

## Goodpasture's syndrome in ageing. An experimental study on the rat. I

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**Summary.** The following hypothesis is proposed: Experimental lung disease in old rats is different from this disease in adult rats. In order to demonstrate this, we performed a morphometrical evaluation of the pulmonary state of two groups of rats at different ages and to which Goodpasture's syndrome had been induced.

115 Wistar rats were used. They were divided into four different groups as follows: 1) Healthy adult rats which had not been subjected to treatment; 2) diseased adult rats to which antipulmonary serum had been administered; 3) healthy old rats; and 4) diseased old rats.

With the help of a computerized system, the length of the alveolar chord, the thickness of the alveolar wall and the surface of the bronchial-associated lymphoid tissue in each group was calculated. We also counted the number of alveolar macrophages (AM) with haemosiderin, the percentage of goblet bronchial cells and that of AM, lymphocytes and polymorphonuclear leukocytes of the broncho-alveolar lavage (BAL).

The following results were obtained. When related to the diseased adult rats, the diseased old rats showed an increase in the alveolar chord and a decrease in the thickness of the alveolar wall, as well as in the number of AM with haemosiderin, goblet cells and BAL lymphocytes.

These results support the proposed hypotheses, since the diseased adult animals showed signs of alveolar inflammation with interstitial edema, while in the diseased old animals these results are compatible with emphysema.

**Key words:** Lung, Old, Morphometry, Rat

### Introduction

Over the last decade, several studies have

been performed on groups of old animals. Comparing young and adult animals, they describe the morphological and functional changes produced by the ageing process. As regards the lung, Pinkerton et al. (1982) observed that the most important changes in a rat's life take place before the fifth month. According to these authors, all the subsequent modifications in the rat's lung are no more than a stable increase in the vital capacity, the residual pulmonary volume, the total lung volume and the size of the alveolus.

Although several studies have been carried out on different kinds of experimental diseases, not many of them relate the experimental disease with senescence and, therefore, very little is known about the subject.

In this study, the following hypothesis is proposed: experimental disease in old rats is different from this disease in adult rats. The experimental disease used is Goodpasture's syndrome, which is a lung affection (Willoughby and Dixon, 1970; Queluz et al., 1990). This disease is easily induced by administering antipulmonary heterologous serum and, at the same time, is not difficult to identify, due to the alteration of the pulmonary parenchyma and the alveolar haemorrhage which occur (Willoughby and Dixon, 1970; Queluz et al., 1990). A group of white rats was used for the experiment. This group was divided into two sub age-groups of adult and old rats. These groups were then subdivided into healthy and diseased rats. The process was as follows: 1) the induction of the disease into the adult rat; 2) the demonstration of the existence of the disease in the old animals to which the disease was induced; and 3) the search for the differences which might exist between the diseased old and adult animals.

### Materials and methods

#### *Experimental groups*

Healthy male Wistar rats, whose mean ages ranged between 5 months (adult rats) and 23 months (old rats)

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were studied. They were divided into four different groups: 1) Healthy adult rats: Thirty adult animals to which no substance was administered. 2) Diseased adult rats: Thirty adult animals to which rat antipulmonary rabbit serum was administered and which were sacrificed 5 days after the first dose. 3) Healthy old rats: Thirty old animals to which no substance was administered. 4) Diseased old rats: Thirty old animals to which antipulmonary serum was administered and which were sacrificed 5 days after the first dose.

### *Antipulmonary serum*

The antipulmonary serum was generously provided by Operon Laboratories®. It was obtained from rabbits to which rat alveolar basal membrane extract had been previously administered (Meezan et al., 1975; Jennings et al., 1981). This rat antipulmonary rabbit serum was administered intravenously to the animals at the rate of a daily dose for a period of three days. The animals were slightly anaesthetized with ether prior to the administration of the serum.

### *Preparation of the animals*

All the animals used in the experiment were sacrificed according to the following method: Once the rats had been anaesthetized with 1% Nembutal® (1ml/100gr) by intraperitoneal injection, access to the inside of the thoracic duct was gained and a bronchoalveolar lavage was performed on the right lung in order that the histological study might be performed. The left lung was fixed by immersion in 10% formalin with tracheal insufflation of the same fixative at a positive pressure of 26 cm of water.

### *Bronchoalveolar lavage*

This was performed by means of the introduction of four aliquot parts of 1 ml of saline serum into the right bronchus principalis, which was subsequently extracted. The aspired substance was centrifuged and the sediment poured onto a glass slide. It was then dyed by means of the Giemsa method, in order to work out the percentage of lymphocytes, polymorphonuclear leucocytes (PMN) and alveolar macrophages (AM).

### *Histological study*

The histological study was always carried out on the same section, which corresponded to the perpendicular plane of the main axis of the lung, where the main bronchus becomes intrapulmonary. The selected block, which included the whole lung section and whose height was 5 mm, was dehydrated by embedding it in several different alcohol solutions, of progressively higher degree, and subsequently in paraffin. Several 7 µm sections were made and these were stained using the PAS-Alcian blue and ferrocyanide methods. A

morphometrical study of the sections was carried out and the variables were divided into the alveolo-interstitial, bronchio-epithelial and BALT categories.

### *Alveolo-interstitial variables*

These were performed using our own method which has already been described (Escobar Castellón et al., 1991), with some modifications. For this purpose, the lung section was divided into thirteen areas of which seven were chosen. The sections which had been stained by means of the PAS-Alcian Blue method were studied using a variant of the linear intersection method, which was adapted to a computer. A microscope with a clear camera (x 10 eye lens, x 40 objective lens) was also used. Its function was to superimpose the image of the histological scope onto a digitizer tablet (Bit Pad Plus®) upon which seven parallel equidistant lines were drawn. The distance found between the two walls of a single alveolus was marked on each of these lines. The digitizer tablet was connected to a Macintosh® II CX computer and two different variables were quantified:

1) The alveolar chord length.

2) The mean alveolar wall thickness. The following formula was obtained:  $(\sum L + \sum L_a)/n$ , where  $\sum L$  = the sum of the lengths of each line,  $\sum L_a$  = the sum of the lengths of each alveolar chord of a microscopic field and  $n$  = number of measurements.

