**EXPERIMENTAL approaches**

**Differential Response in the Male and Female Tracheal Epithelium following Exposure to Tobacco Smoke**

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Histochemical quantification of goblet cells and measurements of the epithelial thickness were made in the trachea of male and female rats exposed to fresh whole tobacco smoke for 30 consecutive days. Exposure to tobacco smoke resulted in an increase in the total goblet cell count in both sexes, with the female rats differentially responding to a greater degree than the male rats. There was a shift from PAS-positive to alcian blue-positive goblet cells, an increase in the number of both "large" and "small" goblet cells, and an increase in the epithelial thickness. PAS-positive "small" cells, the major glycoprotein-reactive cell in the rat tracheal epithelium, increased in the female rats but showed no increase in the male rats. On the other hand, male rats contained a substantially higher proportion of alcian blue-positive cells than did female rats, both before and after exposure to the smoke. The significance of these observations in relation to the prevalence of chronic bronchitis in human smokers is discussed.

**Epidemiologic studies of respiratory diseases in humans indicate that the morbidity and mortality associated with chronic bronchitis and lung cancer are usually several times higher in men than women.** These differences mostly have been explained by differences in the exposure of men and women to potential environmental toxins, especially tobacco cigarette smoke. However, there is some evidence that the surface epithelium of the female human airway, including that of the nose, mouth, pharynx, and trachea, differs from that of the male airway and may vary as a function of the menstrual cycle. We have previously demonstrated that the tracheal epithelium (including the numbers of goblet cells and the epithelial thickness) differs in normal male and female rats and varies in the female rat during the estrous cycle. In an attempt to determine if such sexual differences in the morphologic structure of the tracheal epithelium could modify the potentially adverse effects caused by inhalation of tobacco smoke, histochemical quantification of the level of glycoproteins in the goblet cells and measurements of epithelial thickness were performed in sections of the trachea of male and female rats following identical experimental exposure to tobacco smoke.

**MATERIALS AND METHODS**

Adult rats of the CD strain (Charles River Breeding Laboratories, Inc.), consisting of 18 smoke-exposed and 18 unexposed age-matched male rats and 24 smoke-exposed and 29 unexposed age-matched female rats, were studied. The female rats were chosen from animals that had a normal estrous cycle, as determined by examination of vaginal smears each morning for at least four consecutive days and again at the time of killing. In both the smoke-exposed and the control groups, roughly half of the female animals studied were at the estrous phase at the time of killing, and the other half of the female rats were at the diestrus phase. Experimental exposure to tobacco smoke resulted in the cessation of estrous cycling in some female animals, and these were excluded from our analysis. Rats weighed approximately 110 gm at the start of the experiment.

Smoke-treated animals were exposed three times each day for ten minutes at each exposure for a total of 30 ± 1 consecutive days to fresh whole smoke from standard 2R1 Kentucky reference unfiltered cigarettes. The smoke was generated in a multiportal smoking machine, which was adjusted to generate smoke with a square-wave puff-profile of a two-second duration per puff and a 35-mI volume per puff, as recommended by the report of Hunter's committee. On the average, cigarettes were smoked to a butt length of approximately 23 mm. The fresh smoke aerosol was stabilized by an immediate 1:9 dilution in fresh air and was delivered within two seconds to the animals. In experiments using an identical schedule of exposure, cigarettes were labeled with...
an organic chlorinated hydrocarbon, decachlorobiphenyl, and the amount of smoke actually retained in the lungs of each animal was quantified. With decachlorobiphenyl as a tracer of the particulates in the inhaled smoke, exposures in our study were adjusted to deliver an amount comparable in man to approximately 1k packs of unfiltered cigarettes per day. Animals were killed within (but not before) 18 to 26 hours of the last exposure. The details of our system of exposure to smoke have been published separately.

Rats were anesthetized with intraperitoneal administration of pentobarbital sodium (50 mg/kg of body weight) and were exsanguinated by transection of the abdominal aorta. The trachea of each animal was fixed in situ by infusion with a mixture of a 4 percent solution of formaldehyde and a 2.5 percent solution of glutaraldehyde in 0.09 M phosphate buffer at a pH of 7.4, as described previously. Longitudinal 4μ-thick sections of the trachea were cut from blocks of paraffin and were stained with a combination of alcian blue (pH 2.5) and PAS for counting goblet cells and with Weigert's hematoxylin-eosin for measurement of epithelial thickness. Quantitative analyses were performed on one section for each procedure.

Goblet cells in each trachea were counted continuously for 50 consecutive fields under the oil-immersion microscope, each field being approximately 0.18 mm in length. Data for goblet cell counts were expressed as the absolute number of cells for the accumulative 9 mm of epithelium. This alcian blue-PAS technique allowed identification of granules of acid glycoprotein as alcian blue-positive and those of neutral glycoprotein as PAS-positive. We classified the four types of goblet cells according to the system of Jones et al., i.e., a "small" or "large" PAS-positive cell and a "small" or "large" alcian blue-positive cell. A "small" cell contained granules only at its apex, and a "large" cell was filled with granules above the level of the nucleus, with the total area of granules reaching a size of 50 sqμ or greater. Any cell with a positive reaction for glycoproteins was termed a goblet cell. Any cell that contained only a few granules was excluded from our counting.

Epithelial thickness was measured with an eyepiece graticule as the vertical distance between the basement membrane and the epithelial surface, excluding cilia. Ten measurements were made over the plates of cartilage and an additional ten measurements between the plates of cartilage, and a mean epithelial thickness was calculated for each animal. All rats were examined microscopically to ensure that lymphocyte infiltration in the tracheal wall was minimal.

