. The remain-

ing cell types are mainly lymphocytes and a relatively low percentage of neutrophils. The eosinophils and mast cells usually amount to less than 1%. In smokers, the total numbers of cells are increased several fold compared to nonsmokers [4, 5], due mainly to an increase in the pigment-loaded macrophages.

We have previously reported on a rat model, using concomitant BAL and morphological analysis in the same animal. The BAL data from the non-exposed animals displayed the same cellular distribution as found in humans. In the irradiated animals (30 Gy single dose), a distinct pattern of early neutrophilia, later followed by a tendency towards increased numbers of BAL lymphocytes, was seen.



Moreover, a close correlation was found between the concentration of hyaluronan in rat BAL and the occurrence of interstitial deposition hyaluronan [6].

Parallel data from BAL studies in humans suggest that some of the findings in the rat model are also applicable to the human system. It was recently observed that smoking markedly suppressed the radiation-induced lung reaction in women treated for breast cancer [7, 8], as evaluated by BAL. The aim of the present study was, therefore, to evaluate the significance of these findings in the animal experimental model, and to see whether the smoke-related suppression of radiation-induced inflammation, found in BAL, also reflects a real suppression of the inflammation occurring within the lung tissue. Both light and transmission electron microscopy was used.

### Materials and methods

#### Animals

Thirty two male Sprague-Dawley rats, aged 16 weeks and weighing about 300 g each at the start of the experiment were used. The experiments were approved by the Ethics Committee (No. A 103/90), and conducted according to the regulations of the Swedish Animal Protection Act (SF 1988:534). The animals were given standard rat pellets and tap water *ad libitum*. The rats were divided into four groups of eight animals, with one group serving as nonsmoking non-irradiated controls (C), one group exposed to smoke only (S), one group exposed both to tobacco smoke and radiation to the lung (RS), and finally one group subjected to lung radiation but not smoke (RNS). No mortality occurred in any of the groups.

#### Smoke exposure

The animals were individually exposed, using a nose-only technique, to cigarette smoke interspersed with fresh air. Tobacco smoke (TS) from University of Kentucky high yield reference (2R1) cigarettes was generated, and administered as "nose-only exposure" in a specially designed apparatus described previously [6]. In brief, rats were arranged in restraining tubes, in groups of 16 around a central exposure chamber, allowing nose-only exposure. Smoke was generated from cigarettes fitted into a rotating turntable on the top of the machine. Each cigarette was smoked to a standard length of about 2 cm in 24 puffs, each lasting 5 s, at a flow rate of 2 l·min<sup>-1</sup>, each puff was followed by fresh air lasting 10 s, at a flow rate of 2 l·min<sup>-1</sup>. The total particulate matter (TPM) delivered from each treatment in the system was calculated to be approximately 395 mg (315 mg tar). Exhaust air was drawn from the chamber at a flow rate of 2 l·min<sup>-1</sup>. Throughout the whole exposure period, TS was drawn at a constant flow rate of 0.2 l·min<sup>-1</sup>, corresponding to the expected minute volume of a conscious rat [9], from an outlet from the exposure chamber onto a filter with pore diameter of 0.8 µm (Millipore Corp, MA, USA). The average weight of the smoke particulate, collected on the filter, was 4.1±2.1 mg (mean±SD), assumed to reflect the smoke

concentration the individual rats were exposed to. Groups of rats were exposed to TS for 1 h·day<sup>-1</sup>, from three weeks prior to the radiation treatment, and, thereafter, throughout the observation period. Control rats were exposed to fresh air only.

#### Irradiation

Before irradiation, the rats were anaesthetized by intraperitoneal injections of 0.1 ml·100 g<sup>-1</sup> body weight of equal parts of Dormicum (midazolam, 5 mg·ml<sup>-1</sup>, Roche, Basel, Switzerland) and Hypnorm (fluanizone, 10 mg·ml<sup>-1</sup>, Janssen Pharmaceutical, Beerse, Belgium). The rats were irradiated with X-rays from a medical linear accelerator (6 MeV, 2.19 Gy·min<sup>-1</sup>). The rats were fixed in the dorsal position in a plastic mould, and the lower parts of the lungs were bilaterally irradiated, an irradiated area corresponding to approximately 8×40 mm [6]. The irradiation was performed three weeks after the start of the TS exposure, and the animals were killed seven weeks later. The tobacco-smoke exposed non-irradiated rats (S), and the control rats (C), were all handled in parallel.

#### Bronchoalveolar lavage (BAL)

The rats were anaesthetized by intraperitoneal injections of Dormicum-Hypnorm, 0.3 ml·100 g<sup>-1</sup> body weight. The lungs and trachea were dissected free, and blood samples were collected from the heart. A small plastic tube with a stiff guide inside was inserted into the trachea, and placed in the right main bronchus. This catheter position allowed only the lower and the middle lobe to be lavaged. The guide was removed and the tube attached to a 10 ml syringe. The left main bronchus was compressed, and 2 ml of ice-cold phosphate buffered saline (PBS) was instilled, and gently aspirated. The lavage procedure was repeated three times with 2 ml of PBS.

