

## **The modifications produced in allergic alveolitis and in goodpasture's syndrome due to exposure to cigarette smoke**

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**Summary.** Two groups of rats with experimental alveolitis were exposed to cigarette smoke. After comparing the results, the possible muffling effect of the cigarette smoke related to interstitial lung disease was discussed.

180 rats were divided into 6 groups of 30 animals each: Group 1: untreated controls; Group 2: exposed to cigarette smoke for 2 months; Group 3: sensitized with bovine albumin (BA) and exposed to an atmosphere with this antigen for two months, to reproduce a type of extrinsic allergic alveolitis (EAA); Group 4: treated with a single daily dose of anti-lung serum for three days followed by two days without treatment, to reproduce a type of Goodpasture's syndrome; Group 5: exposed to cigarette smoke and to BA; Group 6: exposed to cigarette smoke and treated with anti-lung serum. The animals were sacrificed and their lungs were treated for: Bronchoalveolar lavage (BAL), percentage lymphocyte count, polymorphonuclear (PMN) and alveolar macrophages (AM); semiquantitative and morphometric histological study. The semiquantitative study determined the area of the studied lung incision, affected by granulomae, increased alveolar aerial spaces, thickened alveolar walls and haemosiderine lung area. The morphometric study, based on the linear integration method, evaluated: the distance between two alveolar walls, the amount of interstice per field; and the number of AM with haemosiderine per field was counted.

From the results we point out that the treated animals had significantly higher lymphocyte and BAL PMN counts than the untreated ones; no significant differences were found between the singly and doubly treated animals. The animals exposed to cigarette smoke and treated with anti-lung serum were those

that showed the least number of lymphocytes and PMN of all the treated animals. The semiquantitative variables studied were all increased in comparison to the control group, most of the increases being significant. The morphometric variables revealed significant differences with respect to the untreated group, except for the animals which were treated with anti-lung serum and cigarette smoke, which showed a minimum decrease in the alveolar size and a slight increase of the interstitial tissue. Only one morphometric variable showed a significant difference between the group treated with anti-lung serum and the one treated with anti-lung serum and cigarette smoke: the number of AM with haemosiderine in the lung.

From the results we conclude that: 1) exposure to cigarette smoke causes alveolo-interstitial alterations which are detected by means of BAL and histology; 2) these alterations have no adjuvant effect when combined with the administration of BA; 3) the alveolo-interstitial affection found in the animals exposed to cigarette smoke and anti-lung serum is lower than in the animals which were only given anti-lung serum.

**Key words:** Lung, Rat, Cigarette smoke, Extrinsic allergic alveolitis, Goodpasture's syndrome, Bronchoalveolar lavage, Morphometry, BA, Anti-lung serum

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### **Introduction**

The immune complexes are considered to be one of the most important causes of the onset of interstitial lung disease (Shlueter, 1974; Harman, 1985). The harmful effect of the immune complexes is a consequence of their deposition on tissue. This deposit can be caused either by the attachment of an antibody to an exogenous antigen, or by the binding of

the antibody to a tissular antigen of the basement membrane. The first type would correspond to extrinsic allergic alveolitis (EAA), and can be experimentally produced by giving a previously sensitized animal an antigen via air (Ratczak et al., 1980). The second type of disease corresponds to Goodpasture's syndrome, which can be reproduced experimentally, depending on the nature of the antibody, in an autologous or heterologous way (Stebay, 1983) and at the same time can affect the lung and the kidney with varying intensity (Holdsworth et al., 1985).

The antigen binding to the antibody causes a series of phenomena, such as complement activation, recruitment of neutrophils, alveolar macrophages, etc., the result of which appears clinically as an illness (McDermott et al., 1982; White and Kaliner, 1987).

Goodpasture's syndrome, which we will call anti-lung disease (ALD), differs from EAA because it present antibodies to the alveolar basement membrane, together with lung haemorrhage (Harman, 1985; Holdsworth et al., 1985; Carré et al., 1988). Morphologically, in both diseases, immune complexes can be found in the lung by means of immunohistological techniques, the linear deposition of immune complexes on the alveolar basement membrane being a characteristic of anti-lung disease (Glasscock, 1989). The other morphological aspects are less specific: septal thickness, cellular infiltrates in the alveoli and interstices, granuloma, etc. (Salvaggio and Karr, 1979; Reyes et al., 1982; Coleman and Colby, 1988).

In these type of diseases, the predisposing agents are very important, and so the appearance of Goodpasture's disease has been linked to the exposure to hydrocarbons, either industrial (Bernis et al., 1985; Yamamoto and Wilson, 1987) or from the combustion of tobacco (Donaghy and Rees, 1983), even though there is no unanimity with regard to the latter (Leaker et al., 1984). In EAA the nature of the antigen is very important, as is the existence of adjuvants, the individual person's characteristics, etc (Hargreave and Pepys, 1972), and unlike Goodpasture's syndrome, there is general agreement among the authors consulted on the action of cigarette smoke; they all agree that smokers have a lower tendency to suffer EAA than non-smokers (Warren, 1977; Andersen and Cristensen, 1983). This suggests that whereas cigarette smoke can cause serious lesions, it could have a damping effect on some kinds of diseases (Warren, 1977).

Furthermore, the negative action of cigarette smoke on the organism has been widely demonstrated (Foxman et al., 1986; Carstensen et al., 1987), so it would be logical to consider the possibility of cigarette smoke having a negative effect on EAA and anti-lung disease; however, the results we have do not bear out this last point.

In this study, we develop from the morphological point of view, the effect of cigarette smoke in

interstitial lung disease, using as a hypothesis, the possibility that cigarette smoke does not have a negative effect on experimental interstitial lung disease. For this purpose, we compared two experimental lung disease models, EAA and anti-lung disease, with other similar ones which have been exposed to cigarette smoke.