Both variables were determined from the mean values obtained in each case and are shown in µm.

The sections dyed with ferrocyanide were also divided into 13 areas and 7 of these were selected. In each of these areas a microscopic field was chosen (x 10 eye lens, x 40 objective lens) where the number of alveolar macrophages (AM) inside which blue lumps were found was calculated (Fig. 2).

### *Bronchioepithelial variable*

This was quantified by the PAS-Alcian Blue staining and is shown as a percentage of goblet cells. The result was obtained by relating all the PAS-Alcian Blue positive cells with all the nuclei of the epithelial cells (Fig. 3).

### *Variables of the bronchial-associated lymphoid tissue*

In order to reach a definition of the BALT morphology, two variables were quantified using the PAS-Alcian Blue method (Figs. 3-6):

1. Lymphatic area (LA): This is defined as the BALT surface of the cut (Figs. 4-6) and is expressed in mm<sup>2</sup>.

2. Lymphatic epithelium (LEp): This is the length of the flat bronchial epithelium that corresponds to the BALT (Fig. 3), and is expressed in mm.

In order to quantify the variables of the BALT, a microscope (x 10 eye lens, x 40 objective lens) with a clear camera, a graphic tablet and a computer were used.

All the computer programmes used in the

**Table 1.** Results of the percentage count of the broncho-alveolar lavage cells.

| GROUP               | LYMPHOCYTES                | PMN                      | M.A.                       |
|---------------------|----------------------------|--------------------------|----------------------------|
| Healthy adult rats  | 11±4.41                    | 3.2±8.88                 | 85.8±8.8                   |
| Diseased adult rats | 19.58±10.74 <sup>a</sup>   | 12.03±15.34 <sup>a</sup> | 68.37±15.34 <sup>a</sup>   |
| Healthy old rats    | 36.78±15.34                | 8.83±18.60               | 54.38±19.75                |
| Diseased old rats   | 15.04±11.18 <sup>b,c</sup> | 9.36±22.11 <sup>c</sup>  | 75.45±20.66 <sup>b,d</sup> |

<sup>a</sup>:  $p < 0.001$  when the group is compared to the healthy adult animals.

<sup>b</sup>:  $p < 0.001$  when the group is compared to the healthy old animals.

<sup>c</sup>:  $p < 0.01$  when the group is compared to the diseased adult animals.

<sup>d</sup>:  $p < 0.05$  when the group is compared to the diseased adult animals.

quantification were devised by ourselves, with the single exception of the MacDraft® programme.

### Statistical study

All the data are expressed as mean  $\pm$  SEM. In order to carry out the distribution study, we made use of the Kurtosis and Skewness indexes. Values of between  $\pm 1$  were considered to be normal distribution values. When the values of the results of the variables came close to normal distribution (this only happened in the case of the alveolar chord length and the alveolar wall thickness), they were compared with parametric tests (variance, Anova, Student-T). Those variables whose results were far from normal distribution were compared with non-parametric tests (Kruskal-Wallis, Mann-Whitney U).

A simple regression study was performed between the alveolar chord/wall thickness variables. The regression index was considered to be good when  $r \geq 0.06$  in absolute values. Probability values of lower than 0.05 were considered significant in all cases. The statistical study was carried out with a Macintosh® computer II CX and the Statview® II programme.

## Results

The mortality rate due to the anaesthesia required for the administration of the antipulmonary serum was 10% in the diseased adult animals and 60% in the diseased old animals.

### BAL (Table 1)

The administration of antipulmonary serum in the adult animals produced an increase in lymphocytes (19.58  $\pm$  10.74) and PMN (12.03  $\pm$  15.34) which was significant ( $p < 0.01$ ) when compared with the untreated adult animals (lymphocytes: 11  $\pm$  4.41; PMN: 3.2  $\pm$  8.88). The old animals to which antipulmonary serum was administered showed a significant decrease ( $p < 0.05$ ) in lymphocytes (15.04  $\pm$  11.18) and an insignificant increase in PMN (9.36  $\pm$  22.11) in relation to the untreated old animals (lymphocytes: 36.78  $\pm$  15.34; PMN: 8.83  $\pm$  18.60). The differences which were found between the diseased adult and old animals were significant ( $p > 0.01$ ).

### Alveolo-interstitial variables (Table 2)

#### Alveolar chord length (Fig. 1)

In the case of the diseased adult animals the length of the alveolar chord decreased (26.4  $\pm$  4.14) and the alveolar wall thickness increased (23.82  $\pm$  8.7). These changes were significant ( $p < 0.001$ ) when these animals were compared with healthy adult animals (alveolar chord length: 28.5  $\pm$  11.07; alveolar wall thickness: 16.86  $\pm$  4.46). The diseased old animals revealed a significant increase ( $p < 0.001$ ) in the length of the alveolar chord (39.85  $\pm$  9.39) and a decrease which was also significant ( $p < 0.05$ ) in the thickness of the alveolar wall (11.1  $\pm$  4.95) when compared with the untreated old rats (alveolar chord length: 36.87  $\pm$  10.3; alveolar wall thickness: 17.45  $\pm$  9.08). When these variables were compared within the groups of diseased animals, both adult and old, the differences were found to be significant ( $p < 0.001$ ). The regression index obtained when the alveolar chord/wall thickness variables were related was only statistically significant in the groups of diseased adult rats ( $p < 0.05$ ) and healthy old rats ( $p < 0.01$ ). In no case was it greater than 0.4.

#### Haemosiderin

The number of alveolar macrophages with haemosiderin, ferrocyanide positive, in the adult animals was greater in the case of the diseased animals (257.63  $\pm$  109). This increase was significant ( $p < 0.001$ ) in comparison with that which was found for the healthy animals (2.43  $\pm$  8.03). The result in the case of the old animals was the opposite: the healthy rats gave significantly ( $p < 0.001$ ) higher values (44.39  $\pm$  20.79) than the diseased rats (7.25  $\pm$  11.37).