#### Analysis of cells

The lavage fluid was kept on ice, and the total numbers of cells were counted in a Bürker chamber. The lavage fluid was centrifuged at 400×g for 15 min at 4°C. The cell pellet from the bronchoalveolar lavage fluid was resuspended in a balance salt solution, at a concentration of 10<sup>6</sup> cells·ml<sup>-1</sup>. Slides were prepared using a cytocentrifuge (Cytospin Shandon, Southern Ltd, Runcorn, UK) at 96 ×g for 5 min, giving approximately 50,000 cells·slide<sup>-1</sup>. The Cytospin slides were stained with May Grünwald-Giemsa, and 200 non-epithelial cells were counted to determine the proportion of polymorphonuclear neutrophils, pulmonary alveolar macrophages (PAM) and eosinophils. The results were presented as a percentage of all cells, and as total cells per ml of lavage fluid.

#### Morphological analysis

Immediately after BAL the right lungs of every other rat were distended *in situ* to the total lung capacity by instillation of a solution of either MILAB fixative (0.2 g CaCl<sub>2</sub>, 0.2 g KH<sub>2</sub>PO<sub>4</sub>, 1.14 g Na<sub>2</sub>HPO<sub>4</sub>, 8 g NaCl, and



40 ml formaldehyde, dissolved in water to 1 l; MILAB AB, Malmö, Sweden) or Omnifix® (Bio-Zac, Järfälla, Sweden). The right main bronchus was then ligated, and the left lung was perfused by the fixative, and then the procedure was reversed so that two different fixatives were used in the same animal. Four of the left lungs were fixed in 3% glutaraldehyde for electron microscopic analysis (see below). The lung and heart were taken out of the thorax *en bloc*. The lungs fixed in MILAB were further processed in a microwave oven for 25 s, which raised the temperature to 40–50°C. They were washed in buffered saline and kept in the buffer solution at +4°C, until dehydrated. The lungs fixed in Omnifix were kept in this fixative until dehydration. After being sliced in the sagittal plane, the slices were embedded in paraffin wax, and 5 µm thick sections were prepared. These sections were stained with Masson's trichrome, Luna and toluidine blue. Since no significant difference in the interstitial cell numbers between the lavage and the non-lavage side was found (data now shown), both lungs were used for morphological analysis. The MILAB fixation was preferred for total cell counting, since it gave a slightly better morphology. For the counting of mast cells, Omnifix® was used. Slides stained with Luna and toluidine blue were used to determine the total cell numbers and total numbers of mast cells in the alveolar interstitium. Ten fields with an accumulated area of 0.113 mm<sup>2</sup> were counted, at a magnification of  $\times 480$ . The results are presented as numbers of cells-mm<sup>-3</sup>.

Differential cell counting could be carried out on BAL cytopsin centrifuge slides and on cells presented in the alveolar space. The macrophage population was divided into two groups; small to medium-sized macrophages (SMC), and big, rich, vacuolized, foam-like alveolar macrophages (BMC). In the interstitial and peribronchial/perivascular tissue it was difficult to differentiate between different mononuclear cells, such as macrophages and lymphocytes, and therefore differential counting could only be made between mononuclear cells and neutrophils. Four areas of lung tissue were defined for total cell counting; alveolar space, lung interstitium (e.g. alveolar septal tissue), peribronchial and perivascular tissue. With the staining and fixation technique used for counting mast cells, it was difficult to differentiate between vascular and bronchial tissue. Therefore, for mast cell counting, no differentiation between peribronchial and perivascular tissue was made.

#### Electron microscopy study

Four left lungs of animals in each group were perfusion-fixed via the trachea by 3% glutaraldehyde, in 0.1 M cacodylate buffer, with 3% polyvinylpyrrolidone (PVP) and CaCl<sub>2</sub> added. The lungs were immersed in the same fixative for a further 24 h. Pieces of central parts of the lung were then dissected and rinsed in buffer, prior to postfixation in 1% OsO<sub>4</sub> overnight at 4°C. After another rinse, the specimens were dehydrated in a graded series of acetone, and then embedded in an epoxy resin. Sections were cut at 1 µm for light microscopy, and 70–80 nm for transmission electron microscopy (TEM). The 1

µm sections were stained with toluidine blue, examined by light microscopy and photographed. For TEM, the sections were contrasted with uranylacetate and lead citrate before examination (Jeol 1,200×).

#### Statistical evaluation

Statistical comparison between the groups were made using Wilcoxon's nonparametric rank-sum test for unpaired samples. A p-value of less than 0.05 was considered to be significant.

### Results

#### Morphological analysis of alveolar space and lung interstitium

*Smoking (S) versus nonsmoking (C) controls.* The total alveolar cell counts in the S-group were increased compared to the C-group (fig. 1). This increase was due mainly to an increase of small-medium sized macrophages (tables 1 and 2). The same difference was found in the peribronchial areas. In contrast, no difference in cell numbers could be seen between these two groups in the perivascular tissue (fig. 1).

Ultrastructurally, the alveolar septa of the S-group were slightly thickened, despite being lined by a gracile alveolar cell epithelium. The alveolar septa were characterized by extremely widened capillaries, containing numerous red blood cells (fig. 2e). No inflammatory cells or mast cells could be detected within the septa.