Statistical analysis of the data was performed using a one-sided Student's t-test, in which the degrees of freedom were adjusted to compensate for possibly unequal population variances. All data are expressed as the mean ± 1 SE.

**Results**

The goblet cell counts of the tracheal epithelium of control and smoke-exposed male and female rats are shown in Table 1, in which the number of goblet cells counted per 9 mm of tracheal epithelium is given for each type. In the trachea of both male and female control rats, PAS-positive "small" cells constituted the majority of the goblet cells observed, accounting for 91 percent of the stained goblet cells in the female rats and 67 percent of the stained goblet cells in the male rats. The unexposed female epithelium had a higher total count of PAS-stained goblet cells than did the unexposed male epithelium (123 cells in female rats vs 109 cells in male rats) but had a lower total count of alcian blue-positive cells than found in the male epithelium (11 cells in female rats vs 49 cells in male rats). Both PAS-positive and alcian blue-positive "large" cells with a typical goblet shape were few in the unexposed male epithelium and virtually negligible in the unexposed female epithelium.

In preparations stained with hematoxylin-eosin, no appreciable qualitative histologic changes, such as squamous metaplasia or dysplasia, were discernible in the tracheal epithelium of the smoke-exposed male and female rats. In smoke-exposed rats the total goblet cell count increased by 62 percent in the female rats (133 to 215 cells) and by 13 percent in the male rats (158 to 178 cells). As a result, the smoke-exposed female rats had a 21 percent higher goblet cell count than did the smoke-exposed male rats.

Table 1—Goblet Cell Counts and Thickness of Tracheal Epithelium and Body Weights in Control and Smoke-Exposed Rats*

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Male Rats</th>
<th>Female Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Smoke-Exposed</td>
</tr>
<tr>
<td>Goblet cell counts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcian blue-positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large cells</td>
<td>6 ± 2</td>
<td>11 ± 2*</td>
</tr>
<tr>
<td>Small cells</td>
<td>43 ± 8</td>
<td>66 ± 8*</td>
</tr>
<tr>
<td>PAS-positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large cells</td>
<td>2 ± 1</td>
<td>5 ± 1*</td>
</tr>
<tr>
<td>Small cells</td>
<td>107 ± 16</td>
<td>97 ± 13</td>
</tr>
<tr>
<td>Total goblet cells</td>
<td>158 ± 14</td>
<td>178 ± 14</td>
</tr>
<tr>
<td>Epithelial thickness, μ</td>
<td>7.91 ± 0.26</td>
<td>8.69 ± 0.27*</td>
</tr>
<tr>
<td>Body weight, gm</td>
<td>340 ± 6</td>
<td>206 ± 6</td>
</tr>
</tbody>
</table>

*Table values are means ± SE.
**Significant difference from smoke-exposed to control (P < 0.05).
The present study indicates that the exposure of rats to tobacco smoke results in (1) an increase in the density of goblet cells in both sexes, (2) a shift in both sexes in tracheal goblet cell mucous production from PAS-positive to the production of alcian blue-positive glycoprotein, and (3) an increase in the thickness of the tracheal epithelium. These results have largely confirmed the observations reported earlier by other investigators.\textsuperscript{17,18}

The importance of our studies is the demonstration that the response of the tracheal epithelium to exposure to smoke quantitatively differed in the male and female rats. Female animals responded more than males through an increase in the number of each type of goblet cell, resulting in a larger overall increase in their total goblet cell count. A striking difference in the response between the two sexes is that PAS-positive “small” cells increased significantly (P < 0.02) in the female smoke-exposed rats but showed no increase in the male smoke-exposed rats.

We have previously demonstrated that PAS-positive “small” cells occurred not only more frequently in the tracheal epithelium of normal female rats than in normal male rats, but also fluctuated significantly in number during the estrous cycle.\textsuperscript{11} Proestrous and estrous rats contained more of these cells than did diestrous animals. Apparently the trachea of the female rat is capable of producing more of these

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**Discussion**

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cells than is that of the male rat under normal conditions, as well as in response to exposure to tobacco smoke. Whether "femaleness" is responsible for production of more PAS-positive glycoproteins in the goblet cells (and, conversely, whether "maleness" is responsible for greater production of alcian blue-positive glycoproteins) is speculative at this point. These aspects of hormonal or other internal factors in the control of the goblet cell population and its glycoproteins remain poorly understood and will be a subject of future study in our laboratory.

A higher proportion of alcian blue-positive cells17 and a greater thickness of tracheal epithelium18 are possible anatomic features which, after exposure to the potentially adverse effects of tobacco smoke, may be important factors in the pathogenesis of chronic bronchitis. Thus, male characteristics of the tracheal epithelium would potentially increase these effects. A recent epidemiologic study has shown that although both men and women showed a linear increase in the prevalence of chronic bronchitis with increased smoking, the risk of chronic bronchitis was greater for men than women in all smoking categories.22 This study also suggested that the excess risk in men did not appear to be due to a difference in consumption of cigarettes and that there existed borderline significance for the association of "maleness" with chronic bronchitis.23 Furthermore, there is some evidence that an androgenic factor also may be significant in the development of bronchogenic carcinoma,24 and its importance relative to our observations deserves further clarification.

It remains to be seen whether these reported sexual differences in the morphologic response of the tracheal epithelium to experimental exposures to smoke also exist in humans and whether the differences contribute to the differential risk of respiratory disease following tobacco smoking in men and women.

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