The experiment set-up was as follows: First, the different experimental disease were induced in five groups of animals. The lungs of these animals were compared to another group of untreated animals. Secondly, the animals with induced EAA and anti-lung disease were compared with those with double treatment («EAA + cigarette smoke» and «anti-lung disease + cigarette smoke»). The lungs were studied with morphological methodology: broncho-alveolar lavage cytology, together with the morphometric and semiquantitative analysis of the lung tissue.

## Materials and methods

180 adult Wistar rats, both male and female, aged between two and eight months, were distributed into groups of thirty animals each, as follows:

- 1) Untreated control.
- 2) Exposed to cigarette smoke.
- 3) Treated with BA (bovine albumin).
- 4) Treated with anti-lung serum.
- 5) Treated with BA and cigarette smoke.
- 6) Treated with cigarette smoke and anti-lung serum.

### *Treatment guidelines*

**Exposure to cigarette smoke:** This was carried out following previous experiences (Maurain Vilorio et al., 1989); using black-blend commercial cigarettes (1.1 mg of nicotine and 19 mg of tar) connected to a source of continuous flowing air that led the smoke into the cages. The dose was of 10 cigarettes per day for two months, five days a week.

**BA:** The animals were sensitized by means of injection in the sole pad of three doses of  $9 \times 10^{-6}$  BA in 0.1 cc of saline and 0.1 cc of complete adjuvant. There was an interval of two weeks between each dose. At the fifth week, after checking the presence of anti-BA antibodies in the animals' blood by means of immunoprecipitation, they were put into cages similar to the ones used for the exposure to cigarette smoke and, by means of a Hudson device, they were exposed to a spray containing BA at a concentration of  $10 \times 10^8$  for two months, 8 hours a day, five days a week. The animals were sacrificed after the last exposure. This was done so as to reproduce a model of experimental extrinsic allergic alveolitis (EAA).

**Anti-lung serum:** Prepared by Operon laboratories, by means of the administration of rat alveolar basement membrane extract (Meezan et al., 1975; Jennings et al.,

1981) to rabbits and subsequent exsanguination. 0.2 cc/day were injected intravenously for three days. The animals were sacrificed on the fifth day after the first dose.

*EAA and Cigarette smoke:* The animals were treated in a similar way to those in the third group and were exposed to cigarette smoke two months before sacrifice.

*Cigarette smoke and anti-lung serum:* The animals were exposed to cigarette smoke for two months. Five days before sacrifice they were given anti-lung serum.

#### Material processing

*Sacrifice:* After putting the animals under anaesthesia with 1% pentathol (1 cc/100gr weight), a catheter (Venocath no. 18) was inserted via a tracheostomy orifice to the right lung for bronchoalveolar lavage. Afterwards, the thoracic cavity was opened and the left lung pedicle was bound and its lung was taken out for histological processing.

*BAL:* Using a technique which has already been described (Maurain Viloria et al., 1989), 4 equal 1 cc aliquots of saline were inserted through the catheter that was situated in the right lung bronchus and then extracted. The product was centrifuged. The sediment was spread on a slide and was stained following the Giemsa method, in order to perform a percentage alveolar macrophage (AM), lymphocyte and polymorphonuclear (PMN) count.

*Histological study:* The left lung was fixed by immersion in 10% formalin with tracheal insufflation of the same fixer at a positive pressure of 26 cm of water. After dehydrating it by successive immersions in increasing concentrations of alcohol, it was put in paraffin wax and was cut at 7  $\mu$ m. The PAS and Pearl Prussian Blue (to detect haemosiderine) staining techniques were used. The histological study was always made on the same section which corresponded to the perpendicular plane of the main axis of the lung, where the main bronchus becomes intrapulmonary (Fig. 1).

#### Morphometric study

The pulmonary section was divided into 13 zones of which 7 were selected (Fig. 2). A field was selected at random in each zone, and the linear integration technique and the alveolar macrophage (dyed with the Pearl Prussian blue method) count were carried out.

For the linear integration, a digital pad (Bid Plad Plus) connected to a Macintosh IIfx computer was used. Seven parallel, equidistant lines were drawn on the graphic pad. A Nikon microscope ( $\times 10$  eye lens and  $\times 40$  objective glass) with an overhead projector was used to superimpose the field image, stained with

the PAS method, on the graphic pad. The distance between the walls of an alveolus (Fig. 3) was drawn on the prefixed parallel lines. Using a computer programme developed by us, the signal produced in the graphic pad was converted into linear distance units on the computer. One unit (equal to one pixel) was equivalent to 0.86  $\mu$ m. Two types of variables were obtained from this study:

*Alveolar variable:* Obtained directly from the distance between two alveolar walls. Its results were transformed to the function  $\text{Log}(1 + \times)$ .

*Interstitial variable:* This refers to the quantity of Interstice per field. It was obtained by subtracting the sum of the alveolar distances in each histological field from the sum of the lengths of the seven fixed lines of the field.

The AM count with haemosiderine was carried out in seven microscopical fields chosen in a similar way to the linear integration method.

*In the semiquantitative study,* the following variables were taken into account:

Percentage of pulmonary surface covered with granulomae. BALT (Fig. 4) is not considered as granuloma.

Percentage of pulmonary surface with increased alveolar aerial spaces (Fig. 5).

Percentage of pulmonary surface with thickened alveolar walls (Fig. 3).

Percentage of pulmonary surface with haemosiderine. Presence of alveolar macrophages (AM) with Pearl Prussian Blue-positive granulations (Fig. 6).

The pulmonary surface affected by these variables was quantified in relation to the total surface of the studied section. A Macintosh IIfx computer with the Mac Draft programme was used.