*Irradiation: smokers (RS) versus nonsmokers (RNS).* A more than twentyfold increase in the total number of alveolar cells was found in the RNS-group compared to the controls (fig. 1). This increase mainly comprised an increased amount of macrophages and lymphocytes (table 1 and 2, and fig 2c). In the RS-group the total cell numbers were also increased considerably, but significantly less than in the RNS-group (fig. 1). In contrast to the RNS-group, the increase was due mainly to an increased number of small-medium sized macrophages, whereas the increase of big macrophages and lymphocytes was less pronounced (table 1 and 2). In the interstitium, and peribronchial area, as well as in the perivascular area, a marked increase of total cell numbers was seen in the RNS-group, and to a minor degree in the RS-group (fig. 1).

Ultrastructurally, the septa were extremely thickened in the RNS-group, and infiltrated with inflammatory cells, mainly polymorphonuclear leucocytes, but also lymphocytes (fig. 2b). Several mast cells appeared in the septal connective tissue, which constituted the bulk tissue of the thickened septa, with fibroblasts, collagenous fibres and capillaries. The alveolar type I cell also seemed thickened, and displayed an increased number of microvilli on their surface. In clear contrast to the finding in the RNS-group, the RS-group displayed a minor septal thickening of the alveolar structure, closely resembling that of the nonsmoking (C) controls (fig. 2d). The alveolar septa were devoid of inflammatory cells, apart from occasional polymorphonuclear



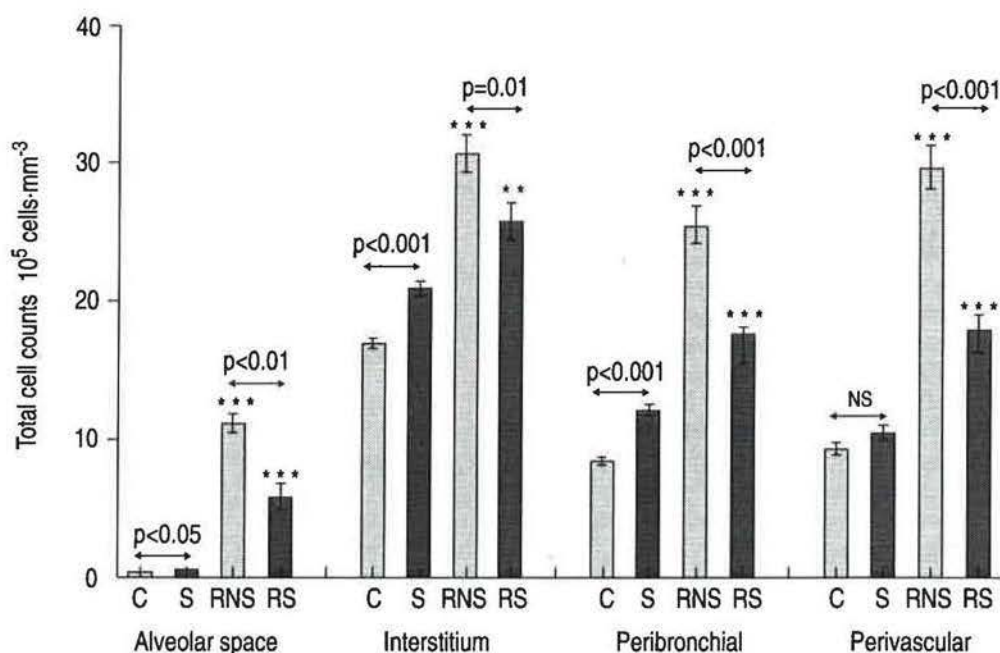


Fig. 1. — Quantification of total cells from alveolar space, lung interstitium, peribronchial and perivascular space. Four groups of rats, eight in each group, were divided into C: controls; S: smoking controls; RNS: irradiated nonsmokers; RS: irradiated smokers. Statistical comparisons among the groups were made by using Wilcoxon's nonparametric rank sum test for independent samples and differences between C versus RNS and S versus RS are flagged as \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; and \*\*\*:  $p < 0.001$ . Comparisons between C versus S and RNS versus RS are shown with arrows. The data are presented as mean bars  $\pm$  SEM. □: nonsmokers; ■: smokers.

Table 1. — Quantitative differential cell counting from bronchoalveolar lavage and alveolar space