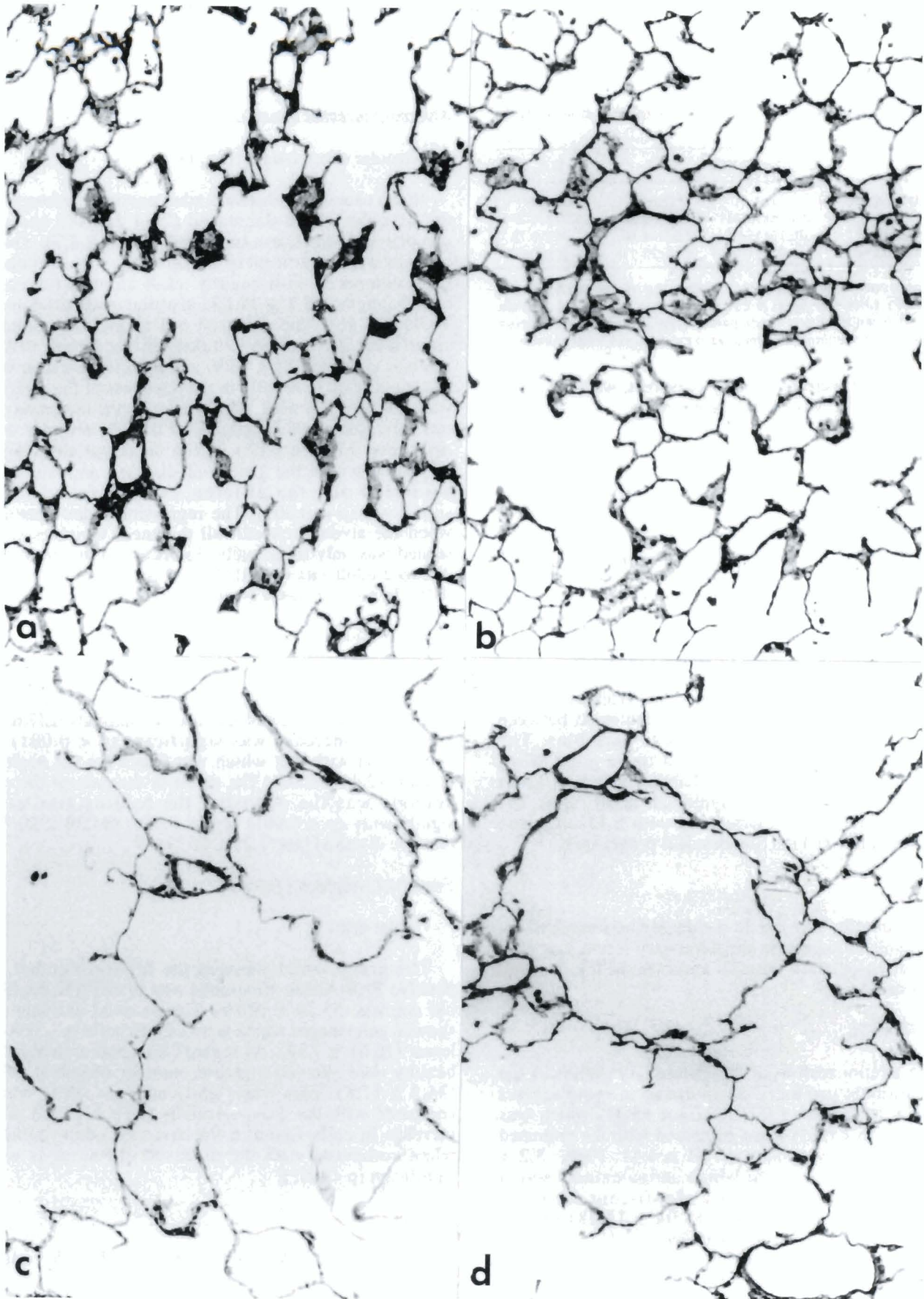
### Bronchial variables (Table 2)

#### Goblet cells

The group which showed the highest number of positive PAS-Alcian Blue cells was that of the healthy old animals (53.39  $\pm$  10.89). The diseased old animals showed percentages which were significantly ( $p < 0.001$ ) lower (16.61  $\pm$  7.37). As regards the adult animals, the healthy ones showed a greater number of goblet cells (34.2  $\pm$  17.8). This was significant ( $p < 0.01$ ) when compared with the diseased adults (20.8  $\pm$  12.19). The increase in cells found in the diseased adult animals when compared with the diseased old animals was significant ( $p < 0.01$ ).

#### LA

The smallest LA was found in the healthy adult animals (0.26  $\pm$  0.71) (Fig. 4). This was statistically different ( $p < 0.001$ ) from the diseased animals (1.35  $\pm$  1.28). There were no significant differences found for



**Fig. 1.** Pulmonary fields taken at random, belonging to the animals from the different groups used: a) lung of diseased adult group; b) lung of the healthy adult group; c) lung of diseased old group; d) lung of healthy old group. PAS-Alcian Blue staining. x 60

the old animals (Figs. 5, 6).

#### Lep

The shortest LEp ( $0.45 \pm 1.01$ ) was found for the healthy adult animals and this was significant ( $p < 0.01$ ) in comparison with the diseased adult animals ( $0.69 \pm 0.83$ ). Among the old animals, no significant differences were registered.

The differences observed between the variables of the

BALT of the diseased adult animals when compared with the diseased old animals were not significant.

