

# SOME LUNG CELLULAR PARAMETERS REFLECTING INFLAMMATION AFTER COMBINED INHALATION OF AMOSITE DUST WITH CIGARETTE SMOKE BY RATS

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## SUMMARY

Cellular changes were followed in lung cell suspensions after 175 day inhalation by rats of concentrations 30 mg/m<sup>3</sup> or 60 mg/m<sup>3</sup> of amosite asbestos every second day combined with daily exposure to cigarette smoke at 30 mg of total particulate matter (TPM)/m<sup>3</sup> air. Concomitantly, lung inflammation was assessed by changes in the bronchoalveolar lavage fluid (BALF). A dose-dependent rise in the BALF inflammatory parameters was found. The rise of the proportion of binucleate (BNC) and multinucleate cells (MNC) in lung cell suspensions was also dose-dependent. It is concluded that, in the experimental assessment of effects of fibrogenic dusts, the number of BNC and of MNC in lung cell suspensions may serve as a useful semiquantitative biomarker of the inflammation.

**Key words:** amosite, asbestos, inhalation, rats, multinucleate cells

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## INTRODUCTION

Multinucleate cells (MNC) may arise by fusion of macrophages involved into cleaning of foreign bodies from tissues. It was confirmed that after inhalation of fibrogenic dusts higher numbers of MNC appear in lungs from immigrated monocytes and macrophages, where these cells can also proliferate and some of them fuse (1-3).

The aim of this study was to assess if a dose-dependence of the induction of MNC number in lungs may exist after inhalation of two doses of amosite dust in combination with cigarette smoke. Concomitantly, some laboratory symptoms of inflammation were followed.

## METHODS

Male Fisher 344 rats weighing 191,2 (S.D.±10.4) grams were grouped by seven per group, maintained under non-infectious laboratory conditions at 22±2 °C. The animals were fed by standard laboratory pellets and water ad libitum. The exposures lasted 175 days (6 months).

Animals inhaled amosite fiber dust of South African origin in a nose-only inhalation device (In-Tox, USA). Amosite is a naturally occurring silicate of the amphibole asbestos species. The fiber size distribution of the used dust is presented in Table 1.

Every exposure was controlled by weighing the dust deposit on membrane filters located at one of the outlets of the chamber. Only Saturdays and Sundays were held free of exposures. The exposure scheme was:

- 60 mg/m<sup>3</sup> amosite fibers for one hour every second day, combined with exposure to mainstream smoke from three cigarettes daily;
- 60 mg/m<sup>3</sup> amosite fibers for one hour every second day;
- 30 mg/m<sup>3</sup> amosite fibers for one hour every second day, combined with exposure to mainstream smoke from three cigarettes daily;
- 30 mg/m<sup>3</sup> amosite fibers for one hour every second day;
- exposure to mainstream smoke from three cigarettes daily plus immobilization stress as for animals exposed to dust;
- immobilisation stress as for animals exposed to dust;

The immobilisation stress at the exposure arose at putting the animals into tight organic glass cylinders. For smoke inhalation standard research cigarettes of the 1R1 type in a whole-body

**Table 1.** Particle size distribution by length

Length (µm)	%	Mean diameter (µm)
< 20	5	0.71
20-30	75	0.71
> 30	20	0.71

exposure chamber were used (Tobacco and Health Research Institute - THRI, Lexington, KY, USA). Smoker animals breathed diluted main-stream tobacco smoke at the concentration of 30 mg of total particulate matter (TPM)/m<sup>3</sup> air for one hour daily requiring to burn three cigarettes. The smoke dose was controlled by weighing "Cambridge" type glass wool filters (THRI, Lexington, KY, USA).

Six months after beginning of the inhalation exposures, the animals were exsanguinated in anaesthesia. Alveolar macrophages (AM) were harvested by bronchoalveolar lavage. The BALF cell distribution was determined on May-Grunwald-Giemsa stained preparations counting 100 cells. BALF binucleate cell counts on the same preparations were assessed counting 500 cells per animal. Protein was measured by the method of Lowry et al (4). Bovine serum albumin was used as standard for the calibration curve.