	Bronchoalveolar lavage $10^4$ cells·ml <sup>-1</sup>				Alveolar space $10^5$ cells·mm <sup>-3</sup>			
	SMC	BMC	LC	PMN	SMC	BMC	LC	PMN
C	3.5 $\pm$ 0.5	0.12 $\pm$ 0.06	0.54 $\pm$ 0.16	0.16 $\pm$ 0.08	33.5 $\pm$ 5.6	0.25 $\pm$ 0.14	0.7 $\pm$ 0.4	0.22 $\pm$ 0.22
S	5.6 $\pm$ 0.9	0.26 $\pm$ 0.04	1.11 $\pm$ 0.67	0.46 $\pm$ 0.20	57.4 $\pm$ 5.9	0.02 $\pm$ 0.02	0.7 $\pm$ 0.1	0.24 $\pm$ 0.10
RNS	7.2 $\pm$ 0.7*	1.65 $\pm$ 0.43**	7.14 $\pm$ 1.72**	14.66 $\pm$ 3.36†	727.2 $\pm$ 6.8†	150.6 $\pm$ 24.1†	180.3 $\pm$ 24.7†	21.5 $\pm$ 9.4†
RS	5.8 $\pm$ 1.1 <sup>NS</sup>	0.71 $\pm$ 0.13*	3.63 $\pm$ 0.82**	5.38 $\pm$ 1.78**	535.1 $\pm$ 112.4†	23.18 $\pm$ 6.39†	17.8 $\pm$ 3.4†	6.82 $\pm$ 1.12†
C vs S	*	*	NS	NS	**	NS	NS	NS
RNS vs RS	NS	NS	NS	*	NS	†	†	**

C: controls; S: exposed to tobacco smoke; RNS: irradiated but not exposed to smoke; RS: irradiated and exposed to smoke; SMC: small to medium-sized macrophages; BMC: big, rich, vacuolized, foam-like macrophages; LC: lymphocytes; PMN: polymorphonuclear neutrophils. Data are presented as mean  $\pm$  SEM. Statistical comparisons between groups were made by using Wilcoxon's nonparametric rank-sum test for independent samples and differences between C versus RNS and S versus RS are flagged and C versus S, and RNS versus RS are indicated as \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; †:  $p < 0.001$ ; NS: nonsignificant.

Table 2. — Results from differential cell counting from bronchoalveolar lavage and alveolar space.

	Bronchoalveolar lavage %				Alveolar space %			
	SMC	BMC	LC	PMN	SMC	BMC	LC	PMN
C	81 $\pm$ 4.0	2.6 $\pm$ 0.9	13 $\pm$ 3.2	3.4 $\pm$ 1.6	97 $\pm$ 1.4	0.7 $\pm$ 0.4	1.6 $\pm$ 0.9	0.4 $\pm$ 0.4
S	80 $\pm$ 3.8	3.8 $\pm$ 0.5	12 $\pm$ 2.8	4.8 $\pm$ 1.7	98 $\pm$ 0.3	0.1 $\pm$ 0.1	1.1 $\pm$ 0.2	0.4 $\pm$ 0.2
RNS	25 $\pm$ 3.2†	5.2 $\pm$ 0.4*	22 $\pm$ 2.1*	48 $\pm$ 3.0†	65 $\pm$ 3.9†	14 $\pm$ 2.6†	16 $\pm$ 2.5†	1.7 $\pm$ 0.7†
RS	39 $\pm$ 8.2**	4.4 $\pm$ 0.6 <sup>NS</sup>	24 $\pm$ 5.5 <sup>NS</sup>	32 $\pm$ 10.1*	90 $\pm$ 1.9†	4.8 $\pm$ 1.2†	3.4 $\pm$ 0.8**	1.2 $\pm$ 0.3*
C vs S	NS	NS	NS	NS	NS	NS	NS	NS
RNS vs RS	NS	NS	NS	NS	†	**	†	NS

Data are presented as mean  $\pm$  SEM. Statistical comparisons between groups were made according to principles outlined in legend to table 1. For abbreviations see legend to table 1.