#### Statistical study

The mean  $\pm$  standard deviation of each variable is given. The distribution curves were analysed with different statistical indexes, and the distribution was taken as not normal when the Kurtosis and Skewness indexes were beyond  $\pm 1$ .

To compare the values obtained with the semiquantitative and morphometric methods of counting the Pearl Blue-positive cells, non-parametric tests (Kruskal-Wallis and the Mann-Whitney U) were used because the distribution of these values were far from normal. To compare the results obtained by means of linear integration, parametric tests were used (comparison of variances, ANOVA and Student's tests). The variables obtained were related by means of linear integration with the simple regression test, and an index was considered to be rateable when the absolute values were greater than 0.6. Values were

considered to be significant when the probability was lower than 5% ( $p < 0.05$ ). Data are expressed throughout as mean  $\pm$  SD.

The statistical study was carried out by means of a Macintosh IIcx computer with the StatView II programme.

## Results

### BAL

All the treated animals had a significantly higher ( $p < 0.05$ ) percentage lymphocyte and PMN count in comparison with the control group (PMN  $3\% \pm 9$ ; lymph.  $11\% \pm 4$ ). There were no significant differences ( $p > 0.05$ ) between the groups of treated animals (Table 1).

When the animals treated with BA (PMN  $14\% \pm 17$ ; lymphocytes  $24\% \pm 17$ ) were exposed to cigarette smoke, the percentage PMN count increased ( $15\% \pm 23$ ) and the percentage lymphocyte count decreased ( $21\% \pm 12$ ). The group of animals treated with anti-lung serum and cigarette smoke had a decrease in PMN ( $8\% \pm 13$ ) and lymphocytes ( $19\% \pm 13$ ) compared to the group treated with anti-lung serum alone (PMN  $12\% \pm 15$ ; lymphocytes  $20\% \pm 11$ ).

### Histological study

#### Semiquantitative study

The animals exposed to BA, either alone or with cigarette smoke, were the only ones that had granulomae in their pulmonary tissues (Table 2). The rest of the semiquantitative variables were higher in the treated animals when compared to the controls, with the exception of «thickened alveolar walls» in animals exposed to cigarette smoke; this increase was significant ( $p < 0.001$ ) (Table 2). When the animals with EAA and ALD were exposed to cigarette smoke, their lungs underwent important variations in terms of «increased alveolar aerial spaces» and «thickened alveolar walls».

The exposure to cigarette smoke of the animals treated with BA produced a significant increase ( $p < 0.01$ ) in the pulmonary surface with «increased alveolar air spaces» (BA:  $9.4 \pm 18.8$ ; BA + cigarette smoke:  $17.8 \pm 19.5$ ); the result being similar to the one obtained in animals exposed to cigarette smoke alone ( $18.7 \pm 13.4$ ) (Table 2). On the other hand, the animals treated with anti-lung serum and exposed to cigarette smoke suffered a slight decrease in the variable (anti-lung:  $18.8 \pm 21$ ; anti-lung + cigarette smoke  $16 \pm 19$ ;  $p > 0.05$ ).

The pulmonary surface with «thickened alveolar walls» decreased, without reaching significant values ( $p > 0.05$ ) with the combinations «BA and cigarette smoke» (BA:  $21.6 \pm 21.9$ ; BA + cigarette smoke:  $22.4 \pm 21$ ), and «anti-lung serum and cigarette smoke» (anti-lung:  $36.4 \pm 25$ ; anti-lung and cigarette smoke

$25 \pm 37.9$ ). These changes were significantly higher ( $p < 0.01$ ) than those of animals exposed to cigarette smoke alone ( $5.1 \pm 8.4$ ).

The pulmonary surface variable occupied by «AM with haemosiderine» did not show significant variations ( $p > 0.05$ ) when comparing animals with EAA and ALD and those exposed to cigarette smoke (Table 2). The highest figures were shown in the two groups which received anti-lung serum (anti-lung:  $37.4 \pm 39.9$ ; anti-lung + cigarette smoke  $35.5 \pm 38.6$ ). The differences between the animals treated with BA were higher, because the mean of those treated with BA and cigarette smoke ( $3.3 \pm 5.9$ ) was less than half that found in the group exposed to BA alone ( $7.6 \pm 14.8$ ).

FIG 1

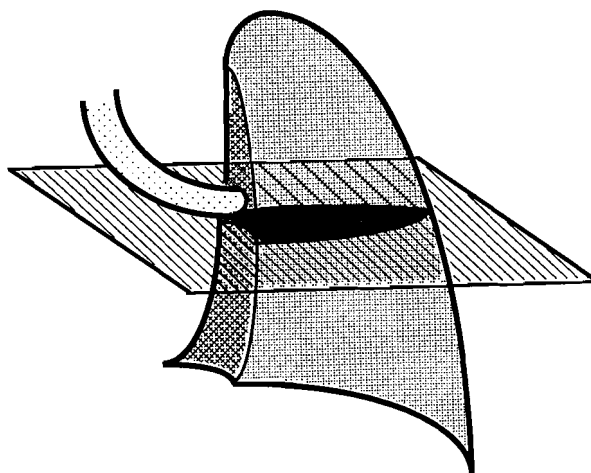


Fig. 1. Schematic representation of the plane of the histological section used in the study, which is perpendicular to the main lung axis in the area where the bronchus becomes intraparenchymatous.

