Tobacco smoke and age as risk factors in emphysema. Morphometrical study on the rat

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Summary. During ageing, a progressive deterioration in the pulmonary function, which can be accelerated by exposure to tobacco smoke, takes place. The hypothesis that the initial age of exposure to tobacco smoke is a factor of utmost importance in the development of emphysema is proposed. Eighty-six rats, aged nineteen months at the time of sacrifice, were used and were ordered into three groups: the first group consisted of unmanipulated animals; the second, of animals which had been exposed to tobacco smoke from the age of twelve months to the age of nineteen months; and the third, of animals which had been exposed to tobacco smoke from the age of nine months to the age of twelve months. The lungs of the animals were histologically processed for light microscopy and were studied morphometrically by computer. Eleven quantitative variables were quantified and ordered into three groups: variables related with alveolar enlargement; variables related with tissue loss; and variables related with the elastic fibre. The number of animals in which alveolar enlargement and tissue destruction concurred was counted, thus enabling the attributable and relative risks of developing emphysema to be calculated in the two groups of manipulated animals. From the results it is clear that, when compared with the unmanipulated group, the two groups which had been exposed to tobacco smoke displayed an increase in the variables which quantified alveolar enlargement and a decrease in those which measured tissue loss; these results were more significant in the third group (p<0.001) than in the second (p<0.05); significant differences were also found between these two groups of animals. The relative risk and attributable risks of developing emphysema were 2.41 and 28.15 respectively in the second group and 3.48 and 34.48 in the third group. Our results lead us to propose that the risk of developing emphysema exists in inverse proportion to the initial age of exposure to tobacco smoke.

Key words: Lung, Morphometry, Tobacco smoke, Age, Emphysema

Introduction

Morphometrical studies performed over 30 years ago on human lungs suggest the existence of a relation between the smoking habit, age and emphysema (Auerbach et al., 1972). From that time up to the present, the evidence accumulated has been sufficient to establish the fact that the progressive loss in the lung function which takes place during ageing is accelerated in the smoker (US Department of Health and Human Services, 1984; Xu et al., 1992).

The senile lung is characterized morphologically by simple distal airspace enlargement, without tissue loss (Snider et al., 1985; Verbeek et al., 1992), unlike pulmonary emphysema in which distal airspace enlargement is found together with tissue destruction (Snider et al., 1985). Several studies have demonstrated that the lungs of smokers display attachment loss (Saetta et al., 1985), an increase in the destructive index and in the mean linear intercept index (Saetta et al., 1985; Eidelman et al., 1991), which suggests distal airspace enlargement and tissue loss, all of which is compatible with the present definition of emphysema (Snider et al., 1985).

Pinkerton et al. (1982), after studying healthy rats of different ages, concluded that from the adult age onwards, a progressive increase of the distal airspaces, without apparent tissue loss, and also a progressive deterioration in the lung function take place. Furthermore, the lungs of animals exposed to tobacco smoke displayed morphological alterations similar to those which take place in emphysema (Auerbach et al., 1967; Park et al., 1977; Heckman and Dalbey, 1982; Hoidal and Niewoehner, 1983; Snider, 1992). All the above coincides with the observations made with regard to the human species (Saetta et al., 1985; Snider et al., 1985; Eidelman et al., 1991).

The number of cigarettes per day and the period during which a given individual has been a smoker have
been proposed as factors which make the individual more likely to develop emphysema (Xu et al., 1992). It has been described that when the smoking habit is broken, a deceleration takes place in the rapid deterioration of the lung function of the smoker (Camilli et al., 1987; Dockery et al., 1988; Xu et al., 1992; Wright and Sun, 1994). In our opinion, there are two very important factors related with the consequences of exposure to cigarette smoke: 1) the age at which the habit starts, which is not frequently reflected in the studies performed on groups of smokers; and 2) the fact that children who live with smokers display alterations in the lung function (Helms, 1994).

In this study, the following working hypothesis is proposed: the initial age of exposure to tobacco smoke is an additional risk factor in the development of emphysema. In order to demonstrate our hypothesis, an experimental model of passive exposure to tobacco smoke in rats was used (Escolar et al., 1995). The study of the lungs is morphometric, and quantitative variables capable of assessing the modifications in the distal airspace size, the interstitial alveolar tissue and the elastic fibre are proposed.