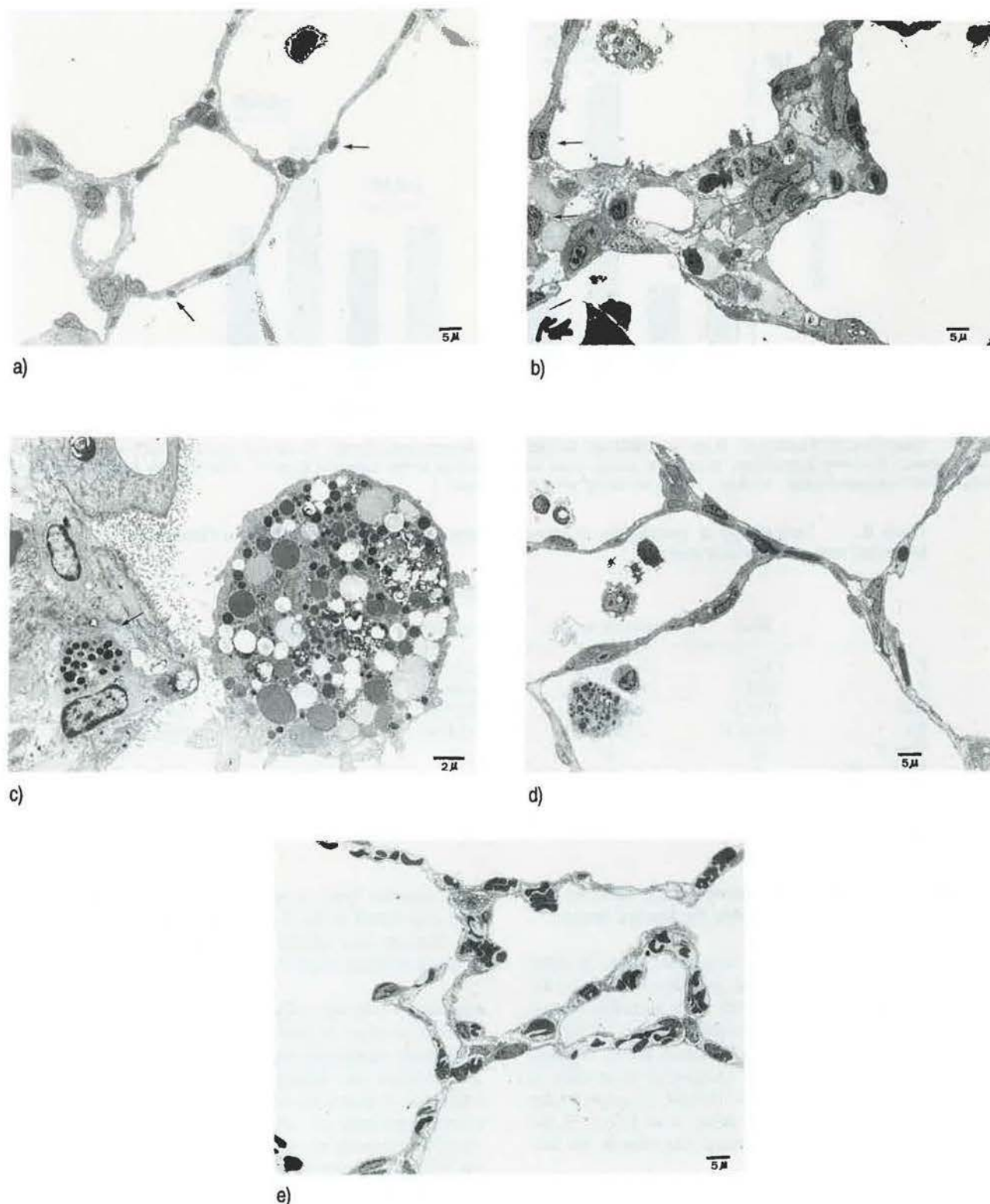


Fig. 2. - Electron micrographs of the alveolar lung septa from the various experimental groups. a) Nonsmoking control (C). The alveolar septa are thin, mainly lined by the alveolar type I cell. Single alveolar type II cells are visible (arrows). (Scale bar = 5  $\mu$ m). b) Irradiated nonsmoking rats (RNS). The septa are extremely thickened. Many inflammatory cells; lymphocytes and polymorphonuclear leucocytes are located within the connective tissue. Mast cells are observed (arrows). (Scale bar = 5  $\mu$ m). c) Irradiated nonsmoking (RNS). A macrophage is seen within the alveolar lumen. The epithelium is thickened, and a mast cell is located in the alveolar connective tissue (arrow). (Scale bar = 2  $\mu$ m). d) Irradiated smoking animals (RS). Except for a slight thickening of the alveolar septa the structure resembles that of nonsmoking controls. Inflammatory cells are noted in the alveolar lumen but only occasionally in the septal connective tissue. (Scale bar = 5  $\mu$ m). e) Smoking control (S). The alveolar septa are thickened because of extremely widened thin-walled capillaries. Otherwise the septal structure is very gracile. (Scale bar = 5  $\mu$ m).



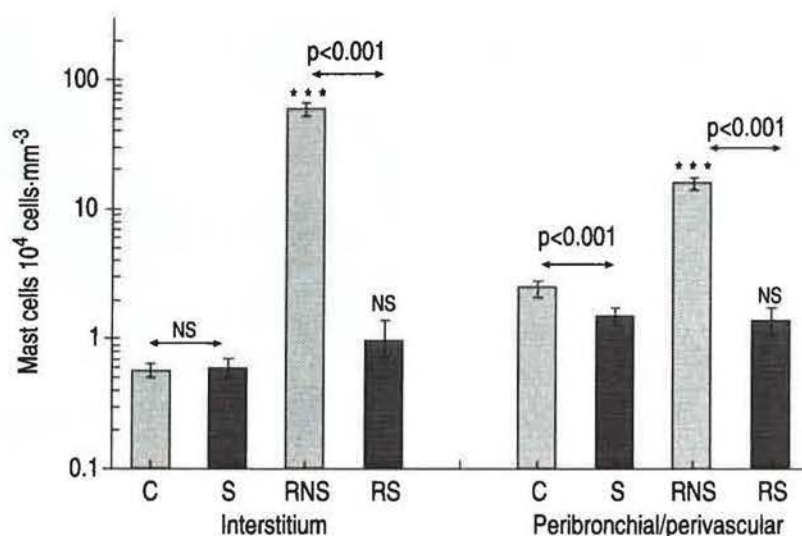


Fig 3. — Quantification of mast cells in the interstitial and the peribronchial/perivascular tissue. No or very few mast cells were seen in the alveolar space. Statistical comparisons between the groups were made according to the legend in figure 1. The data are presented as mean bars $\pm$ SEM.  $\square$ : nonsmokers;  $\blacksquare$ : smokers. For abbreviations see legend to figure 1.

