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Pulmonary response to bovine albumin. A morphometric study in rats

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Summary. The following hypothesis is proposed: that hypersensitivity pneumonitis (HP), experimentally induced in rats, is the cause of a thickening in the alveolar wall, a decrease in the size of the alveole, hyperplasia in the bronchus-associated lymphoid tissue (BALT) and hypertrophy in the goblet cells.

Wistar rats were classified into two different groups, namely, non-treated animals and animals exposed to bovine albumin (BA).

A morphometric study was carried out and the following variables were quantified: a) percentage of lymphocytes, neutrophils and alveolar macrophages of the bronchio-alveolar lavage (BAL); b) the interstice of the alveole, the alveolar chord length, the alveolar wall thickness and the number of alveolar macrophages with hemosiderin within its cytoplasm; c) the size of lymphatic area (LA) in BALT, the length of the lymphatic epithelium (LEp) in BALT and the percentage of goblet cells in the bronchial epithelium.

The following results were obtained from the animals exposed to BA: 1) a significant increase in both lymphocytes and neutrophils of BAL, and of alveolar macrophages with hemosiderin in its cytoplasm; 2) a significant thickening of the alveolar walls and the BALT elements, which confirms the above mentioned hypothesis; 3) a significant increase in the alveolar chord and a significant decrease in the number of goblet cells of the bronchus, which contradicts the above mentioned hypothesis.

The increase in alveolar macrophages with hemosiderin is related to an increase in the capillary alveole permeability, which, together with the variations in the BAL formula, lead us to consider the existence of an inflammation in the interstitial alveole; this interstitial alveole inflammation explains the alveolar wall thickening and the BALT hypertrophy.

Key words: Rat, Hypersensitivity pneumonitis, Bronchus-associated lymphoid tissue, Morphometry, Bronchioalveolar lavage, Bovine albumin

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Introduction

Hypersensitivity pneumonitis (HP) is a process that includes only those alveolar fillings and interstitial diseases produced after intense or prolonged exposure to finely dispersed organic dust of appropriate particle size (Salvaggio and Karr, 1979). In relation to its morphometry, interstitial and alveolar infiltrations and mononuclear cells, granuloma formation (Salvaggio and Karr, 1979) and a degeneration of alveolar pneumocytes and bronchiolar epithelia (Kohno et al., 1989) have been observed in the HP.

Many experimental models have been developed in different kinds of animals. The method of inducing the experimental pneumonitis consists of administering an antigen into an animal, causing the union to its antibody in the pulmonary alveole. This antibody can be either autologous or heterologous.

Infiltrations of intraalveolar and interstitial neutrophil leucocytes, intraalveolar haemorrhage and edema have been observed in rats (Warren et al., 1989). In rabbits, other phenomena have been described: in models of HP performed in these animals, the presence of interstitial and bronchial signs has been reported; the airway has also been affected by the existence of inflammatory infiltration in the walls (Richerson et al., 1982; Schuyler et al., 1983) hyperplasia in the goblet cells and hypertrophy in the bronchus-associated lymphoid tissue (BALT) (Jakab et al., 1983; Schuyler et al., 1983). Similar results have been obtained in guinea pigs (Hutson et al., 1988) and mice (Curtis et al., 1980; Du et al., 1991; Schuyler et al., 1991).

To explain the results of these experimental HP, many authors, such as Richerson et al. (1982), have reported the great variability of the morphologic results obtained when comparing different lungs within the same group of animals in morphologic models. In view of these circumstances, we suggest the necessity of carrying out a morphometric valoration of these experimental HP, which could add a degree of objectivity to the large number of studies carried out on the subject. These are the hypotheses that we propose: firstly, the interstitial inflammation produced in the HP can be estimated

objectively by measuring the thickness of the alveolar walls; secondly, this could imply a diminution of the alveolar volume, which could be calculated by measuring the length of alveolar chord; thirdly, the exposure to the antigen can produce BALT hypertrophy; and lastly, can produce hyperplasia in the goblet cells in relation to the airway.