Materials and methods

Animals

Eighty-six disease-free Wistar rats (Iffa Credo®) were used; the rats were aged nineteen months at the time of sacrifice. The animals were housed in F1-type cages (Iffa Credo®), which were equipped with a filter cover to avoid contamination, for the entire duration of the experiment; water and food was available ad libitum to the animals, which were divided into three groups in the following manner:

1. Control group: 30 unmanipulated animals.
2. Group exposed to tobacco smoke: 27 animals exposed to tobacco smoke from the age of twelve months. They were sacrificed sixteen hours after the last cigarette.
3. Group withdrawn from exposure to tobacco smoke: 29 animals exposed to cigarette smoke from the age of nine months and withdrawn at the age of twelve months. These animals were sacrificed seven months after the last cigarette.

Exposure to tobacco smoke

A protocol which has already been described was followed (Escolar et al., 1995). The cages containing the animals were introduced, without the filter cover, into a chamber of 75x75x75 cm for ninety minutes per day. There was an atmosphere of tobacco smoke within the chamber at a concentration of CO 41.7 mg/m³ (35 ppm). The smoke was generated by the burning of a commercial Virginia cigarette (16 mg of tar and 1 mg of nicotine) and was introduced into the chamber by the airflow generated by a compressor. In order to well ventilate the chamber, air produced by two compressors was introduced. The chamber was equipped with six 3 mm holes which enabled the air to flow out and thus be well renewed. The dosage was of one cigarette every half hour, three cigarettes per day for five days per week.

Sacrifice

The animals were anaesthetized with pentobarbital intraperitoneally (0.1 mg/g body weight). Once the absence of reflexes had been established, the costal pad was removed. The left lung was extracted for morphometric study.

Morphometric study

This was performed according to a protocol already described (Escolar et al., 1994b). The left lungs were fixed in formalin at 10% with a transbronchial pressure of 25 cm of H₂O, for 48 hours. The block chosen was taken from the area immediately below the main bronchus, where it becomes intraparenchymatous and is perpendicular to the main axis of the lung. The block was dehydrated and embedded in paraffin. Seriated sections of 7 µm were made. The stainings performed were Prussian-blue, methylene blue and orcein. A different quantification protocol was used for each of the stainings.

Prussian blue

This staining was used to demonstrate the presence of hemosiderin inside the alveolar macrophages since it contrasts the Fe⁴⁺ in blue. The quantification of the alveolar macrophages was performed according to a protocol already described. The histological section was divided into thirteen areas, of which seven were always chosen; from each of these seven areas, a field was chosen at random (x100) (Escolar et al., 1991) and all the Prussian blue-positive alveolar macrophages in the field were counted. The results are expressed as the number of Prussian blue-positive alveolar macrophages per cm².

Methylene blue

Seven fields were chosen, according to the protocol outlined for the previous staining. The processing of the images was systematized in three stages: Capture, treatment and quantification (Escolar et al., 1995).

Capture. This was performed by means of a black and white video camera (Hitachi Kp-110®) adapted to a microscope (x 100); the microscopic image (600x450 µm) was captured by a computer (Fig. 1).

Treatment. The image captured was transformed by means of the threshold option into another image, consisting of two colours: white (air) or black (tissue).
The background noise was eliminated by means of a median filter. Lastly, any part of the image which was smaller than two hundred μm and completely surrounded by the opposite colour was eliminated by the stain filter.

Quantification. The following variables, which have already been described, were quantified on the resulting image (Fig. 2):

1. - Tissue Density (TD): this represents the percentage of tissue (black) in relation to the total of the image (black and white).
2. - Wall Thickness: measurement of the mean thickness of the alveolar walls of a histological field, expressed in μm.
3. - Alveolar Chord: distance between two walls of a single alveolus, expressed in μm.

For the quantification of the last two variables, the computer traced thirty parallel equidistant lines on the histological field. All the measurements were performed according to a criterion which has already been described (Lum et al., 1990).

4. - Internal alveolar perimeter (IAP): the computer measured the length of the white-black interphase, expressed in μm.

Two further variables were determined from these values.