FIG 2

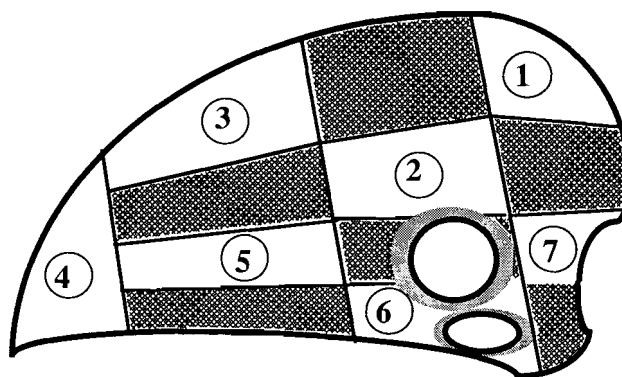
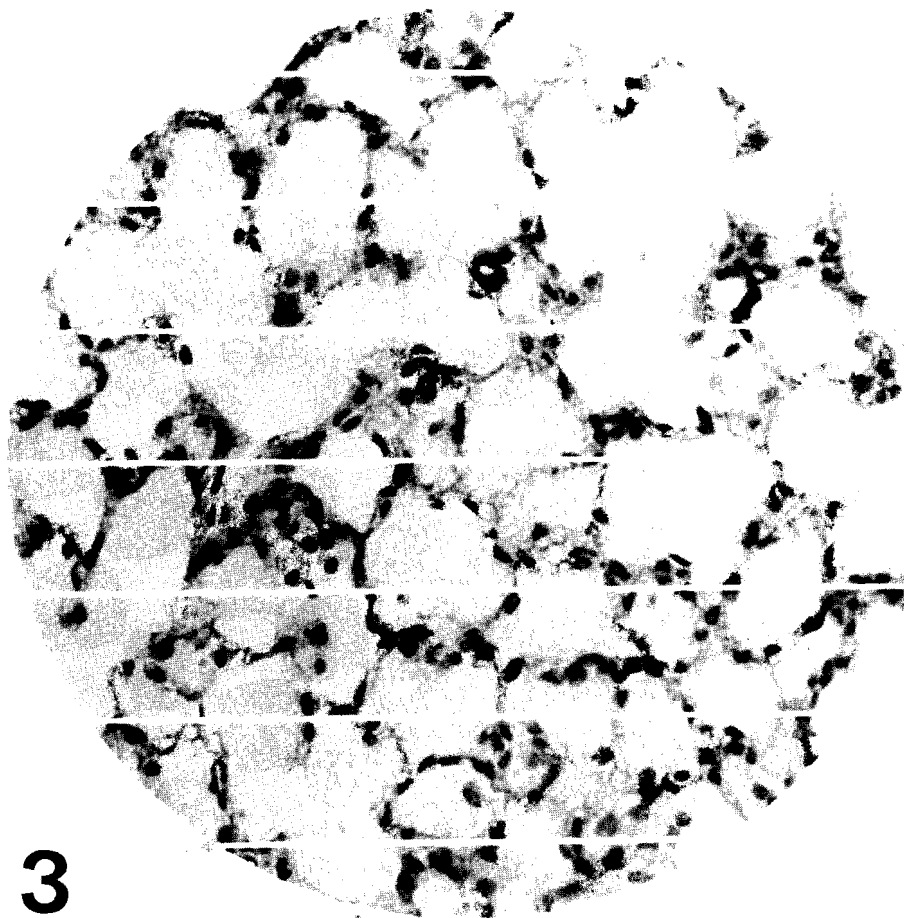


Fig. 2. Schematic representation of the histological section divided into thirteen areas. The morphometric study was carried out on the seven numbered areas.



**Fig. 3.** Superimposition on the microscopic field of the seven equidistant parallel lines. The distance between the two alveolar walls was calculated by drawing on the lines. The histological section corresponds to the semiquantitative variable «thickened alveolar walls». Control group animal. PAS stain.

**3**

The last result proved to be very similar to the figure for animals exposed to cigarette smoke alone ( $7.6 \pm 1.48$ ).

#### Morphometry

##### Linear integration

All the animals treated showed a decrease in the mean alveolar variable and an increase in the interstitial (Table 3) when compared to the control group (alveolar  $1,404 \pm 35$ ; interstitial:  $646 \pm 180$ ). These variations were significant in all the groups ( $p < 0.001$ ) except in the one treated with anti-lung serum and cigarette smoke (alveolar:  $1,397 \pm 36$ ; interstitial:  $686 \pm 217$ ).

Animals exposed to cigarette smoke and BA showed a non-significant decrease in the alveolar distance ( $1,320 \pm 227$ ) and an increase in the interstitial variable ( $782 \pm 227$ ) when compared to those treated with BA alone (alveolar:  $1,330 \pm 38$ ; interstitial:  $758 \pm 208$ ;  $p > 0.05$ ). After exposing the animals treated with anti-lung serum (alveolar:  $1,320 \pm 34$ ; interstitial:  $838 \pm 221$ ) to cigarette smoke, alterations of a different sign to those found in the previous case were

discovered: the alveolar component increased ( $1,397 \pm 36$ ) and the interstitial one decreased ( $686 \pm 217$ ) ( $p > 0.05$ ).

When relating the amount of interstice per field with the mean alveolar distance per field, negative regression indexes were obtained; only the animals treated with BA reached an absolute value higher than 0.6 ( $r = -0.62$ ); the indexes of the rest of the groups did not exceed an absolute value of 0.55.