Table 3. — Percentage of neutrophils in bronchoalveolar lavage, alveolar space, interstitial, peribronchial and perivascular tissue

	BAL	Alveolar space	PMN % Interstitial	Peribronchial	Perivascular
C	3.4 $\pm$ 1.6	0.43 $\pm$ 0.43	1.64 $\pm$ 0.43	0.46 $\pm$ 0.17	0.11 $\pm$ 0.08
S	4.8 $\pm$ 1.7	0.44 $\pm$ 0.18	1.60 $\pm$ 0.34	1.69 $\pm$ 0.27	0.59 $\pm$ 0.18
RNS	48 $\pm$ 3.0 <sup>†</sup>	1.67 $\pm$ 0.69 <sup>†</sup>	5.89 $\pm$ 1.02 <sup>†</sup>	3.31 $\pm$ 5.20 <sup>†</sup>	2.60 $\pm$ 0.77 <sup>†</sup>
RS	32 $\pm$ 10.1*	1.20 $\pm$ 0.29*	5.22 $\pm$ 0.62 <sup>†</sup>	2.68 $\pm$ 0.61 <sup>ns</sup>	0.60 $\pm$ 0.16 <sup>ns</sup>
C vs S	NS	NS	NS	**	*
RNS vs RS	NS	NS	NS	NS	**

Data are presented as mean $\pm$ SEM. Statistical comparisons between the groups were made according to principles outlined in legend to table 1. For abbreviations see legend to table 1.

leucocytes. Inflammatory cells, mainly macrophages, but also some lymphocytes, were seen within the alveolar lumen.

**Mast cells.** No, or very few, mast cells could be seen in the alveolar space and in the interstitial tissue of the C- and S-group. A few mast cells were normally located in the peribronchial and perivascular tissue in the C-group, markedly more than could be seen in the S-group. A more than one hundred fold increase of mast cells in the interstitium, and more than tenfold increase in the peribronchial and perivascular areas, was found in the RNS-group. In contrast, no increase was seen in the RS-group (fig. 3).

#### BAL versus alveolar space

**Smoking (S) versus nonsmoking (C) controls.** The total numbers of cells in BAL in the S-group was approximately twice that in the C-group (table 1). The differential counts did not distinguish between the groups, with approximately 80% being macrophages, mostly small to medium-sized, 10–15% being lymphocytes, and a few neutrophils (table 2).

In the alveolar space, a significantly higher number of total cells was found in the S-group than in the C group, and this increase was mainly due to an increased proportion of small-medium sized macrophages (table 1).

**Irradiation: smokers (RS) versus nonsmokers (RNS).** A fivefold increase in total cells in BAL was seen in the RNS-group, significantly higher than in the RS-group ( $p<0.04$ ). This increase was mainly due to an increased number of neutrophils, lymphocytes, and to a lesser degree large "foamy" macrophages (table 1). The total amount of neutrophils was 14.7 $\pm$ 3.4 (mean $\pm$ SEM) in the RNS-group, and 5.4 $\pm$ 1.8 in the RS-group, respectively (table 1).

In contrast to the BAL findings, the relative numbers of neutrophils was low in the alveolar space (tables 2 and 3). Whilst a more than twentyfold increase of total cells was seen in the RNS-group, which was mainly due to an increase of lymphocytes and macrophages with an increased proportion of large foamy macrophage (table 1 and 2). In the RS-group, a marked increase in the total number of cells in the alveolar space was also seen. It was, however, considerable less than in the RNS-group ( $p<0.007$ ).



### Discussion

It is obvious from the present data that smoking markedly suppressed the radiation-induced inflammatory reaction in the rat lung, as has been shown in humans [7, 8]. Structurally, the irradiated nonsmoking lungs were characterized by dramatically thickened alveolar septa, mainly consisting of newly formed connective tissue, containing many inflammatory cells and mast cells. In contrast, the irradiated smoking group showed hardly any changes at all; especially striking was the absence of mast cell response. The time-point for morphological analysis was chosen according to experiences from our previous studies, where we made a more detailed analysis on the time kinetic after irradiation induced pneumonitis in the rat lung [6]. The time kinetic studies were, however, performed solely on irradiated animals not exposed to smoke, and whether the time kinetic in smoke-exposed animals, in this respect, should be expected to act differently is partly an open question. One explanation for the differences between the smoke-exposed and nonsmoke-exposed animals could, therefore, hypothetically be that the inflammatory reaction occurred at different time-points after the irradiation, *i.e.* that the findings in the smoke-exposed group represented a later, more advanced, stage of reparation. This is, however, highly unlikely. We know, from the earlier studies, that the reaction with morphological changes, such as those seen in the nonsmoke-exposed irradiation group, is more likely to end in extensive formation of fibrosis and derangement of the tissue, than being repaired and restituted towards normal [6].