5. - Mean linear intercept (Lm), which was obtained from the following formula (Escolar et al., 1995):

\[ Lm = \bar{XA} + \bar{XW} \]

\( \bar{XA} \): mean of alveolar chords; \( \bar{XW} \) mean of thickness of alveolar walls.

6. - Air density/IAp (ADIAP): this was calculated taking AD=100-TD. The decrease in this variable suggests that the shape of the alveolar space is similar to that of a sphere.

Orcein

This staining contrast the elastic fibre in brown. It was performed according to Fränkel’s criterion, omitting the final background contrast (Escolar et al., 1994b). For the capture, treatment and quantification, a protocol similar to that used for methylene blue was observed, with the following modifications:

Capture. The image was captured at an increase of 200, obtaining a size of 350×262.2 μm (Fig. 3).

Treatment. The threshold option was used to colour
the elastic fibres black and the rest of the image white and the median filter to eliminate background noise (Fig. 4).

Quantification

The computer quantified two variables:
7. Elastic Fibre Density (EFD): proportion of elastin in relation with the rest of the field.

Two variables were found from these data:
9. EFD/EFP: this was considered as a shape coefficient. When this variable increased, the elastic fibre acquired a circular shape.
10. EFD/TD: this was obtained from the following formula: EFD x 100/TD. It indicates the variations in the elastin in relation to the tissue density.

The treatment and quantification programme was designed by ourselves (Escolar et al., 1995).

Statistical Study

Mean values ± one standard deviation are presented. The central tendency and dispersion values were found. A study of the dispersion curve morphology was made and the Kurtosis and Skewness indices calculated. The results were compared with each other by means of the Anova, the T or Kruskal-Wallis test and Mann Whitney's U test; the latter being used where the results were far from normal distribution. Taking as a reference the hypothesis of the study, which proposes that the risk of developing emphysema increases when the subject is exposed to cigarette smoke from an early age, the attributable risk and the relative risk were calculated; the following protocol was established for this purpose:

1. Calculation of the sensitivity, specificity and misclassified indices:
   1.1. The criterion of unmanipulated animals (control group) and manipulated animals (2nd and 3rd groups) was used to establish the gold standard.
   1.2. In the values obtained for each variable, the cut-off point was established, following the criterion of the mean value (Escolar et al., 1994b):

\[
\frac{XC + X2 + X3}{2}
\]

Where:
XC: Mean of the values obtained for the control group.
X2: Mean of the values obtained for the second group.
X3: Mean of the values obtained for the third group.

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The specificity index applies to the percentage of animals of the control group for which the results were those expected in relation with the cut-off point established. The sensitivity index applies to the percentage of manipulated animals for which the results were those expected in relation with the cut-off point. The mis-classified index applies to the percentage of manipulated and unmanipulated animals for which the results were outside the cut-off point. In the definition of emphysema the possible modifications which the elastic fibre is liable to undergo are not included; for this reason, no index related with the variables which quantify the elastic fibre is calculated.

2. The emphysema criterion was established when The TD and IAP variables for a given animal were below the cut-off point and the alveolar chord variable above it.

3. The two groups of animals which had been exposed to tobacco smoke were compared by means of the $\chi^2$ test, with the aim of demonstrating that the number of emphysematous animals was significantly different in both groups.

4.1. The attributable risk in the groups of manipulated animals was calculated by subtracting the percentage of emphysematous animals obtained in the control group from the percentage of emphysematous animals in the 2nd and 3rd groups; the confidence interval was calculated (level 95%).

4.2. The relative risk was obtained by dividing the percentage of emphysematous animals corresponding to each of the manipulated groups by the percentage of emphysematous animals of the control group; the confidence interval was calculated.

The data were processed on a Macintosh II Lc® computer, with the StatView® programme. Values of $p<0.05$ were considered to be significant.