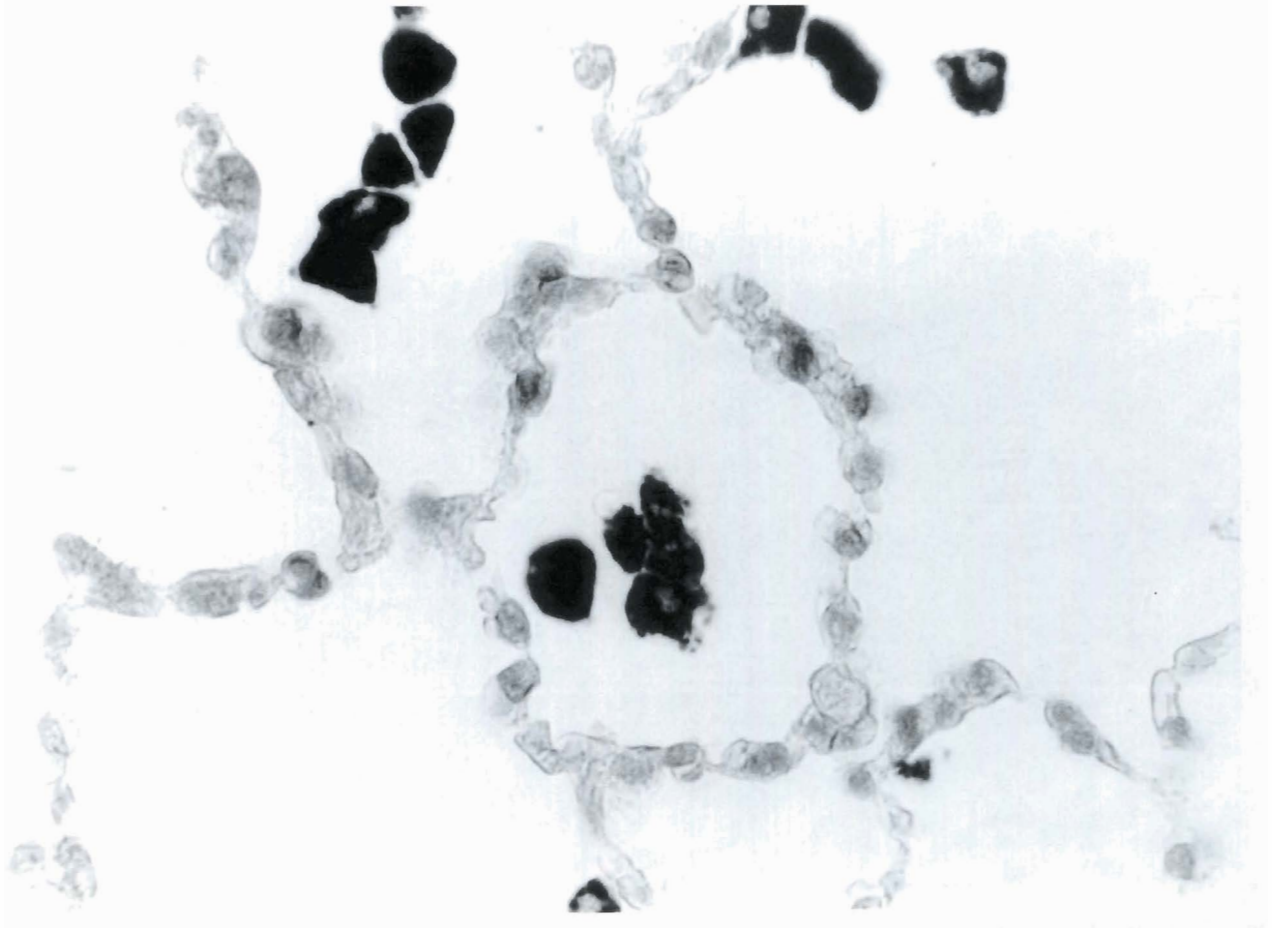
#### Discussion

The data obtained suggest that there are great differences between the two groups of diseased adult and old animals, and this is in favour of the hypothesis of this paper. Nonetheless, we must find out if Goodpasture's disease of lung affection has in fact been

**Table 2.** Results of the values obtained after quantifying the different variables.

| GROUP               | ALVEOLAR CHORD            | THICKNESS OF WALL        | HAEMOSIDERIN              | GOBLET CELL               | LYMPHATIC AREA         | LYMPHATIC EPITHELIUM   |
|---------------------|---------------------------|--------------------------|---------------------------|---------------------------|------------------------|------------------------|
| Healthy adult rats  | 28.5±11.07                | 16.86±4.46               | 2.43±8.03                 | 34.2±17.8                 | 0.26±0.71              | 0.45±1.01              |
| Diseased adult rats | 26.4±4.14 <sup>a</sup>    | 23.82±8.7 <sup>a</sup>   | 257.63±109 <sup>a</sup>   | 20.8±12.19 <sup>b</sup>   | 1.35±1.28 <sup>a</sup> | 0.69±0.83 <sup>c</sup> |
| Healthy old rats    | 36.87±10.3                | 17.45±9.08               | 44.39±20.79               | 53.39±10.89               | 1.26±1.21              | 1.57±1.19              |
| Diseased old rats   | 39.85±9.39 <sup>d,e</sup> | 11.1±4.95 <sup>e,f</sup> | 7.25±11.37 <sup>d,e</sup> | 16.61±7.37 <sup>d,g</sup> | 1.1±0.76               | 0.95±0.49              |

<sup>a</sup>:  $p < 0.001$  when the group is compared to the healthy adult animals; <sup>b</sup>:  $p < 0.01$  when the group is compared to the healthy adult animals; <sup>c</sup>:  $p < 0.05$  when the group is compared to the healthy adult animals; <sup>d</sup>:  $p < 0.001$  when the group is compared to the healthy old animals; <sup>e</sup>:  $p < 0.001$  when the group is compared to the diseased adult animals; <sup>f</sup>:  $p < 0.05$  when the group is compared to the healthy old animals; <sup>g</sup>:  $p < 0.05$  when the group is compared to the diseased adult animals.



**Fig. 2.** Ferrocyanide-positive alveolar macrophages in the dark within the alveolar lumens. x 400

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produced in both the adult and old animals.