Very few mast cells were seen in the RS-group compared to the RNS-group. Moreover, the smoking controls had significantly fewer mast cells situated peribronchially and perivascularly than the nonsmoking controls. The reason for this is not clear. The reduction in mast cell numbers seen in the RS-group could be due either to a decreased recruitment of mast cells (or mast cell precursors) or degranulation of mast cells induced by tobacco smoke. Increased numbers of degranulated mast cells have been reported in the monkey lung exposed to smoke [10]. Complement C5A has been shown to release histamine from mast cells [11], and increased levels of C5A, C9 and C1-inhibitor have been reported in serum from human smokers [12]. However, in the present study, very few partly degranulated mast cells were found in the interstitial tissue in the RS-group, suggesting that degranulation was not a major reason for the absence of cells. Further studies are necessary to outline whether the mast cells are directly attacked by tobacco smoke inhalation or, indirectly, by the effect of smoke on other cellular targets in the inflammatory response.

The majority of mast cells involved in the radiation-induced lung reaction, at least in rat lung, are more likely to be of the connective tissue type [6]. Connective tissue mast cells are considered to exhibit little or no dependence on T-lymphocyte-derived factors for the recruitment to the tissue [13]; however, their proliferation seemed to be T-cell regulated [14]. Thus, an enhanced production of T-cell-derived cytokines may contribute to the pronounced infiltration of mast cells in the RNS-group. Mast cells are

known to interact with fibroblasts and, as such, may stimulate fibroblast proliferation as well as secretion of connective tissue components [15]. A close correlation between mast cell increase and fibroblast secretion of hyaluronan in the irradiation-induced pneumonitis, has been reported previously [16]. In sarcoidosis patients, a close correlation was found between increased numbers of mast cells and hyaluronan in BAL [17], and this increase was closely related to the disease activity and prognosis [18]. The findings were similar in patients with acute extrinsic allergic alveolitis [19]. Increased numbers of mast cells are also found in the interstitial tissue from patients with cryptogenic fibrosing alveolitis [20].

One major cellular target for the effect of tobacco smoke is most probably the alveolar macrophages. A significant increase in the total number of cells in the alveolar space as well as in the lung tissue was seen in the RNS-compared to the RS-group. This increase was mainly due to an increase of macrophages, and to a lesser degree of lymphocytes. Moreover, in the macrophage population an increased percentage of large, rich, vacuolized, "foamy" macrophages was seen. Alveolar macrophages from smokers release an increased amount of reduced  $O_2$ -radicals, compared to nonsmokers [21].

One other possibility that could explain parts of the assumed immunosuppressive effect, is the effect by smoke exposure on the lymphocytes. Chronic smoke exposure is known to cause a decreased ratio of CD4/CD8 positive lymphocytes [22, 23]. Moreover, smoking has also been reported to suppress the natural killer cell activity in the lung.

A substantial increase in BAL neutrophils was seen in the RNS-group, but also to a lesser degree in the RS-group. This increase was not reflected morphologically in the tissue, or in the alveolar space. A significant but modest increase in neutrophils was seen in the peribronchial area. Thus, the data might suggest that a significant portion of the neutrophils are derived from the bronchial and peribronchial regions. However, this may not be the only explanation. Variation in surface adherence and, thus, recoverability by BAL must also be considered. In irradiated rats, there was a more than twentyfold increase in total cell numbers in the alveolar space. In contrast, only a fivefold increase in total cells in BAL fluid was found. The explanation for this discrepancy between BAL and alveolar space, is more likely to be due to an increased adhesiveness in activated macrophages, and possibly also lymphocytes, making the cells less recoverable by BAL.

In addition to the absence of mast cells, the most striking features of the smoking controls [5] were markedly widened capillaries. It is difficult to assess to what extent these vascular changes may participate in the process of reducing inflammatory reaction in smoking-mediated animals compared to the nonsmoking controls [6]. An increased flooding of the alveolar tissue in the smoking controls [5] should be possible, and that would reduce the accumulation of inflammatory substances and cells within the tissue. Other possible mechanisms could include changed surface properties of the capillaries, interfering with *e.g.* the adhesion of inflammatory cells to the vessel wall.



The occurrence of hyaluronan seemed to be reduced in the smoking group following irradiation (CRS) [24]. It is feasible to suggest that reduced levels of hyaluronan could also lessen the connective tissue reaction after irradiation in the smokers. It has been suggested that accumulation of hyaluronan in the alveolar interstitial tissue plays a role in the fibrosis process in bleomycin-induced alveolitis [25]. The histopathological features of the alveolar septa in bleomycin-induced alveolitis resemble those of the irradiated nonsmokers.

In summary, the present study supports earlier observations that tobacco smoke can interact with irradiation-induced pulmonary inflammation. The lack of mast cells in the lung tissue of smokers might play an important role in the prevention of the development of irradiation-induced fibrosis. However, reduced numbers of other inflammatory cells and matrix components could also be of importance. Interactions between these elements in smokers and nonsmokers during irradiation should be further explored, as an increased knowledge in this field could be of value in explaining the process of the development of pneumonitis and normal tissue reaction following irradiation.