Results

Alveolar architecture variables (Table 1)

In relation to the control group, the Lm and the alveolar chord increased significantly ($p<0.05$) in the groups of animals exposed to tobacco smoke. The results for the variable IAP showed it to have decreased significantly in these two groups ($p<0.05$). The TD decreased when the animals were exposed to tobacco smoke but significance was only found in the third group ($p<0.001$). The variations in wall thickness and AD/IAP did not reach statistical significance in either of the groups of animals exposed to tobacco smoke. When compared with the second group, the alveolar chord and

Fig. 3. Image of a histological section stained with orcein and digitalized in 16 shades of grey. $\times 200$
the Lm of the animals of the third group were found to have increased (p<0.001) and the TD (p<0.05) and the IAP (p<0.001) to have decreased.

*Elastic fibre variables* (Table 1)

When compared with the control group and the third group, the animals which were sacrificed immediately after having inhaled the smoke of the last cigarette (second group) displayed an increased in the EFD, the EFP and the EFD/TD, and a decrease in the EFD/EFP; all the results were statistically significant (p<0.001). The animals of the third group, when related with the control group (Table 1), displayed a decrease in the EFP and the EFD/EFP and an increase in the EFD/TD; all the differences were statistically significant (p<0.001). The EFD showed little variation in these animals (p>0.05).

*Prussian blue* (Table 1)

The number of Prussian blue-positive macrophages increased significantly in the two groups of animals exposed to tobacco smoke (p<0.001) when compared with the control group. On comparing the results of the second and third groups with each other, the latter displayed an increase, although significant values were not reached.

*Sensitivity, specificity and mis-classification indices* (Table 2)

Several mis-classification indices were obtained, ranging between 29.33 (IAP) and 50.04 (AD/IAP). We
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![Graphical representation of the relative risk of developing emphysema](image)

**Fig. 5.** Graphic representation of the relative risk of developing emphysema, with confidence intervals at 95%, obtained in the groups of animals which were exposed to tobacco smoke. The black vertical bars show the index risk of developing emphysema. The scratched area shows the confidence intervals.

![Graphical representation of the attributable risk of developing emphysema](image)

**Fig. 6.** Graphic representation of the attributable risk of developing emphysema, with confidence intervals at 95%, obtained in the groups of animals which were exposed to tobacco smoke. The black vertical bars show the index risk of developing emphysema. The scratched area shows the confidence intervals.

### Table 2. Sensitivity, specificity and mis-classified indices obtained in the variables related with the alveolar architecture.

<table>
<thead>
<tr>
<th>SPECIFICITY</th>
<th>SENSITIVITY</th>
<th>MIS-CLASSIFIED</th>
</tr>
</thead>
<tbody>
<tr>
<td>TD</td>
<td>73.33</td>
<td>58.15</td>
</tr>
<tr>
<td>Wall thickness</td>
<td>50</td>
<td>48.15</td>
</tr>
<tr>
<td>Alveolar chord</td>
<td>73.33</td>
<td>59.26</td>
</tr>
<tr>
<td>LM</td>
<td>63.33</td>
<td>37.04</td>
</tr>
<tr>
<td>IAP</td>
<td>70</td>
<td>59.26</td>
</tr>
<tr>
<td>AD/IAP</td>
<td>50</td>
<td>48.15</td>
</tr>
<tr>
<td>Emphysema</td>
<td>80</td>
<td>48.15</td>
</tr>
</tbody>
</table>

Specificity index: percentage of animals of the control group for which the values obtained in the variables tissue density (TD), wall thickness and internal alveolar perimeter (IAP) were above the cut-off point and in the variables alveolar chord, Lm and TD/IAP were below the cut-off point.

Sensitivity index, Group 2, and sensitivity index, Group 3: percentage of animals in each of the groups, for which the results were above the cut-off point in the variables alveolar chord, Lm, AD/IAP, and below the cut-off point in the variables TD, wall thickness and IAP.

Miss-classified index: percentage of the total of the animals for which the results were other than those expected for each group.

Sensitivity emphysema: percentage of animals of the control group for which the morphometric study was not compatible with emphysema.

Sensitivity emphysema: percentage of animals of the second and third groups which were considered emphysematous.

Would point out that the lowest indices applied to the variables IAP (29.33), alveolar chord (30.52) and TD (33.18). The sensitivity indices of the second group were lower than those of the third group and than the specificity indices.