#### *Diseased adult animals*

Experimentally, it is considered that a heterologous model of Goodpasture's disease has occurred when, after the administration of heterologous antipulmonary serum (Willoughby and Dixon, 1970; Queluz et al., 1990), the animal is found to have alveolo-interstitial inflammation and diffuse alveolar haemorrhage. The alveolo-interstitial inflammation was objectified by means of the counting of the BAL cells and the calculation of the thickness of the alveolar wall. In fact, the percentage increase of lymphocytes and PMN obtained in the diseased adult animals is related to a great number of inflammatory processes of the distal part of the lung (Crystal et al., 1984; Reynolds, 1987). In the subjective morphological studies, there is thought to be inflammation of the alveolar parenchyma when there is an increase in the thickness of the alveolar wall. In our case, the increase in the thickness of the alveolar wall was quantified and statistically compared and was found to be significant with regard to the untreated adult animals. This fact confirms the alveolo-interstitial

inflammation suggested by the increase in lymphocytes and PMN obtained in the BAL. The alveolar chord is a variable which is used, above all, for the diagnosis of emphysema (McCartney et al., 1988), since its increase or decrease suggests the existence of a dilation or reduction of the alveolar size respectively (McCartney et al., 1988; Lum et al., 1990). The decrease in the length of the alveolar chord which was found in the diseased adult animals suggests that the alveoli of these animals have decreased in size. The quantification of the size of the alveolus is not directly of use for the morphological study of pulmonary inflammatory pathology unless one wishes to relate it to the respiratory function. As far as we are concerned, it seems important to point out the relationship which can exist between the size of the alveolus and the thickness of its wall. The existence of a high incidence of correlation between these two variables suggests that the size of the alveolar lumen is related to the thickness of the alveolar wall. It is proposed that the increase in thickness in the alveolar wall might be of influence in the size of the alveolus in two ways: firstly, in that the reduction in the size of the alveolus is a consequence of the inflammation of the alveolar wall; and secondly that the pulmonary tissue

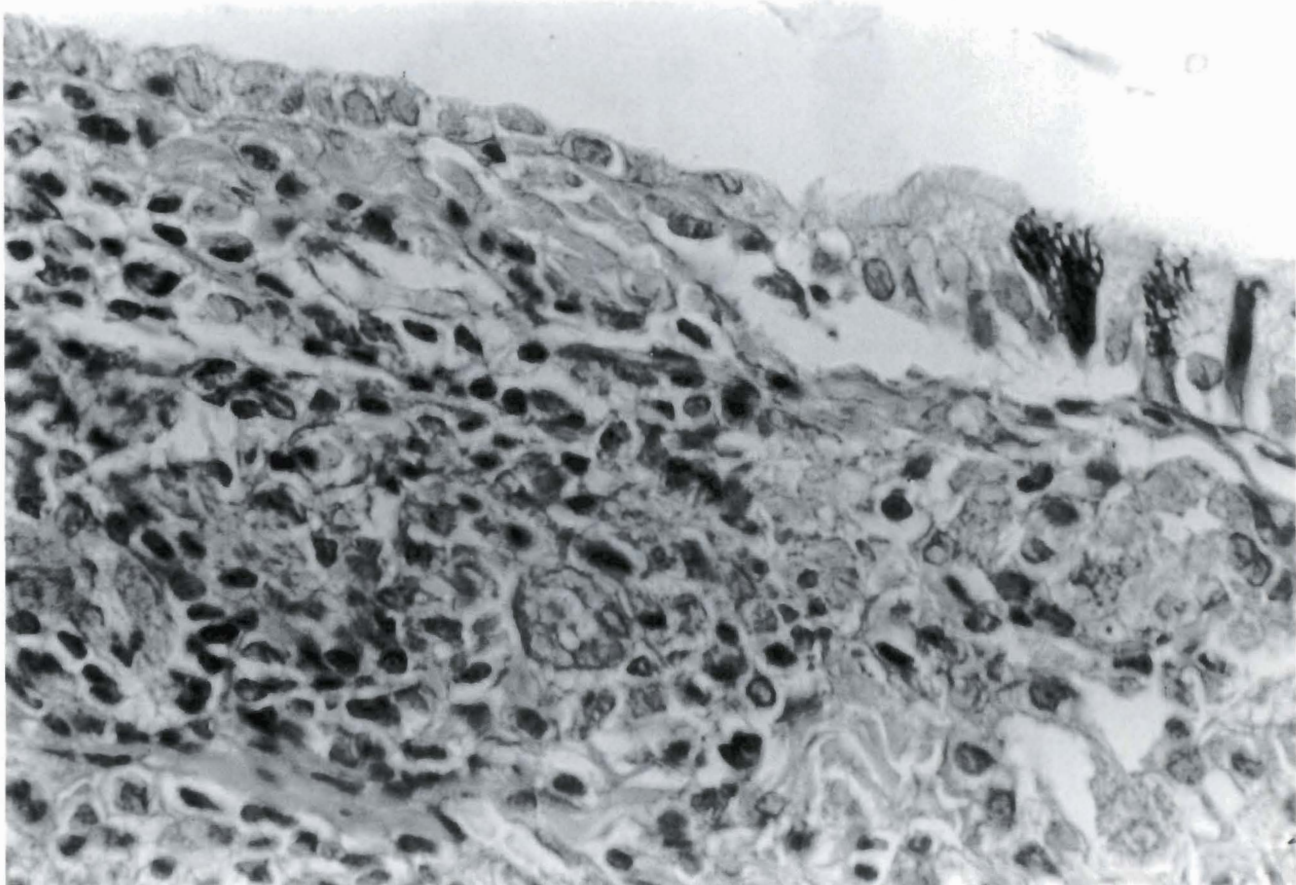
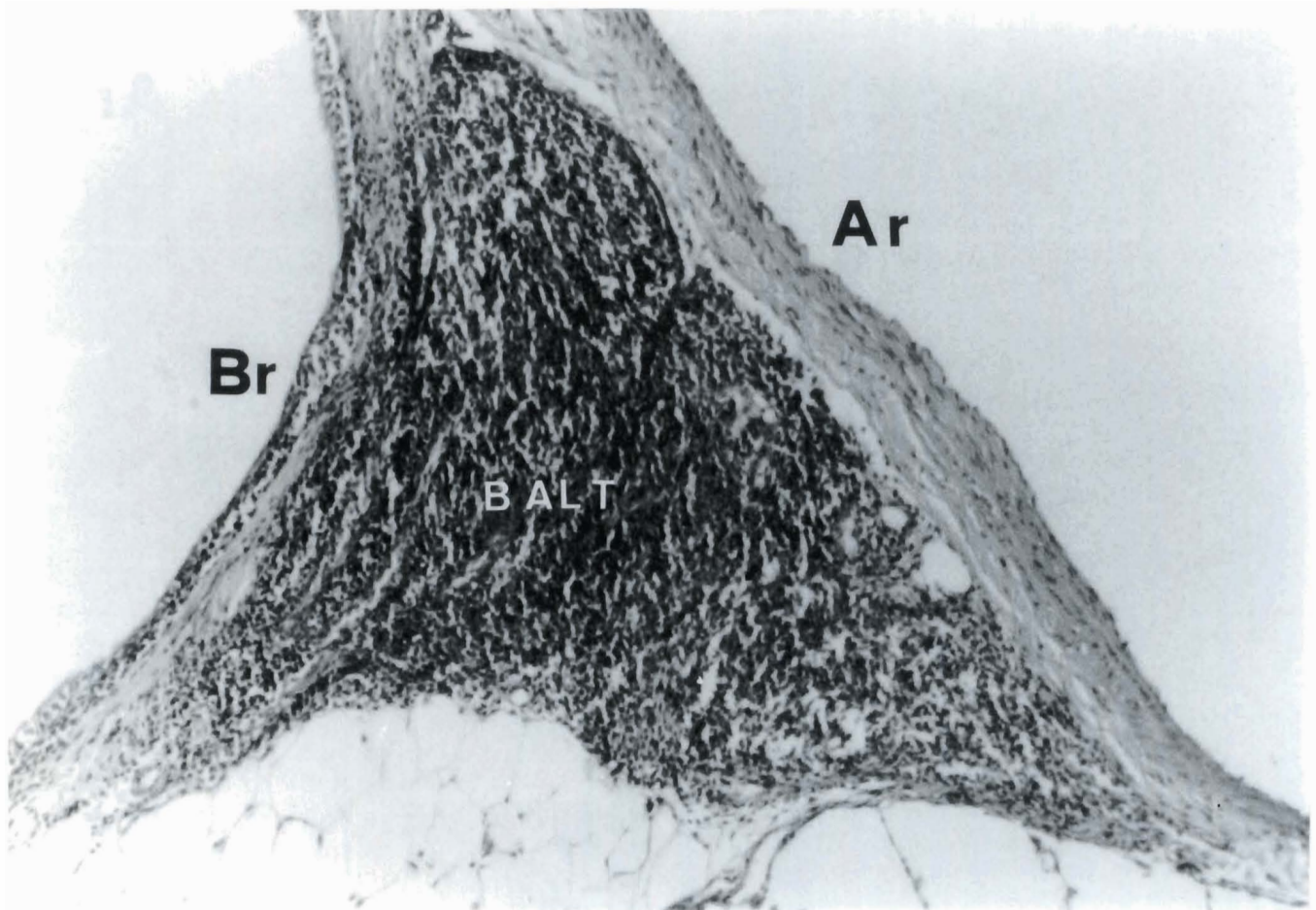