Materials and methods

Animals

We made use of 60 Wistar rats, aged 4-6 months and chosen at random from our animalarium. Throughout the experiment the animals were housed at a constant temperature of 22 °C, and had access to water and commercial feed «ad libitum». The animals were divided into two groups: 1) Healthy adult rats: 30 animals aged 4-6 months, who received no treatment. 2) Sick adult rats (BA): 30 animals aged 4-6 months, with induced HP.

Treatment

HP was induced by injecting 0.2 cc bovine albumin (BA) 9 x 10^{-6}%, in complete Freund adjuvant at 50% into the foot pad; three administrations were given at intervals of 2 weeks. Once anti-BA antibodies were detected by immunoprecipitation in agarose gel, the animals were placed in a BA environment for 8 hours per day over a period of 1 month. For this purpose, sealed laminar flow cages were used (internal dimensions: 23 x 35 x 57 cm). The BA environment was achieved with a Hudson apparatus whose tank contained a solution of BA 18 x 10^{-18}% in saline.

Sacrifice of the animals

The animals were anaesthetized with 1% Nembutal® (1cc/100gr) by intraperitoneal injection and sacrificed according to the following procedure: first a catheter (Venocath® 14G) was introduced, via a tracheotomy orifice, into the main right bronchus. A central laparotomy was then performed in order to extract 5 cc of blood from the lower cava vena. Then, thoracotomy was performed to gain access to the thoracic cavity. The left pulmonary hilus was tied, its lung was separated and then used for the histological study.

BAL

This was performed by perfusing four 1 cc aliquots of saline, via a catheter lodged in the right bronchus, followed by aspiration. The material obtained was then centrifuged. The precipitate was extended and contrasted using the Giemsa method. The percentage of alveolar macrophage (AM), lymphocyte, and polymorphonuclear leucocyte (PMN) counts were taken (Escolar Castellón et al., 1991).

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. The central block of the lung was cut and dehydrated by successive immersions in alcohol concentrations of an increasing degree, and was then placed into paraffin. 7-micron sections were made, and contrasted using the PAS-Alcian blue and the Pearl's Prussian Blue methods (in order to detect hemosiderin). The histological study was always carried out on the same section, namely the plane perpendicular to the main axis of the lung, where the main bronchus becomes intrapulmonary.

Morphometric study

The variables studied were classified as: alveolo-interstitial; bronchial; and BALT, as indicated in previous studies (Escolar Castellón et al., 1991, 1992).

Alveolo-interstitial variables. The lung sections chosen were divided into 13 zones, from which the 7 numbered zones were selected. In each of the 7 zones, a microscope field which did not contain airways or important vessels was chosen at random. The following elements were quantified in each field: in the sections dyed using the PAS-Alcian blue method, the alveole chord length and the width of the alveolar wall, and in the sections dyed using the Prussian Blue method, the number of alveolar macrophages with hemosiderin.

The alveole chord length was obtained by measuring the distance between two walls of the same alveole. For this purpose, seven parallel equidistant lines were placed on a graphics pad (Summaghaphics® Bit Pad® Plus) connected to a Macintosh IIcx computer, and superimposed on the microscope image (Fig. 1). A projector attachment was fitted to a Nikon® microscope with a x10 eye lens and a x40 objective lens. The distance between the two alveolar walls was drawn on these lines.

The linear interstitial length was obtained by using the following formula: $\sum L \times \sum L_a / n$, where $\sum L$ = the sum of all the lengths of each line, $\sum L_a$ = the sum of all the lengths of each alveolar chord of a microscopic scope and n = the number of measurings.

The macrophages with hemosiderin were counted in each microscope field of the sections stained with Pearl's Prussian Blue. This variable was expressed as the number of alveolar macrophages with hemosiderin per section (Fig. 2).