### Risk

The relative risk of developing emphysema (Fig. 5) in the animals of the second group was 2.41 (confidence intervals 1.97 and 2.94). The relative risk of developing emphysema in the animals of the third group was greater: 3.48 (confidence intervals 2.69 and 4.49). The attributable risk (Fig. 6) of developing emphysema in the animals of the second group was 28.15 (confidence intervals 21.72 and 34.58) and was lower than the attributable risk found for the third group: 34.48 (27.4 and 41.56). When the different groups were compared with each other by means of the $\chi^2$ test, significant differences were found between all of them ($p<0.05$).

### Discussion

Our results show that exposure to tobacco smoke causes substantial modifications in the lung tissue. Greater differences were found between the third group and the control group than between the second group and the control group. In order to verify the working hypothesis, we must first demonstrate that our results coincide with the definition of emphysema and subsequently compare the risks of developing emphysema obtained for the two groups of animals exposed to tobacco smoke.

Taking the present definition of emphysema «abnormal permanent enlargement of air spaces distal to the terminal bronchioles, accompanied by destruction of their walls and without obvious fibrosis» (Snider et al., 1985) the results are grouped into variables which are related to tissue destruction.

The variables related with the alveolar enlargement are the Lm and the alveolar chord. The Lm is the most frequently used variable in the morphometric study of the lung; it is related to the number of times a line of a specific length is superimposed on the alveolar walls (Dunnill, 1962). The alveolar chord is defined as the distance which exists between the two walls of a single alveolus, taken at random; this was always determined with the help of the computer. As has been established in the section on material and methods, Lm = alveolar chord + wall thickness; over 15% of the Lm applies to the wall thickness and under 85% to the alveolar chord. The variables Lm and alveolar chord were found to have increased significantly in the two groups of animals which had been exposed to tobacco smoke, when compared with those of the control group; the misclassification index for the alveolar chord was the lowest. This leads us to consider that an increase in the size of the distal airspaces has taken place and to...
propose the alveolar chord as the most sensitive variable for the detection of the enlargement of the distal airspaces.

Tissue loss is suggested by any of the following three variables: TD, wall thickness or IAP.

The variable TD has been used in several morphometric studies and is quantified by the point grid method (Pinkerton et al., 1982). The TD decreased in both groups of manipulated animals but only displayed significant differences in the third group. If the histological preparation is extrapolated to a volume, the animals expressed earliest to tobacco smoke presented 26% less lung tissue volume than the animals of the control group.

Emphysema as a result of exposure to tobacco smoke has been related with wall destruction (Eidelman et al., 1990). In a previous study on rats of five months old which were exposed to tobacco smoke, the wall thickness was found to have decreased (Escolar et al., 1995). In a descriptive study, in which the lungs of adult and middle-aged rats were compared, the wall thickness was less in the older animals (Escolar et al., 1994b). From these studies it can be deduced that the alveolar wall can become thinner as a consequence of exposure to tobacco smoke and with age. In this experiment, the wall thickness was found to have decreased in the two groups of animals exposed to tobacco smoke. However, significant differences were not reached. We propose that, even if both factors - age and tobacco - are combined, the thinning of the alveolar wall obtained for each factor taken singly is not equivalent to the result of the factors taken together.

The significance of the IAP is similar to that of the internal alveolar surface (IAS). Up to the present, the IAS has been considered as the only quantitative morphometric variable related to the destruction of lung tissue (Thurlbeck, 1967). The IAS is deduced from the lung volume and the Lm. The development of the formula for obtaining the IAS is based on the relation surface area/unit volume (Campbell and Tomkeieff, 1952). We propose the IAP per surface unit (the histological field); if the IAP per surface unit (the histological field) and the surface of a histological field by the thickness of the histological field and the surface of a histological field is considered as the only quantitative variable related to the destruction of lung tissue (Thurlbeck, 1967). The variable TD has been quantified, it was concluded that the elastic fibre (Wright et al., 1960). From this study, it could be deduced that in pulmonary emphysema, the possible quantitative variations in elastic fibre cannot be detected through biochemical study. More recent morphometric studies have demonstrated a shortening of the elastic fibre once mild emphysema has been established (Niewoehner and Kleinerman, 1977). When these studies are related with the results obtained for the third group, it can be proposed that in experimental models of microscopic emphysema, there is little chance of being able to demonstrated that loss of elastic fibre has taken place, because the tissue destruction is very slight. However, one must not lose sight of the fact that experimental elastase-induced emphysemas are also microscopic and loss of elastic fibre has been demonstrated in them (Kuhn et al., 1976; Goldstein, 1982). Biochemical determinations of desmosine, performed more recently on emphysematous human lungs with the aim of establishing the possible destruction of elastic fibre, are not conclusive, since the information obtained varies greatly among the different studies published (Snider, 1992).