Lungs were perfused and minced in 4 ml of PBS, then digested in 0.25% trypsin solution (Difco). The cells were gained by filtering through 2 layers of gauze into ice cold foetal calf serum. Then, 3 x 10<sup>4</sup> cells were cytocentrifuged on one slide by a Cytospin (Shandon, USA) centrifuge. Cells were stained for DNA by Feulgen (5) procedure, the cytoplasm counterstained by 1% Light Green. Mononucleate, binucleate (BNC) and multinucleate (MNC) cells were counted using a 100x objective.

#### Statistical Methods

The differential cell counts from individual rats were grouped according to the exposure type and the differences between the proportions of cells in all 6 groups were tested. Binomial distribution

was the base for calculation of confidence intervals ( $\alpha = 0.05$ ) at using Bonferroni modification (6,7). Testing the possible appearance of a synergic effect after combined exposures was performed by a test based on counts of non-BNC or non-MNC (8).

#### RESULTS

Smoking alone caused a statistically significant rise in the proportion of BNC in lung cell suspension by a factor of about 1.3 and a rise in the proportion of MNC by a factor of 2.5 (Table 2 and 3) as compared with the control - unexposed group.

A clear-cut dose-response trend could be observed in the change of the proportion of BNC in both, amosite-only and combined amosite plus cigarette smoke exposed groups (Table 2). Comparison with the control group showed that the proportion of BNC rose by a factor of about 6.5 ( $p < 0.05$ ) in the 30 mg/m<sup>3</sup> group, and by a factor of about 9 ( $p < 0.05$ ) in the 60 mg/m<sup>3</sup> group, the difference between the exposed groups was significant ( $p < 0.05$ ). A positive, statistically significant correlation ( $p < 0.01$ ;  $r = 0.47$ ) between the proportions of the BNC in lung tissue suspensions and in the BALF was observed.

The rise in the proportion of MNC after amosite inhalation has had trends similar to the rise of BNC (Table 3). This parameter showed much greater sensitivity especially after combined dust plus cigarette smoke exposure in that at comparing with controls the proportion of MNC rose in the 30 mg amosite/m<sup>3</sup> plus smoking

**Table 2.** Percent and 95% confidence intervals (C.I.) of BNC in lung cell suspensions of rats after amosite inhalation combined with smoking

Group	No. of rats	No. of BNC	No. of total cells counted	Average %	95%C.I.		S*
					lower	upper	
C	7	225	15,507	1.45	1.27	1.65	$\alpha\beta\gamma\delta\epsilon$
S	7	383	20,415	1.88	1.69	2.07	$\alpha\phi\eta\kappa\lambda$
30	7	1,665	17,088	9.74	9.30	10.20	$\beta\phi\mu\nu$
60	7	1,701	12,999	13.09	12.51	13.68	$\delta\kappa\mu\theta$
30S	7	1,566	15,370	10.19	9.71	10.68	$\gamma\eta\theta\rho$
60S	7	2,501	20,185	12.39	11.94	12.85	$\epsilon\lambda\nu\rho$

Legend: C - control; S - smoking only; 30-30 mg amosite/m<sup>3</sup>; 60-60 mg amosite/m<sup>3</sup>;

30S - 30 mg amosite/m<sup>3</sup> + smoking; 60S - 60 mg amosite/m<sup>3</sup> + smoking;

S\* - significance of the difference: pairs of Greek characters denote pairs with significant differences at  $p < 0.05$ .

C.I. -  $\pm 95\%$  confidence interval

**Table 3.** Percent and 95% confidence intervals (C.I.) of MNC in lung cell suspensions of rats after amosite inhalation combined with smoking

Group	No. of rats	No. of MNC	No. of total cells counted	Average %	95%C.I.		S*
					lower	upper	
C	7	12	15507	0.08	0.04	0.14	$\alpha\beta\gamma\delta\epsilon$
S	7	40	20415	0.20	0.14	0.27	$\alpha\phi\eta\kappa\lambda$
30	7	340	17088	1.99	1.79	2.21	$\beta\phi\nu$
60	7	272	12999	2.09	1.85	2.35	$\delta\kappa\mu$
30S	7	340	15370	2.21	1.99	2.46	$\gamma\eta\rho$
60S	7	597	20185	2.96	2.73	3.20	$\epsilon\lambda\mu\nu\rho$

Legend: the same as in Table 2.

group by a factor of 28.5 ( $p < 0.05$ ). The proportion of MNC in the higher 60 mg/m<sup>3</sup> plus smoke level rose by a factor of about 38 ( $p < 0.05$ ) and testing showed a statistically significant ( $p < 0.05$ ) presence of synergy (more than expected additivity) of combined effects. In samples from non-smoker groups there was observed a rise from control level at the group exposed to 30 mg/m<sup>3</sup> by a factor of about 25.5 ( $p < 0.05$ ) but further rise at the 60 mg/m<sup>3</sup> dust dose was not significant.