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## References

1. Douglas JG, Midleton WG, Gaddie J, et al. - Sarcoidosis: a disorder commoner in nonsmokers? *Thorax* 1986; 41: 787-791.
2. Warren CPW. - Extrinsic allergic alveolitis: a disease commoner in nonsmokers. *Thorax* 1977; 32: 567-569.
3. Jakab GI. - Adverse effect of cigarette smoke component, acrolein, on pulmonary antibacterial defence and on viral-bacterial infections in the lung. *Am Rev Respir Dis* 1977; 128: 33.
4. Harris JO, Swenson EW, Johnson JE. - Human alveolar macrophages: comparison of phagocytic ability, glucose utilization, and ultrastructure in smokers and nonsmokers. *J Clin Invest* 1970; 49: 2086-2096.
5. Hunninghake GW, Crystal RG. - Cigarette smoking and lung destruction; accumulation of neutrophils in the lung of cigarette smokers. *Am Rev Respir Dis* 1983; 128: 833-838.
6. Nilsson K, Henriksson R, Hellström S, Tengblad A, Bjermer L. - Hyaluronan reflects the prefibrotic inflammation in irradiated rat lung: concomitant analysis of parenchymal tissues and bronchoalveolar lavage. *Int J Rad Biol* 1990; 58(3): 519-530.
7. Bjermer L, Hällgren R, Nilsson K, et al. - Radiation-induced increase in hyaluronan and fibronectin in bronchoalveolar lavage from breast cancer patients is suppressed by smoking. *Eur Respir J* 1992; 5: 785-790.
8. Bjermer L, Franzén L, Nilsson K, Littbrand B, Ångström T, Henriksson R. - Bronchoalveolar lavage or irradiated breast cancer patients; effects of smoking and irradiated volume on inflammatory response. *Cancer Res* 1990; 50: 2027-2030.
9. Chen BT, Weber RE, Yeh HC, Lundgren DL, Snipes MB, Mauderly JL. - Deposition of cigarette smoke particles in the rat. *Fundam Appl Toxicol* 1989; 13: 429-438.
10. Walter A, Walter S. - Mast cell density in isolated monkey lungs on exposure to cigarette smoke. *Thorax* 1982; 37: 699-702.
11. Hugli TE, Muller-Eberhard HJ. - Anaphylatoxins: C3a and C5a. *Adv Immunol* 1978; 26: 1-53.
12. Wyatt RJ, Bridges RB, Halatek DG. - Complement levels in cigarette smokers: elevation of serum concentrations of C5, C9 and C1-inhibitor. *J Clin Lab Immunol* 1981; 6: 131-135.
13. Kitamura Y, Shimada M, Go S, Matsuda H, Hatanaka K, Seiki M. - Distribution of mast cell precursors in hematopoietic and lymphopoietic tissues of mice. *J Exp Med* 1979; 150: 482-490.
14. Dy M. - Increase in histamine production and mast cell proliferation during allograft rejection. *Transplant Proc* 1982; 14: 575-580.
15. Choi KL, Claman HN. - Mast cells, fibroblasts, and fibrosis. New clues to the riddle of mast cells. *Immunol Res* 1987; 6: 145-152.
16. Nilsson K, Hellström S, Henriksson R, Hällgren R, Bjermer L. - A mast cell secretagogue, compound 48/80, prevents the accumulation of hyaluronan in the lung tissue injured by ionizing irradiation. *Am J Respir Cell Mol Biol* 1990; 2: 199-205.
17. Bjermer L, Hällgren R, Engström-Laurent A, Thunell M. - Hyaluronic acid in bronchoalveolar lavage fluid in patients with sarcoidosis: relationship to lavage mast cells. *Thorax* 1987; 42: 93-98.
18. Bjermer L, Rosenhall L, Hällgren R. - The predictive value of bronchoalveolar lavage cell analysis in sarcoidosis. *Thorax* 1988; 43(4): 284-288.
19. Bjermer L, Engström-Laurent A, Lundgren R, Rosenhall L, Hällgren R. - Bronchoalveolar mastocytosis in farmer's lung is related to disease activity, and signs of fibroblast activation. *Arch Intern Med* 1988; 148(6): 1362-1365.
20. Kawanami O, Forram VJ, Fulmer JD, et al. - Ultrastructure of pulmonary mast cells in patients with fibrotic lung disorders. *Lab Invest* 1979; 40: 717-734.
21. Hoidal JR, Niewoehner DE. - Lung phagocyte recruitment and metabolic alterations induced by cigarette smoke in humans and in hamsters. *Am Rev Respir Dis* 1982; 126: 548-522.
22. Costabel U, Bross KJ, Reuter C, Rühle K-H, Matthys H. - Alterations in immunoregulatory T-cell subsets in cigarette smokers. A phenotypic analysis of bronchoalveolar and blood lymphocytes. *Chest* 1986; 90(1): 39-44.
23. Lawrence EC, Fox TB, Teague RB, Bloom K, Wilson RK. - Cigarette smoking and bronchoalveolar T-cell populations in sarcoidosis. *Ann NY Acad Sci* 1986; 465: 657-664.
24. Nilsson K, Henriksson R, Yiqing C, Hellström S, Hörnqvist-Bylund S, Bjermer L. - Effects of tobacco smoke on radiation-induced pneumonitis in rats. *Int J Radiat Biol* (in press).
25. Nettelblad O, Bergh J, Schenholm M, Tengblad A, Hällgren R. - Accumulation of hyaluronic acid in the alveolar-interstitial tissue in bleomycin-induced alveolitis. *Am Rev Respir Dis* 1989; 139: 759-762.