The results obtained for the variables EFD and EFD/TD in the animals exposed to cigarette smoke may appear to be contradictory. However, experiments referred to in the literature ratify our findings. In one study performed on human lungs, in which the type of emphysema was described at the same time as the desmosine was quantified, it was concluded that the elastic fibre does not decrease in all types of emphysema (Cardoso et al., 1993). In an experimental model of passive exposure to tobacco smoke, carried out on adult rats, dilation of the distal airspaces and lung tissue destruction were described, but loss of elastic fibre was not (Escolar et al., 1995). It has been proposed that emphysema brought on by exposure to cigarette smoke takes place through wall destruction, while elastase-induced emphysema comes about through elastic fibre destruction (Eidelman et al., 1990). Clinical studies performed on the lungs of smokers have proposed that the loss in the elastic recoil pressure is caused by an increase in the alveolar size (Hogg et al., 1994). In the light of all the above, we propose that, in our model of emphysema caused by exposure to cigarette smoke, tissue loss occurred, but elastic fiber destruction did not.

Deposits of hemosiderin and other substances present in tobacco smoke have been found in the lung (Ghio et al., 1994). The increase of hemosiderin in the alveolar macrophages is evidence of the fact that molecules of considerable size, such as hemoglobin and even red corpuscles have passed through the
alveolo-capillary membrane. This suggests an increase in the alveolo-capillary permeability (Escolar et al., 1994a), the cause of which may be the inhalation of cigarette smoke. Large quantities of hemosiderin were also found in the lungs of the animals which were withdrawn from exposure to tobacco smoke at the age of twelve months, which leads us to consider that the permeability increase caused by inhalation of tobacco smoke is irreversible.

The tests performed to check the results obtained in respect of each variable indicate that in the two groups of manipulated animals the alveolar size increases and the lung tissue is destroyed; these modifications suggest the existence of emphysema. However they do not provide information on whether tissue destruction and airspace enlargement coexist in a single lung. For this reason we have proposed the emphysema variable. This variable indicates that in 20% of the lungs and airspace enlargement coexist in a single lung. According to the concept of normal distribution, only 5% of the animals of the control group should have displayed alterations which are compatible with emphysema and, therefore, that the specificity index should have been 95. A specificity index of 95 could have been obtained by modifying the cut-off point of the variables. By increasing the cut-off point in the case of variables TD and IAP and by decreasing that of the variable alveolar chord, we calculated that a sensitivity index of 41 for the third group applies to a specificity index of 96. In order to obtain these indices, we manipulated the results in a non-random fashion and we therefore consider that they are not acceptable. On considering the mis-classification index obtained (34.29), our diagnostic method appears to be effective. The emphysematous alterations were more remarkable in the animals of the third group, which could lead one to consider that the lung deterioration continued, despite the fact that the animals were withdrawn from exposure. However, studies performed on humans (Dockery et al., 1988; Xu et al., 1992) and on guinea pigs (Wright and Sun, 1994) are unanimous in considering that smoking cessation appears to stop the aggression of emphysema.

All the animals which were exposed to cigarette smoke were subjected to the risk of developing emphysema. The confidence intervals of the attributable and relative risks obtained in the two groups of manipulated animals are related and the $\chi^2$ proved to be significant when the two groups of animals were compared. Our results would confirm the hypothesis of the study: age is a risk factor in developing emphysema. The experimental model presented in this study is not comparable with what occurs in the human. We have developed a model of exposure to tobacco smoke in middle-aged rats in the belief that this could increase the possibility of demonstrating the hypothesis, since with age, the elastic capacities of the individual decrease and are more sensitive to the aggressions of the environment. However, this research should be completed by extending the study to include younger animals. While this would almost certainly be more laborious, the experimental results would be closer to the situations found in the human clinic.

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