The bronchial variable studied was the percentage of goblet cells as a function of the total number of bronchial epithelial cells. This was done by counting the PAS-Alcian Blue-positive cells + (goblet cells) and the nuclei of all the epithelial cells (Fig. 3) in the main bronchus. The count was made at bronchial levels I, II and III, as suggested by Olesen et

al. (1987).

The BALT variables were as follows: Peribronchial lymphatic area (LA) was defined as the lung area occupied by bronchus-associated lymphatic tissue, expressed in mm^2 (Fig. 4). Lymphatic epithelium (LEp) was defined as the length of bronchial epithelium associated with the lymphatic area which had flat cells with absence of cilia, expressed in mm (Fig. 4). In order to quantify these variables, the BALT was drawn using a projecting microscope and a graphics pad. The images obtained were then quantified with the computer using the Mac Draft® and Mac Space® programs.

Statistical analysis

All data in the text are expressed as mean ± SD. The figures obtained from the alveole chord length and the amount of interstice per field were compared using Student's t test. The results of the other variables were compared using the non-parametric and Mann-Whitney U-tests. Probability values lower than 0.05 were considered significant in all cases. The statistical analysis was performed on a Macintosh® IIcx computer with the StatView® II program.

Results

LBA (Table 1)

The animals treated with BA showed a percentage increase in lymphocytes (18.43±17.93) and polymorphonuclears (5.63±7.87) which was significant ($p< 0.001$) when compared with that of the non-treated animals (lymphocytes: 11±4.41; polymorphonuclears: 3.2±8.88).

Interstice (Table 2)

In the animals that had been treated, a significant increase in the alveolar chord (66.03±15.61) and in the alveolar wall thickness (35.71±10.06) was observed in relation to the other group of non-treated animals (alveolar chord 36.07±7.30; wall thickness: 21.06±5.58).

The number of alveolar macrophages with hemosiderin increased (37.76±33.91) quite considerably ($p< 0.001$) in the group of animals treated with BA when compared with the non-treated group (2.43±8.03).

Bronchus (Table 3)