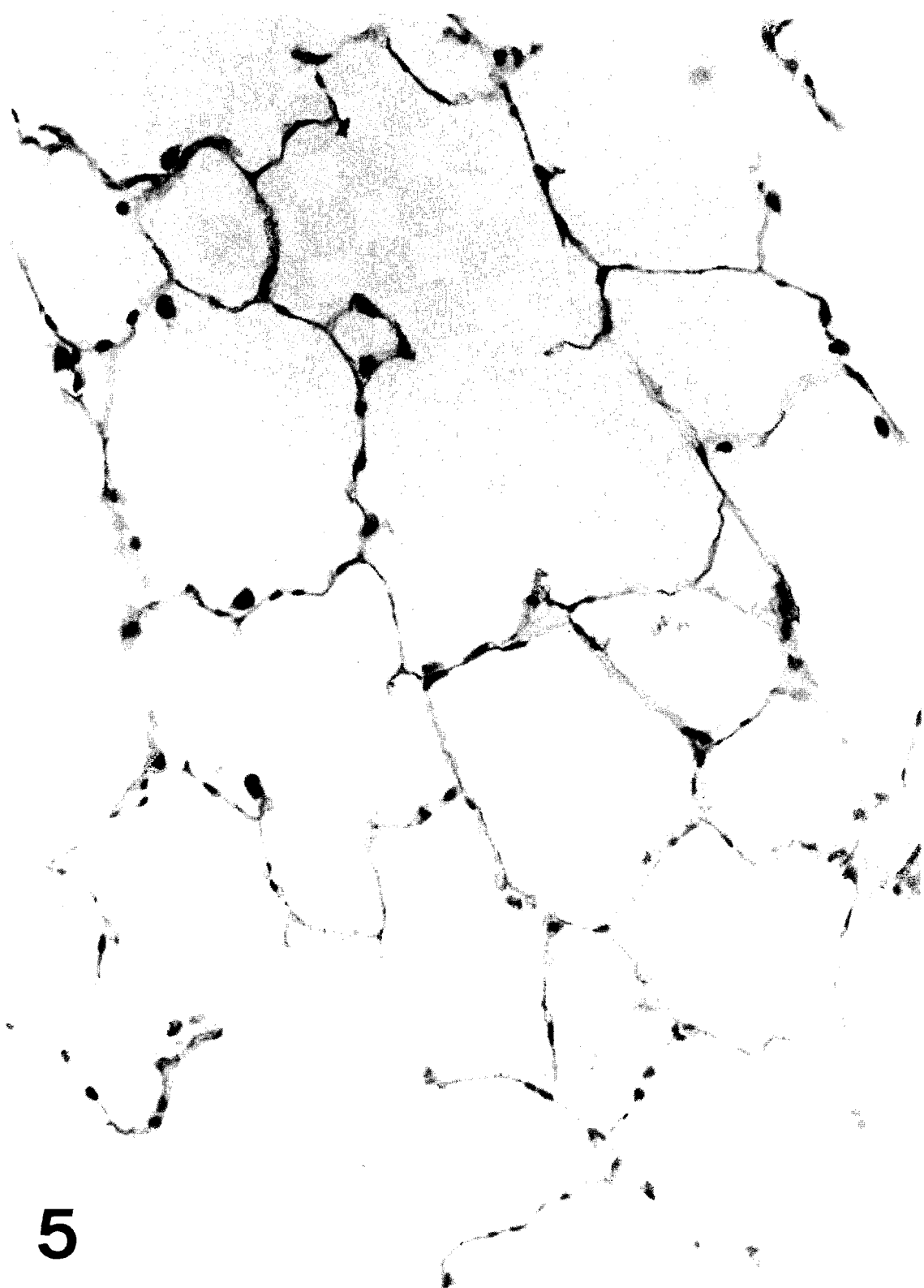
##### Pearl's Prussian Blue

The least amount of AM containing haemosiderine was found in the untreated group ( $2.43 \pm 8.03$ ), where they were found in only 6 animals, with up to 40 marked cells in one case; these values were statistically significant ( $p < 0.001$ ) in comparison to the rest of the groups (Table 3).

After comparing the two groups of animals treated with a single agent (BA:  $25.27 \pm 28.11$ ; ALD:  $257.63 \pm 109.01$ ) and those treated with that agent plus cigarette smoke, it was possible to quantify a decrease in the number of Prussian Blue-positive AM (Table 4), which was only significant ( $p < 0.01$ ) in those treated with anti-lung serum and cigarette