Fig. 3. Bronchial epithelium, pseudostratified on bronchial-associated lymphoid tissue. Goblet cells, in the dark continuing on the lymphatic epithelium of lower height. PAS-Alcian Blue staining. x 400

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may have been altered during the manipulation of the lung, since the fixation, perfusion, etc, could cause the pulmonary wall to retract in some cases and to dilate in others, thus affecting the size of the alveolus. The regression index obtained after relating the alveolar chord to the thickness of the wall, when this was significant, was very low, which suggests only a very slight dependence between these two variables and considerably reduces the possibility of alteration. It is suggested that the inflammation of the alveolar wall, caused by the administration of heterologous rat antipulmonary serum to the adult animals is not a conditioning factor in the decrease of the size of the alveolar lumen. Diffuse alveolar haemorrhage is a fact which must be demonstrated in the diagnosis of Goodpasture's disease of lung affection, whether it be directly or indirectly. The haemosiderin which is present in the alveolar macrophages originates in the haemoglobin, which is transferred from the capillaries of the alveolus, and its increase is related to the possibility of an alveolar haemorrhage having been produced. The increase in the number of alveolar macrophages with

haemosiderin which was demonstrated in the diseased adult animals is an indirect sign that an alveolar haemorrhage has taken place (Willoughby and Dixon, 1970; Jennings et al., 1981; Queluz et al., 1990). The goblet cells were quantified with the object of establishing the possible degree of involvement of the airways in this disease. It has been generally accepted that goblet cells proliferate when faced with bronchial aggression (Olesen et al., 1987). Semiquantitative studies have related the experimental administration of antigens to the increase of goblet cells (Nygren and Ahlstedt, 1983). However, more recent experiments in which O<sub>2</sub> in high concentrations (Wiswell and Wiswell, 1990) or antigens (Du et al., 1991) have been administered to laboratory animals have not been able to demonstrate an increase either of goblet cells or mucus. In previous experiments carried out by ourselves on models of extrinsic allergic alveolitis and Goodpasture's disease (Escobar Castellón et al., 1992) the results were similar to those obtained for the diseased adult animals in the present study; i.e., a decrease in goblet cells. The study of the BALT was performed with the object of



**Fig. 4.** Bronchial-associated lymphoid tissue (BALT) situated between an artery (Ar) and a bronchus (Br). Healthy animal. LA: Lymphatic area. PAS-Alcian Blue staining. x 25

relating the nature of the disease provoked in the group of diseased animals with a possible immunological origin, as is the case with Goodpasture's disease.