The BALT elements showed a significative ($p<$

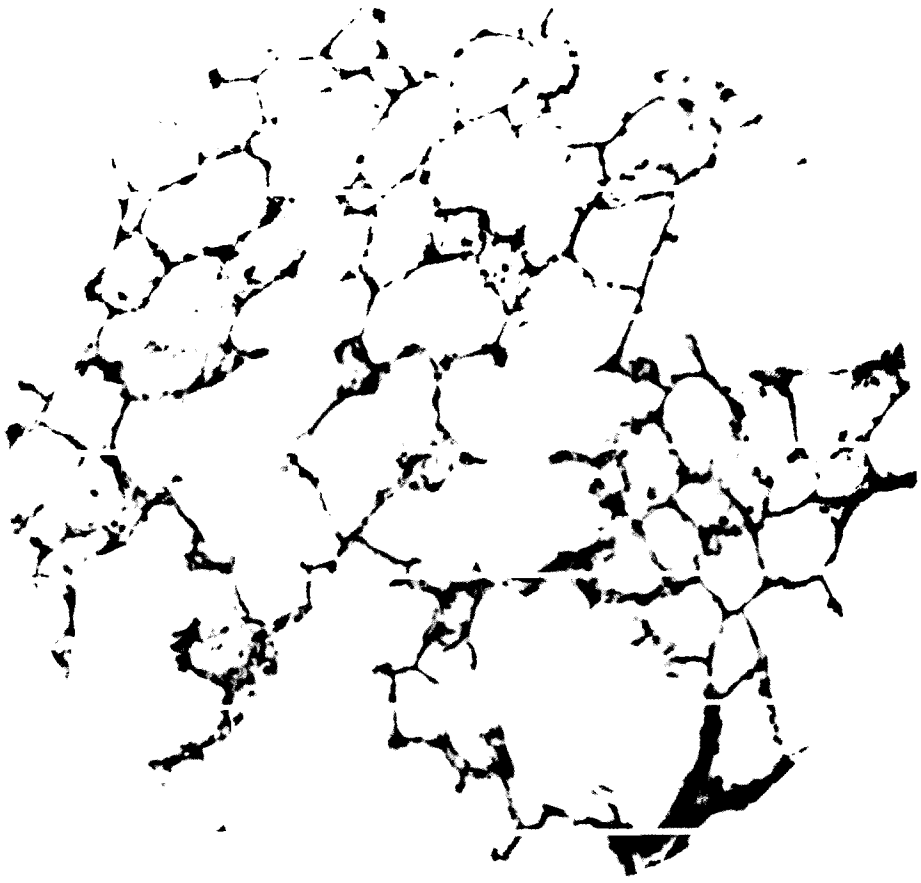

Fig. 1. The alveolar chord method. Overlap of the seven straight lines in a histologic field. PAS-Alcian blue. x 200

0.001) increase (LA: 1.55±1.09; LEp: 1.83±1.33) in relation to those of the control group (LA: 0.26±0.71; LEp: 0.45±1.01).

In the group of treated animals, the goblet cells showed a percentage decrease ($p< 0.01$) (80±10.83) when compared with non-treated animals (34.69±17.03).

Table 1. Standard deviation ± mean of the cytologic results obained from the bronchioalveolar lavage.

GROUP	LYMPHOCYTES	NEUTROPHILS	ALVEOLAR MACROPHAGES
Non-treated	11±4.41	3.2±8.88	85.8±8.7
BA	18.43±17.93*	5.63±7.87*	75.94±8.91*

*: $p< 0.001$.

Table 2. Standard deviation ± mean of the alveolar results obtained.

GROUP	ALVEOLAR CHORD	WALL ALVEOLAR	ALVEOLAR MACROPHAGES HEMOSIDERIN
Non-treated	36.07±7.30	21.06±5.58	2.43±8.03
BA	66.03±15.61*	35.71±10.06*	37.76±33.91*

*: $p< 0.001$.

Discussion

BAL

HP is a disease caused by immuno complexes, in

Table 3. Standard deviation ± mean of the results obtained from the bronchial variables.

GROUP	LYMPHATIC AREA	LYMPHATIC EPITHELIUM	GOBLET CELLS
Non-treated	0.26±0.71	0.45±1.01	34.69±17.03
BA	1.55±1.09*	1.83±1.33*	80±10.83**

*: $p< 0.001$; **: $p< 0.01$.

Fig. 2. Alveolar macrophage with hemosiderin (arrow). Ferrocianure and neutral red. x 600

which several humoral and cellulary mechanisms of a diverse inflammatory and immunologic nature are involved and whose activity is centred in the lung (Jakab et al., 1983; Gehrke and Pabst, 1990). The accumulation of lymphocytes and neutrophils in the pulmonary alveoles is a consequence of the inflammatory process that the lung undergoes (Richerson et al., 1982; Berman et al., 1990). The percentage increase in lymphocytes and polymorphonuclears in the BAL is a non-specific sign of pulmonary alveolointerstitial alteration, which is typical of HP (Haslam et al., 1987; Soda et al., 1988).

Interstice

Hemosiderin

The increase of the capillary permeability is a phenomenon that inflammation conveys. With the aim of quantifying the variations in the alveolocapillary permeability which was produced in the group of animals with HP, the number of alveolar macrophages with blue lumps in the pulmonary tissue was assessed. The ferrocyanure technique used as dyes the divalent iron with blue colour. This divalent iron is caused by the destruction of hemoglobin and is included in the hemosiderin molecule, whereas the trivalent iron has a different origin, and is usually linked to carrying enzymes, such as transferrin, which is not stained blue with the ferrocyanure. Our results suggest that, in the animals treated with BA, an increase in the permeability to larger molecules, such as the hemoglobin is produced. In the same way, the presence of hemosiderin in the alveole can imply the existence of an alveolar haemorrhage. Although alveolar haemorrhage has been shown in the experimental HP (Warren, 1973; Johnson and Ward, 1981; Schuyler et al., 1991) we believe that, if a blood extravasation towards the alveoles had occurred, this could not have been quantitatively significant, since red blood cells in the alveolar area have not been observed.