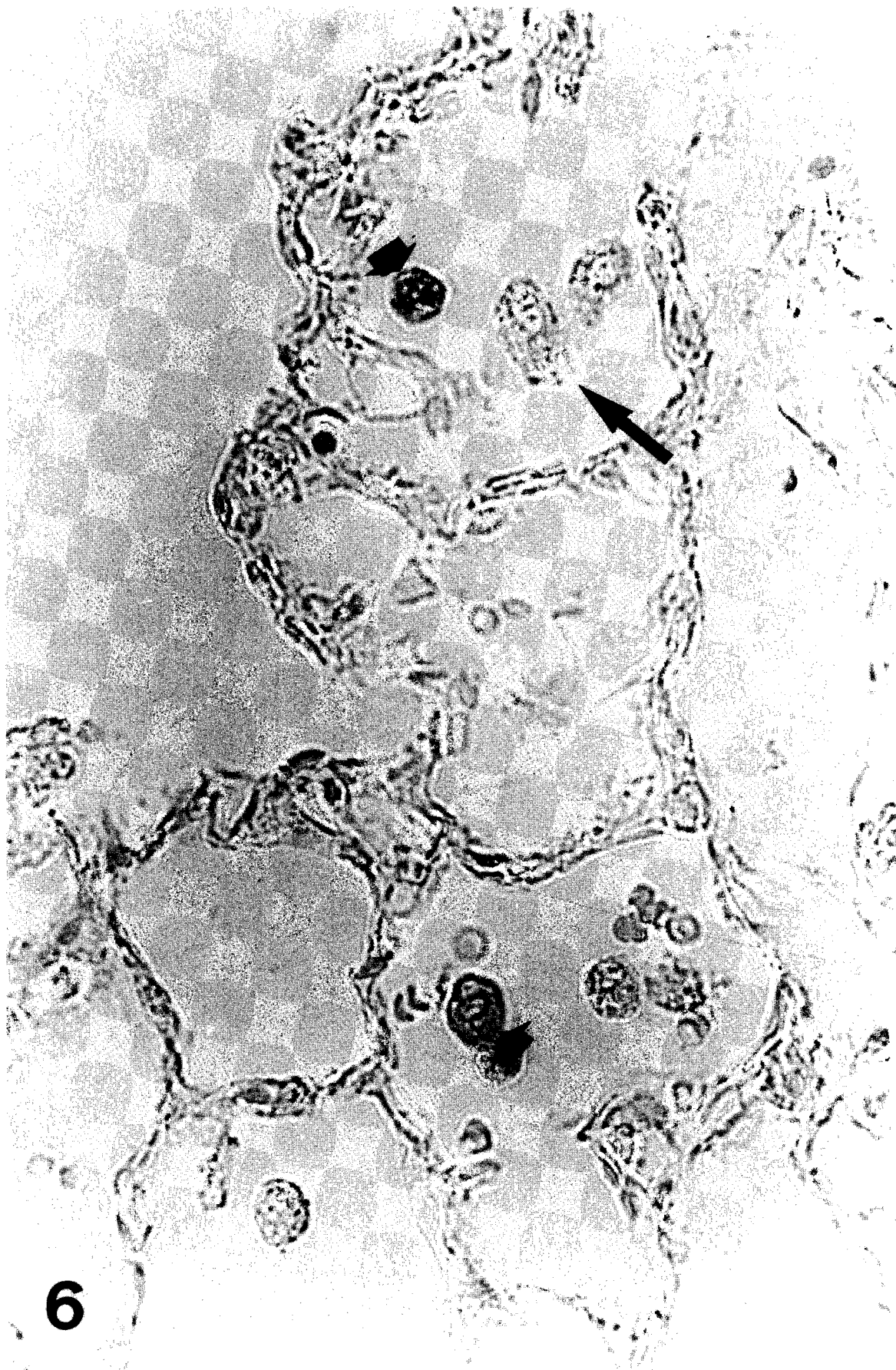


**4** Fig. 4. Semiquantitative variable granuloma. Animals exposed to BA. PAS stain.  $\times 400$



**Fig. 5.** Affected lung surface with a semiquantitative variable «increased alveolar aerial spaces». PAS stain.  $\times 400$





**Fig. 6.** Alveolar macrophages with haemosiderine (thick arrows) and without haemosiderine (long arrow).  
Pearl Prussian Blue stain.  $\times 600$



**Table 1.** Bronchoalveolar lavage percentage counts

	PMN	lymphocytes	AM
CONTROL	3 ± 9	11 ± 4	86 ± 9
CIG. SMOKE	†† 6 ± 10	†† 27 ± 18	††† 69 ± 17
BA	††† 14 ± 17	††† 24 ± 17	††† 62 ± 18
ANTI-LUNG	††† 12 ± 15	††† 20 ± 11	††† 68 ± 15
BA + CIG.	††† 15 ± 23	††† 21 ± 12	††† 64 ± 20
CIG + ANT-L.	† 8 ± 13	††† 19 ± 13	††† 73 ± 19

Result obtained after the percentage cytological count of the bronchoalveolar lavage expressed as mean ± 1 SD. (†††:  $p < 0.001$  compared with the control group; ††:  $p < 0.01$  compared with the control group; †:  $p < 0.05$  compared with the control group).

**Table 2.** Semiquantitative results

	Granulomae	Enlarged alv. air space	Expanded alv. septal	Haemosiderine
CONTROL	0	2.5 ± 6.9	3.1 ± 6.3	0.1 ± 0.8
GIG. SMOKE	0	††† 18.7 ± 31.4	††† 5.1 ± 8.4	††† 7.6 ± 19
BA	0.28 ± 1.2	††† 9.4 ± 14.8	††† 21.6 ± 22.9	††† 7.6 ± 14.8
ANTI-LUNG	0	††† 18.8 ± 21	††† 22.4 ± 21	††† 37.4 ± 39.9
BA + CIG.	0.15 ± 3	††† ΔΔ 17.8 ± 18.5	††† 36.4 ± 25	††† 3.3 ± 5.9
CIG + ANT-L.	0	††† 16 ± 19	††† *** 25 ± 37.9	††† 35.5 ± 38.6

Results obtained with the semiquantitative method expressed as mean ± SD (††:  $p < 0.001$  compared with the control group; ΔΔ  $p < 0.01$  compared with the group treated with BA; \*\*\*:  $p < 0.001$  compared with the group exposed to tobacco smoke).

smoke (BA + cigarette smoke:  $10.95 \pm 15.80$ ; anti-lung + cigarette smoke:  $37.41 \pm 47.03$ ).

## Discussion

The results obtained with the different methods used here suggest that the treated animals showed interstitial-alveolar alterations compatible with inflammatory processes, where inflammatory cells take part and are characterized by a destruction of the distal parts of the lung.

### BAL

The BAL cell percentage count is a technique that affords data about the course of interstitial lung disease, according to the proportion in which the different cell groups are found (Cormier et al., 1987; Stoller et al., 1987). In this test, it has been possible

to distinguish between the control group and the rest of the animals, and we therefore only propose that the lymphocyte and the PMN increase suggests the existence of interstitial-alveolar alterations that the histologic study should clarify.

### Histology

The histological results will be described first, method by method. Then the experiment will be discussed conceptually.