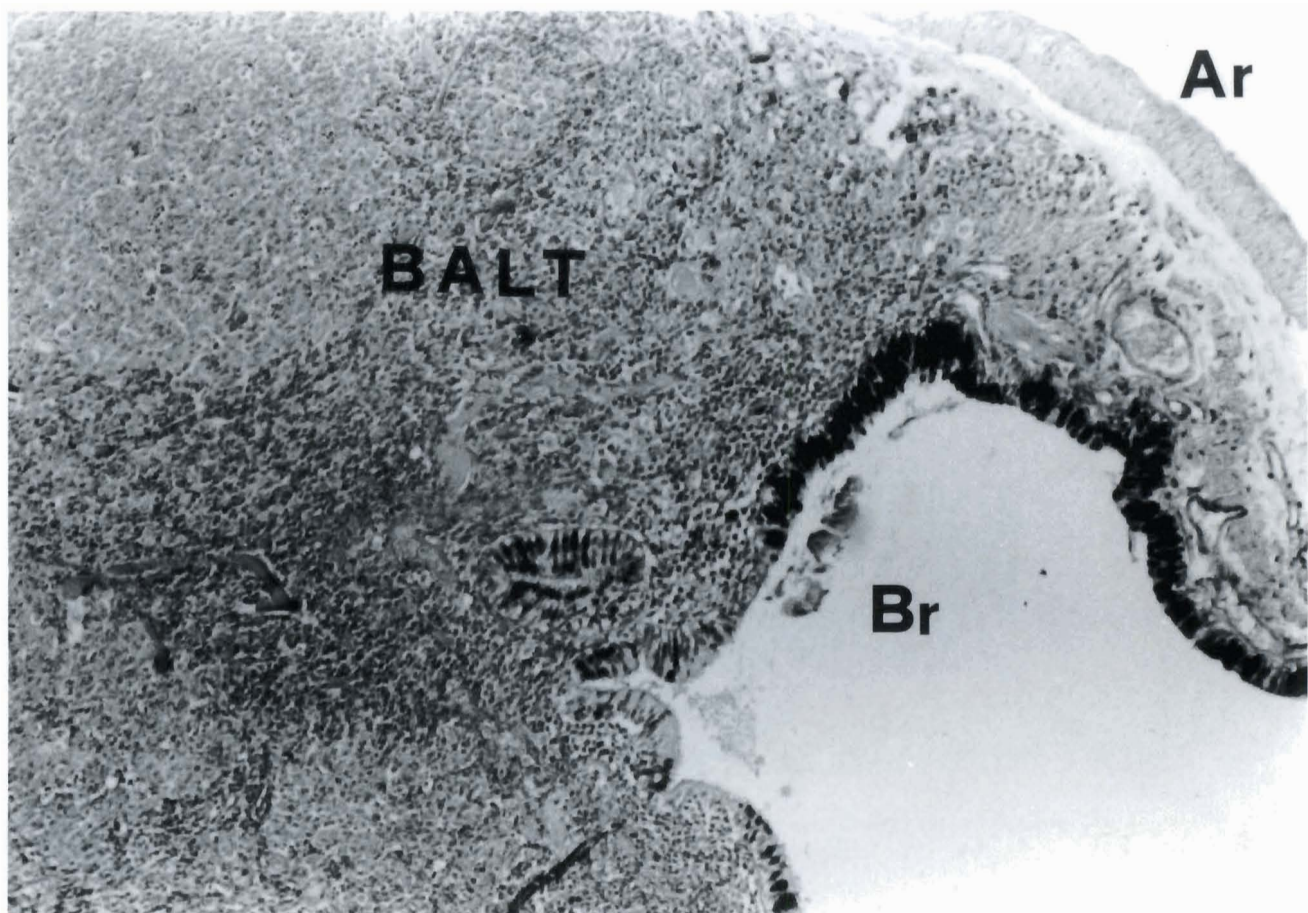
Indeed, it has been claimed that, in order that an alveolar haemorrhage should take place in Goodpasture's disease, the presence of the IL-2 and IFN gamma alveolar macrophages is necessary (Proust et al., 1988; Lan et al., 1991). The BALT was considered to be a key in pulmonary immunological defence (Sminia et al., 1989; Van der Bruffe-Gamelkoorn et al., 1985). Morphologically speaking, two parts have been differentiated: one epithelial part, whose flat cells are different from the rest of the bronchial epithelium and which specialize in the capturing of antigens (Sminia et al., 1989); and another lymphatic part where the antigen is brought into contact with the cells which are receivers of antigens (Van der Bruffe-Gamelkoorn et al., 1985). Experimentally, it has been described that BALT proliferates when faced with different diseases such as extrinsic allergic alveolitis and Goodpasture's disease (Escobar Castellón et al., 1992). In the case of diseased adult animals, we consider that the proliferation of BALT, demonstrated by the increase of the LA and EPp

variables is a fact which is in favour of the initiation of immunological mechanisms.

With regard to the diseased adult animals, all of our data are in favour of the hypothesis that the alterations produced by the administration of heterologous antipulmonary serum are compatible with the pulmonary affection Goodpasture's disease.

#### *Diseased old animals*

The results obtained for diseased old animals do not in any way suppose the presence of a pulmonary affection model of Goodpasture's disease, since neither alveolo-interstitial inflammation nor alveolar haemorrhage have been demonstrated. Alveolo-interstitial inflammation is related to an increase of lymphocytes of LBA (Reynolds, 1987) but never with a decrease. It has been a cause of surprise for us to have found a decrease in the percentage of lymphocytes in the diseased old rats. The increase in the length of the alveolar chord and the decrease in the thickness of the wall suggest that in the diseased old animals the size of the alveolus is greater and that part of the distal



**Fig. 5.** Bronchial-associated lymphoid tissue (BALT) situated between an artery (Ar) and a bronchus (Br). Healthy old animal. LA: Lymphatic area. PAS-Alcian-Blue staining. x 25

pulmonary tissue component has disappeared, all of which is characteristic of emphysema (Snider et al., 1985; McCartney et al., 1988; Llum et al., 1990) and not of inflammation. Our data suggest that the possibility of the diseased old animals having suffered an alveolar haemorrhage are nil and we would suggest that such a low number of ferrocyanide-positive alveolar macrophages removes the suspicion of the existence of diffuse alveolar haemorrhage, as is described in all the versions of the pulmonary affection Goodpasture's disease (Willoughby and Dixon, 1970; Queluz et al., 1990).