Alveolar wall

The increase in the alveolocapillary permeability is the cause of an accumulation of substances in the alveolar interstice which, together with the infiltration of

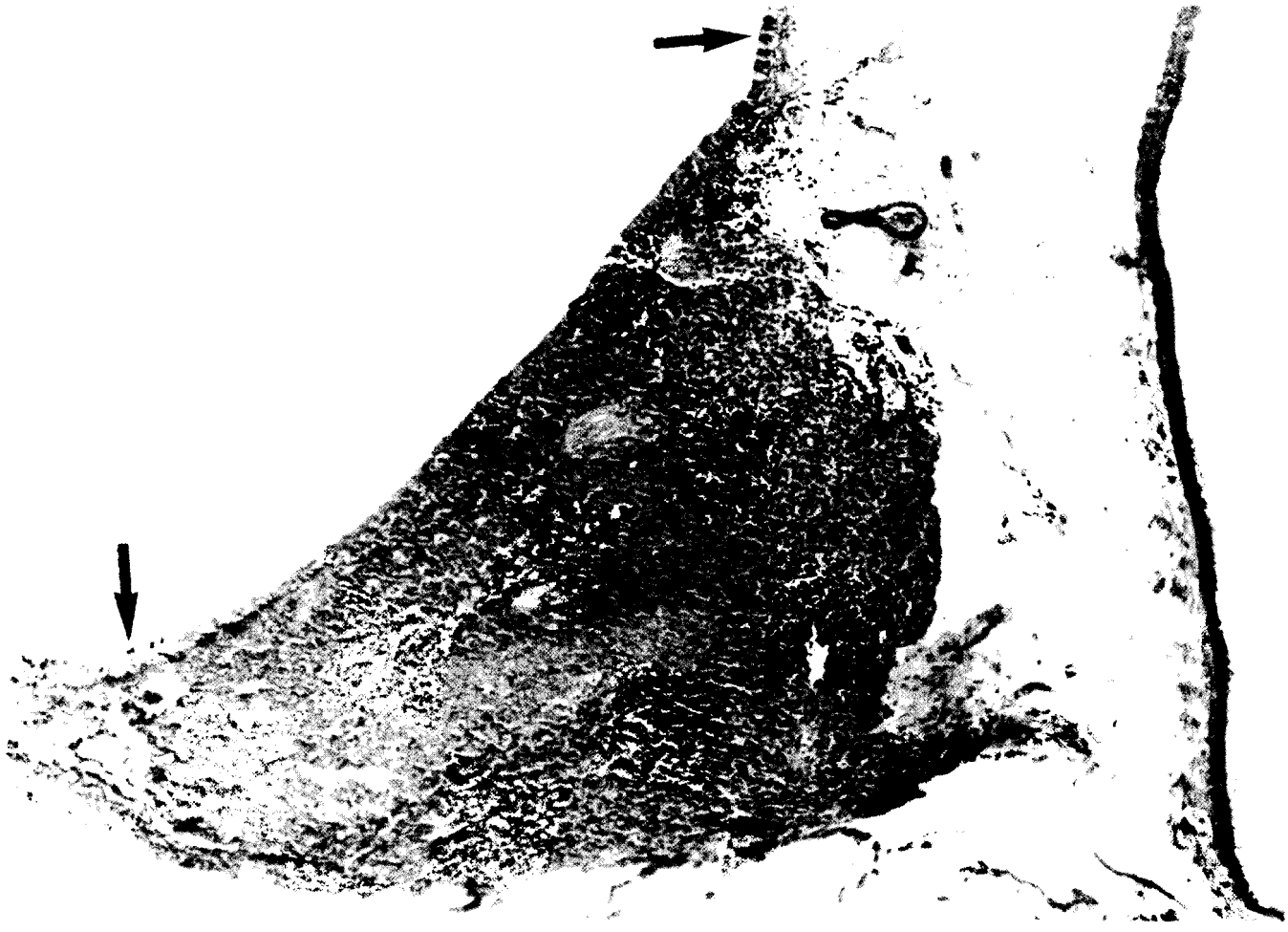

Fig. 3. Bronchial-associated lymphoid tissue (BALT) and bronchial epithelium. Goblet cells (arrows). PAS-Alcian blue. x 200