First of all, it must be pointed out that semiquantitative methods are very often used in the clinical diagnosis, because they are considered much more accurate than a visual examination of the whole sample. The method we have called semiquantitative has two aspects: 1) subjective, that qualifies the affected lung area with a certain variable; 2) objective, which includes the exact measurement of

Table 3. Morphometric results

	Log (1 + x) Alv.	Interstice	AM Haemosiderine
CONTROL	1.404 ± 0.35	646 ± 180	2.43 ± 8.03
CIG. SMOKE	††† 1.336 ± 0.36	††† 787 ± 176	††† 13.03 ± 23.80
BA	††† 1.330 ± 0.38	††† 758 ± 208	††† 25.27 ± 28.11
BA + CIG.	††† 1.320 ± 0.34	††† 782 ± 227	††† 25.27 ± 28.11
ANTI-LUNG	††† 1.352 ± 0.34	††† 838 ± 221	††† 257.63 ± 109.01
CIG. + ANTI-L.	*** 1.397 ± 0.36	*** 686 ± 217	†††    ●●● 37.41 ± 47.03

Results obtained with the morphometric method expressed as mean ± SD. The alveolar results show a distribution curve that is assymetrical and so they are expressed as the function log (1 + x). The results of the alveolar macrophage count with haemosiderine has an assymetric distribution because, except for the group treated with antilung serum, in all the rest of the groups, the mode value is very close to zero.

(†††:  $p < 0.001$  compared with the control group; ●●●:  $p < 0.001$  compared with the group treated with anti-lung serum; \*\*\*:  $p < 0.001$  compared with the group exposed to tobacco smoke).

the mentioned lung area. We think that this method would be more objective if it distinguished the degree of affectation of each defined variable, which would make the process considerably more complicated.

The advantage of morphometric methods is based on objectivity. For us the greatest difficulty is in designing the test, especially when choosing the variables that should define the tissue to be studied. We have considered that from the morphometric methods that Weibel (1963) developed for studying lung tissue, the linear integration method is the best one for us in view of the resources available in our laboratory. After Weibel, this method was modified by McCartney et al. (1988) who suggest its computerization for speed and objectivity. The same authors propose that 200 measurements per case should be carried out and they advised the use of the non-parametric Kolmogorov-Smirnov test for the comparison. We adapted the linear integration technique to our computer system, studying seven fields per lung, in which we made a minimum amount of 215 measurements per case. Starting from the above-mentioned alveolar variable, we defined a second one, the quantity of interstice per field. Llum et al. (1990) recently developed a computer programme where the microscopic field image is digitalized, which makes it possible to carry out the linear integration method automatically; these authors only propose one variable that must be taken into account: the distance between the alveolar walls. The distribution of the results obtained when measuring the distance between two alveolar walls in a random way has the form of an assymetric curve. For this reason, we have brought the distribution closer to the normal by applying a logarithmic function, as Llum et al. (1990) propose. We point out here that what the

authors meant was to approximate the distributions of all the results to the normal, but no mathematical function was found that could do this.

Pinson et al. (1986) advise that the morphometric study be carried out after making a subjective study of the tissue, because they consider that morphometry is useful in clarifying the results obtained with more subjective methods. This procedure is the one used in this work.

*Semiquantitative study.* The results obtained for the variable «thickened alveolar walls» suggest the presence of interstitial inflammation in the treated animals. The increase in the lung area with «increased alveolar air spaces», together with «thickened alveolar walls» suggests the loss of the normal alveolar architecture, this being a phenomenon that accompanies interstitial alveolar inflammation (Salvaggio and Karr, 1979; Reyes et al., 1982; Coleman and Colby, 1988). The haemosiderine found in the alveoli in the cases treated with anti-lung serum is an expression, of course, of induced ALD (Willoughby and Dixon, 1970); but not so in the rest of the animals treated, where the presence of haemosiderine has been considered as an alveolo-capillary indicator, increased due to the inflammation (Mishkin et al., 1987). Lastly, the presence of granulomae is clinically associated with EAA (Coleman and Colby, 1988) and experimentally with the administration of adjuvant (Stebly and Rudofsky, 1983).

*Morphometric study.* The increase of the interstitial linear measurement together with the decrease of the alveolar measurement in the animals treated led to the

conclusion that the size of the alveoli in these animals is smaller and the amount of interstice greater. These data as well as the ones obtained by the semiquantitative method point to an interstitial alveolar inflammation (Salvaggio et al., 1979; Reyes and Karr, 1982; Coleman and Colby, 1988).

The fact that the alveolar variable is decreased and the interstitial one increased suggests the existence of an interdependence between the two. It seems logical to assume that interstice increases at the expense of the alveolar space. Nevertheless, the results obtained by the simple regression test do not bear out this reasoning. We think that to accept that the size of the alveolus decreases due to the increase in the amount of interstice, we should have obtained regression indices with absolute values greater than 0.6 in all cases. We infer from this that the amount of lung interstice is not closely related to the alveolar size, even though in some cases this ratio can increase, as in the case of the animals treated with BA.

When proposing the linear integration method, McCartney et al. (1988) pointed to the presence of emphysema by measuring only the distance between alveolar walls. When developing this method, we discussed whether the result showed the real alveolo-interstitial state as a whole. For this reason, we had to define a variable related to the lung interstice. In the beginning, we proposed the length of the septal thicknesses per field, but it was very difficult for us to determine this variable. Secondly, we proposed the possibility that the number of measurements made could be a good index, because by multiplying this value by the mean length of each field, we would obtain the total interstitial length of the field. The interstitial variable proposed, the amount of interstice, is related to the number of measurements and it is complementary to the alveolar magnitude, because it is obtained by subtracting the sum of the alveolar lengths measured from the 7 straight lines of the pattern, in the same field.