The results obtained when the BALT of the two groups of old animals were compared lead us to consider that the immunological response of the lungs of the diseased old animals might be modified. Existing experience related to the behaviour of the BALT suggests that this proliferates either totally or partially when faced with experimental disease (Escolar Castellón et al., 1992). However, all these experiments were performed on adult groups. As regards the BALT of the old animal, it is only known that it is of greater size than that of the adult (Anderson et al., 1986) and that the

continuous exposure to antigens which occurs throughout life is thought to be responsible for this increase. As for our own experiment, we would postulate that the increase in the size of the BALT observed in the group of healthy old rats is as much as can be attained, which leads us to propose that neither the administration of antigens nor the induction of disease could increase the size of the BALT in the old group.

The decrease in goblet cells has been described in experimental models of Goodpasture's disease (Escolar Castellón et al., 1992). However it is a very unspecific sign and only serves in this experiment to mark the differences between the healthy and diseased animals.

Since we were unable to induce Goodpasture's disease in the old animals, we propose that the fundamental hypothesis of the study is possible. The results obtained in the diseased adult animals suggest that, in effect, the pulmonary affection Goodpasture's disease has been induced while the results in the diseased old animals are compatible with emphysema. These behavioural differences could be related to the immunological nature of Goodpasture's disease since the behaviour of the immune system of the adult animal is

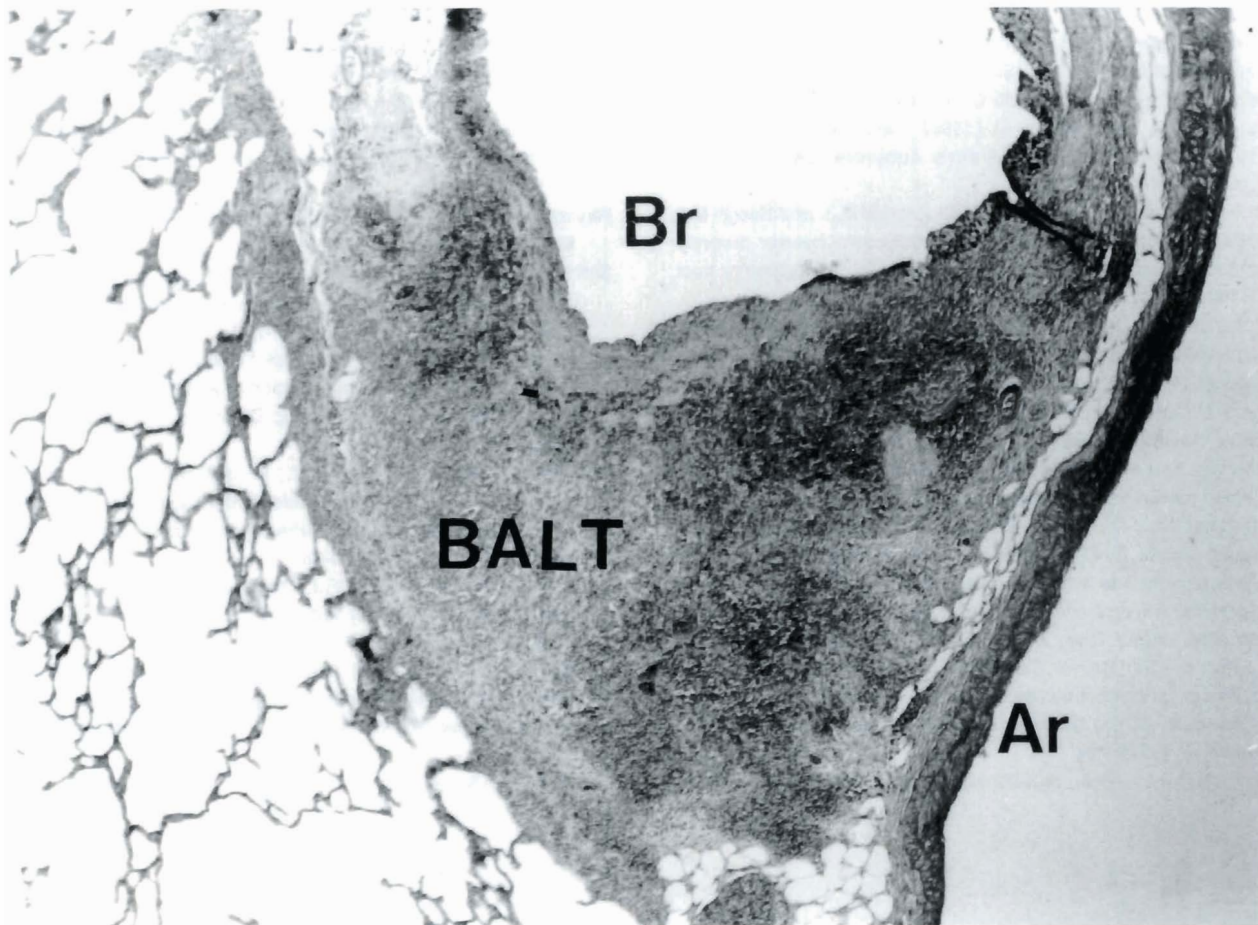


Fig. 6. Bronchial-associated lymphoid tissue (BALT) situated between an artery (Ar) and a bronchus (Br). Diseased old animal. LA: Lymphatic area. PAS-Alcian Blue staining. x 25

different to that of the old animal. While immune deficiency is not a characteristic of ageing *per se* (Barcellini et al., 1988), it has been experimentally demonstrated that the reactive capacities of the T- and B-lymphocytes in the old animal are decreased (Barcellini et al., 1988; Matour et al., 1989). With regard to our results, it cannot be considered that the behaviour of the lung of the old rat in the face of immunological disease is similar to that of the young rat. This study is merely descriptive and does not claim to explain the mechanisms which have conditioned its results. We believe that this experiment should be continued, together with others in which the cause of the different behaviour between old and adult rats might be proposed as a hypothesis.

**Acknowledgements.** The authors of this study would like to thank Laboratorios Operón®, for their help in the elaboration of the rat antipulmonary rabbit serum. This study was financed by a research grant from the Spanish Ministry of Education No. PM88-096.

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Accepted April 5, 1993