inflammatory cells in the alveolar wall described in the HP, can be observed as an increase in the alveolar wall thickness. In this paper, we have morphometrically demonstrated that the group of animals exposed to BA showed an increase in alveolar wall thickness, which confirms the first mentioned hypothesis, and at the same time, coincides with the other experimental methods which we have studied (Warren et al., 1973; Richerson et al., 1982).

Alveole

Our attention was particularly drawn to the increase in the alveolar chord length of the animals exposed to BA. Making use of a similar technique to that which was used in this paper, it has been reported that the administration of BA for a period of two months decreases the alveolar chord length and increases the size of the interstice (Escolar Castellón et al., 1991). In this study the statistical regression test was performed, with the purpose of finding any relation between the size of the alveole and the quantity of alveolar interstice, but no significant results were observed. As a consequence of this, we think that the reasoning used to justify the second hypothesis of this study is not valid and therefore, we postulate that the increase in the alveolar wall thickness is not related to the decrease in the alveole size.

The reported increase in the alveolar chord can be caused by both technical and organic factors. Among the technical causes we should emphasize an experiment in which the lungs were insufflated with gerbils with a positive pressure of 30 cm of water; this pressure was maintained in the right lung for 10 minutes longer than in the left. As a consequence, the right lung, morphometrically, showed a higher number of alveoles. It was also noted that they were of a smaller size and that their walls were subjectively thinner than those of the left lung (Lum et al., 1990). The reason for this was thought to be the longer period of pressure, which caused a better insufflation of the lung and made the walls of the collapsed alveoles unfold. This obviously could not have been possible with a shorter period of pressure (Lum et al., 1990). This study is complemented with a later work,