The last morphometric variable was the AM count contrasted with ferrocyanide. The reasoning that explains the presence of haemosiderine in the tissue is the same as that proposed when discussing the semiquantitative method; we consider that the haemosiderine present in the animals treated with anti-lung serum is a consequence of the capillar alveolar membrane lesion when a specific antibody is fixed (Willoughby and Dixon, 1970); in the rest of the cases, it was produced by the increased permeability due to inflammation (Mishkin et al., 1987).

*Cigarette smoke:* Concentrating on the conceptual part of the test, we will start with the effects of cigarette smoke in the lung, which have been discussed in a large number of papers, based on all the possible points of view (immunological, enzymatic, functional, clinical, radiological, etc.). We begin emphasizing only the results obtained with a morphological method: cigarette smoke causes non-specific alveolo-interstitial

alterations in the rat (Huber et al., 1981) even though emphysema has been induced in larger animals such as pigs and sheep (Snider et al., 1986).

*EAA and ALD:* The morphological alterations found experimentally after causing alveolo-interstitial diseases EAA and ALD, were also non-specific (Cormier et al., 1988); it is only possible to make some qualifications, such as in the case of ALD, which presents blood and blood derivatives such as haemosiderine in the lung tissue (Willoughby and Dixon, 1970).

The non-specific alveolo-interstitial alterations are related to the septal thickening caused by the inflammation, which we have defined semiquantitatively and morphometrically. We think that it is very important in the study of alveolar inflammation to objectively measure the permeability increase by using tracers. Classically, exogenous tracers such as peroxide are usually used (Hulbert et al., 1981); however, we propose using endogenous tracers such as haemosiderine, which is not usually found in significant quantities in the lung and was found in higher amounts in the animals we treated.

*Alveolitis and cigarette smoke:* Once the presence of alveolo-interstitial alterations has been accepted in the animals subject to only one treatment, it is necessary to prove the working hypothesis: cigarette smoke has no detrimental effect on EAA or ALD. The different authors consulted, besides discussing the effect of cigarette smoke in relation to these two diseases, link other lung diseases with the administration of different substances. From all these we have reached the conclusion that, in relation to lung disease, cigarette smoke can have three effects: predisposition, adjuvant and protection.

The only authors consulted who consider cigarette smoke as a predisposing agent for ALD are Donaghy and Rees (1983). In a detailed clinical study they arrived at this conclusion, suggesting that the lesion caused by cigarette smoke in lung tissue could make it easier for the tissular antigen to bind to the circulating antibody. Afterwards, their arguments were discussed by Leaker et al. (1984) who considered that cigarette smoke has no effect on this disease. Many papers give cigarette smoke a role in EAA and almost all the authors consulted believe that cigarette smoke reduces the risk of suffering this disease. The majority of clinical studies report that the serum antibody count is lower in smokers. Decreases in de IgG, IgD and IgA (McSharry et al., 1985) as well as IgG (Anderson et al., 1988) have been detected among smokers. With regard to people with a risk of suffering from EAA, it has been proven that smokers have fewer or have no specific antibodies to the antigens causing EAA (Morgan et al., 1975; Andersen et al., 1982; Andersen and Cristensen, 1983). Our experimental design does not enable us to infer whether cigarette smoke has a predisposing effect or not.

We consider that the adjuvant or additive effect of cigarette smoke counters the protective effect. It has not been possible to demonstrate an adjuvant effect of cigarette smoke in EAA and ALD; the available evidence points to the opposite. Warren (1977) suggested that cigarette smoke has a damping effect in EAA. In EAA and ALD, an antigen binds to an antigen, for which one of them has had to go through part of the alveolo-capillary barrier at least. The reasoning that considers cigarette smoke as an adjuvant factor in the experimental disease is based on the increased permeability caused by cigarette smoke, which would make the binding of the antigen and the antibody more probable. This reasoning helps to justify the adjuvant effect of oxygen in experimental ALD in the rabbit (Jenning et al., 1981). As stated before, the lower incidence of EAA among smokers may be due to the fact that cigarette smoke reduces the production of specific antibodies against the antigen responsible for EAA. Our animals were sensitized before being exposed to cigarette smoke and they had antibodies against BA at the moment of the exposure, and for this reason the rats exposed to cigarette smoke and BA had the same antibodies as those exposed to BA alone. It seems logical that exposure to cigarette smoke should produce an increase in the pathology in the double treatment group compared to the group exposed to BA only, although we have not been able to prove this, nor that cigarette smoke has a protective effect in EAA. In an experimental EAA model, that was carried out by giving lyophilized *micropolyspora faeni* to guinea pigs, Cormier et al. (1988) were not able to demonstrate that cigarette smoke prevented the production of specific antibodies against the *micropolyspora faeni*, even though they found an initial neutrophilic response.

In our experiment the possible protective effect of cigarette smoke was suggested in animals treated with anti-lung serum, as the ones with double treatment showed a great morphometric similarity to the untreated ones. Rhodes et al. (1988) administered anti-lung serum to rats exposed to cigarette smoke and they did not find that cigarette smoke was harmful, and so they proposed that cigarette smoke could have a protective effect on the lung, at least with regard to ALD. We have not found any author with a clinically based methodology who considers that cigarette smoke has a protective effect in ALD.

In conclusion, different authors have suggested that cigarette smoke in EAA causes modifications that can be demonstrated morphologically and immunologically, but we have not been able to demonstrate this. The effect of cigarette smoke in ALD is subject to argument among the few authors who have approached the subject, and this is precisely where this paper suggests a possible damping effect due to cigarette smoke. Cigarette smoke can modify the response of any disease and, depending on the technique used, the modifications can be revealed in

different ways. Considering only our results, cigarette smoke has no adjuvant effect on EAA. In ALD it damped the results obtained. However, due to the cultural importance of tobacco, we dare not state that cigarette smoke has a protective effect because this, in our opinion, must depend on the administration guidelines, intrinsic circumstances of the person, etc.

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