In BALF, the total number of cells/ml was found to be increased significantly ( $p < 0.01$ ) after combined 60 mg/m<sup>3</sup> plus smoking exposure, in comparison with the control as well as with the corresponding dust-only exposure group. The proportions of lymphocytes showed significantly ( $p < 0.05$ ) increased values in the dust-only exposed groups as compared to the non-exposed controls. In the combined dust plus smoking groups lymphocyte proportions rose significantly ( $p < 0.05$ ) only after higher dust dose. The percentages of polymorphonuclear leukocytes showed a statistically significant ( $p < 0.05$ ) rise in the amosite-only 60 mg/m<sup>3</sup> group, and in both combined dust plus smoking groups, as compared with the control group values. The reverse trend, to reduced proportions of AM with increasing exposure dose, was observed in both dust-only and combined dust plus smoking groups of animals. Moreover, the 60 mg/m<sup>3</sup> dust plus smoking group showed also a significantly ( $p < 0.05$ ) lower suppression of AM proportions as compared with the smoking-only group.

The proportion of BNC in BALF showed a tendency to increase with the dust dose, but statistically significant ( $p < 0.05$ ) increase, as compared to controls, was seen only in the group exposed to 60 mg/m<sup>3</sup> amosite alone. A statistically significant ( $p < 0.05$ ) increase, relative to the control group, in the total BALF protein levels in animals exposed either to dust or to smoking alone was found. The total protein levels in BALF in groups inhaling combined amosite and smoke rose significantly ( $p < 0.01$ ) also in comparison to the smoking only group. Other inflammation biomarkers followed in this experiment are described elsewhere in this journal issue (9).

## DISCUSSION

The higher than control values of the proportions of PMN, of lymphocytes and of protein concentration in the BALF proved the presence of inflammation in the lung at sacrifice. Such findings were described previously by our and also by other laboratories (10-12). The magnitude of the increase of these parameters was dose-dependent.

Associated with inflammatory changes, a dose-dependent increase in BNC proportions was found in the BALF as well as in the lung tissue suspensions. Both BNC and MNC counts increased in lung cell suspensions after exposure (separate or in combination) to tobacco smoke as well as both fibre concentrations. Furthermore, a significantly positive correlation between the proportions of BNC in BALF and in lung cell suspensions suggested significant immigration of BNC from lung interstitium into alveoli. This is the first time that such correlation has been reported, as no corresponding data could be found in the accessible literature.

There is wide agreement that the vast majority of BNC and MNC found in vivo are derived from cells belonging to the macrophage population. Binucleate cells may appear in normal, control lung tissue suspensions at frequencies close to 1%, and are also

often found in BALF. Their number may increase after various genotoxic stimuli and also after exposure to various types of fibre dusts. In vitro experiments by Hong and Choi (13) showed a dose-dependent response of the number of MNC cells after exposure of hamster V79 cells to asbestos fibers.

Studies of the phagocytic capacity of "giant" MNC formed in vivo suggest that the mechanisms of macrophage fusion may operate generally in a similar way at granulomatous inflammation (14-15). These observations suggest that the MNC are an unspecific marker of subchronic- and chronic-type inflammation. Nevertheless, in specific experimental situations corresponding to a specific exposure to known fibrous particles, this marker becomes a specificity.

In conclusion, the findings of the study reported here indicate that the count of BNC and, especially, of MNC in lung suspensions from experimental animals exposed to fiber dusts may reflect the presence of inflammation and, as such, may be considered as a useful semiquantitative biomarker of the specific inflammation effect.

## Acknowledgement

The work was supported by an E.U. grant, contract, No. QLK4-CT-1999-01629 (FIBRETOX project). We wish to thank to Professor Dr A. Collins for carefully reading the manuscript and to Mrs. H. Bobeková, Mrs. D. Čepcová, Mrs. G. Dragúňová, Ms. M. Valentová, Ms. L. Miklošková, Mr. D. Klamo for skilful technical assistance.

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