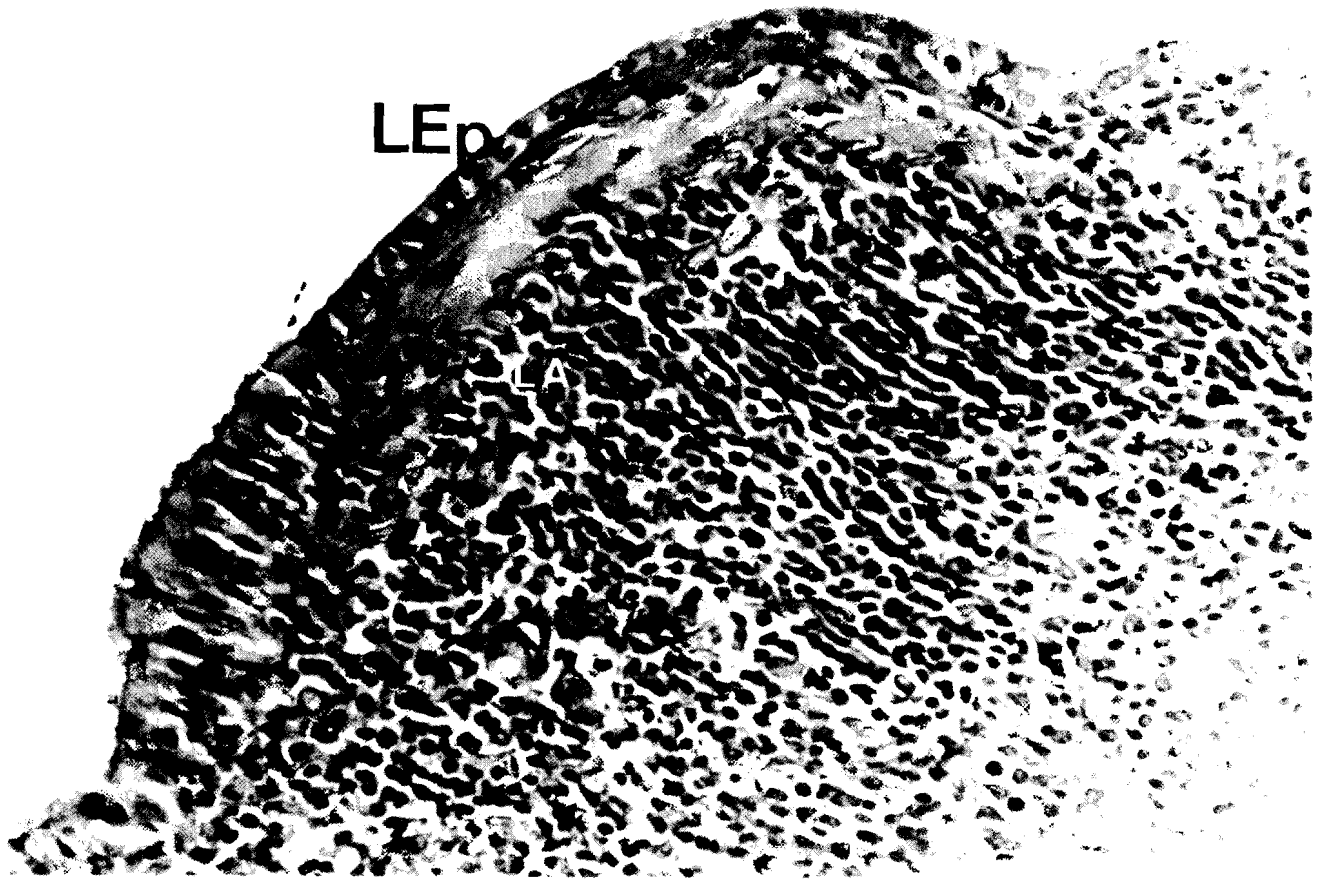

Fig. 4. Bronchial-associated lymphoid tissue (BALT). LA: lymphatic area; LEp: Lymphatic epithelium. PAS-Alcian blue. x 400

also experimental, where it is postulated that the pressure in the fixation is irrelevant, since the absence of the alveolar septal folding depends on a determined pressure range, which corresponds to concepts of critical opening and closing pressure (Oldmixon and Hoppin, 1991). In order to support our findings, we report that the diseased lung showed firstly, an inflammatory process in the alveole, which could modify the physical properties of the alveolar surface; secondly, that the inflammation of the pulmonary interstice modifies, both qualitatively and quantitatively, the tissular components of the alveolar wall, which could also alter the elastic properties of the tissue. These two hypotheses lead us to state the third: that the pressure for a correct insufflation of both a healthy and a diseased lung, does not necessarily have to be the same, since their structural components are different. Among the possible organic causes, we should point out that BA administration to rats can provoke an alveolar dilatation in the first month, and a decrease in its size in the second. More than one single cause has possibly contributed in this experiment. We accept that the relation between the fixation pressure and the alveolar insufflation is not sufficiently clear, and at the same time, we do not know of any other more convincing argument that could provide an explanation of what has happened with the animals exposed to HP. Taking into account exclusively the method used and the results obtained, we believe that we are not contradicting any earlier authors when we state that, after administering BA to sensibilised rats for a month and fixing their lungs with a transtracheal pressure of 26 cm of H_2O, the alveolar chord length increases.

Bronchus

BALT

All the authors we have consulted that have studied the experimental HP airway, describe the presence of inflammatory infiltrates around it. These infiltrates have been typified in different ways: as diffuse infiltrates (Jakab et al., 1983; Schuyler et al., 1983; Hutson et al., 1988); as granulomas (Richerson et al., 1982); and as BALT hyperplasia (Curtis et al., 1980; Jakab et al., 1983; Schuyler et al., 1983). The BALT hyperplasia caused after the administration of antigens has been proved in different studies, where the BALT is related to the lung immune response (Otsuki et al., 1989). The BALT epithelium has the function of attracting antigens (Biennenstock and Befus, 1984; Van der Brugge-Gamelkoornm et al., 1985) and ejecting carrying substances from the alveole (Haslam et al., 1987). The cells of the BALT epithelium are flat, deprived of cilia and, unlike the rest of the epithelial bronchial cells, the secretory component of IgA (Gehrke and Pabst, 1990) has not been demonstrated in their cytoplasm, a fact which corroborates their specialization.

It has been reported that after the administration of antigens the dome epithelium, which is a BALT protrusion towards the bronchus, is formed (Roy et al., 1987). The fact that the dome epithelium is covered with the lymphatic epithelium, causes us to relate the appearance and growth of the dome epithelium with the increase of the lymphatic epithelium, as described in the animals treated with BA. The increase in the flat lymphatic epithelium could be derived from the fact that, since this epithelium acts as a selective barrier, then, if its surface expands, it also increases its interchanging capacity, its antigens and its alveolar clearing. The increase in the lymphatic epithelium can be related to two circumstances: first, the great quantity of antigens administered and deposited in the alveolar walls of the airway and that have to be carried to the inside of the BALT by these epithelial cells; secondly, the alveolar clearing towards the interstice and from this to the BALT where it is drained through the lymphoepithelium towards the light of the bronchus. The mechanisms which control the BALT hyperplasia are still unknown, although in an experimental model of ozone exposition, the presence of some vacuoles in the lymphoepithelium has been related to the proliferation of lymphocytes (Dziedzic et al., 1990).

Goblet cells

The diffuse accumulation of lymphocytes around the airway described in the experimental HP models can also be considered as a sign of bronchial inflammation (Schuyler et al., 1983). In bronchial inflammation, the hypertrophy of the goblet cells has also been shown (Schuyler et al., 1983). The goblet cells are very sensitive to aggressions and react against them by means of hypertrophy (Olesen et al., 1987). The increase of goblet cells has also been related in a semiquantitative study to the hyperplasia observed in the BALT (Nygren and Ahlstedt, 1983). Our results do not coincide with these two previous works (Nygren and Ahlstedt, 1983; Olesen et al., 1987), and we believe that the experimental findings of several authors found in the literature are not conclusive. So the O_2 administration at high concentrations has, as a consequence, a metaplasia of the scaly cells and a decrease in the goblet cells (Wiswell and Wiswell, 1990). Together with this, the existence of a considerable degranulation in the goblet cells of the bronchus has been described in an HP experimental model using guinea pigs (Hutson et al., 1988). The reason for the decrease in goblet cells ir our study could be derived from an escape in the content of goblet cells so quick that the visualization of PAS-Alcian blue positive-cytoplasm was not possible. However, other authors (Du et al., 1991) assessed the quantity of mucus existing in the light of the rat airway after exposing it to antigens, and did not find any significant increase. We have not observed a quantity of mucus sufficient to attract our attention.

In the HP model described, a series of pulmonary inflammatory mechanisms which justify the alveolar wall thickening and the BALT hyperplasia, have

occurred. All these signs coincide with those reported in the experimental HP. However, the decrease found in the percentage of goblet cells was not expected. The increase in the goblet cells agrees with the bibliography as consulted, since most of the studies differ in methodology and do not coincide in their results. Finally, the alveolar dilatation reported by measuring the alveolar chord of the animals exposed to BA, seems to contradict what has been traditionally accepted, namely that the thickening of the alveolar wall reduces the size of the alveoles; however, the development of new techniques, such as morphometry raises once again questions that are evident with respect to other techniques. It is clear, both from the data contained in the bibliography and from that provided by this study, that we cannot come to the conclusion that there is a close relationship between the size of the alveole and the thickening of the alveolar wall.

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