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Cell Distribution in Tracheal Surface Epithelium and the Effects of Long-Term Pilocarpine and Atropine Administration

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ABSTRACT Cell distribution and the effects of 12 daily injections of 80 mg/kg pilocarpine or 5 mg/kg atropine were studied in rat tracheal epithelium. Ciliated, periodic-acid-Schiff-positive (PAS+), Alcian blue–positive (AB+), nonstaining, and basal cells were counted and their order of occurrence was recorded. Pilocarpine caused a decrease in ciliated and an increase in PAS+, basal, and nonstaining cell numbers. Atropine caused similar changes, although to a much lesser extent. AB+ cells were rare. Cell occurrence was randomized by computer, and comparisons with nonrandomized counts were made to discern between 1) differences in cell arrangement owed to variations in cell numbers, and 2) actual biases in cell distribution. In general, ciliated areas amounted to a few cells and were separated by nonciliated patches of comparable size. The grouping characteristics of cells supported the notion that basal cells were surrounded by their progeny and that daughter cells were displaced by siblings. It was concluded that the cells were not randomly distributed. Basal cells were dispersed, and probably immediately related to PAS+ cells but not to ciliated cells. A bias toward grouping implied concurrent differentiation of clusters of sibling cells. With drug treatment, a substantial increase in PAS+ cells without increase in cell concentration suggested a decrease in ciliated cell differentiation. Larger groups of secretory cells with treatment suggested cell division without differentiation through the basal cell pathway. Cholinergic agents were not the predominant modulators of this epithelium, and their effect was probably secondary to influence over mucociliary function.

The upper respiratory tract is lined by a mixed epithelium composed of basal, ciliated, and nonciliated cells. Cells that secrete mucus can be identified with the periodic acid–Schiff and Alcian blue combination, which stains neutral mucus in pink and acid mucus in blue (McCarthy and Reid, 1964). The trachea is mostly ciliated, and the mucus-secreting cells appear arbitrarily distributed and may occur in large patches (Andrews, 1974).

The distribution of cells in this epithelium varies widely and unpredictably, with cells of different types appearing singly or in groups, randomly mixed with those of other types (Marin et al., 1979). It has not been shown, however, that the cells are randomly distributed. Ciliated cells are arranged in irregular groups interspersed among numerous nonciliated cells at all levels of the trachea (Greenwood and Holland, 1972). It has been noted that the occurrence of regional variations in epithelial organization along the pars membranacea indicates that secretory cells cannot be considered randomly distributed throughout the trachea (Wilson et al., 1984). Further, there is evidence of bias in the distribution of at least one cell type in the rat nasal cavity, as brush cells reportedly occur individually at the junction of three or more cells but never between only two adjacent cells, usually between nonciliated cells, but not adjacent to goblets (Popp and Martin, 1984). There is no previous report of a systematic study of near-neighbor and grouping patterns of the cell types in the respiratory tract.

The cells of the tracheal epithelium migrate from basal to superficial portions and are lost after a life span of 6–7 days (Shorter et al., 1966). There is evidence that a basal cell divides to form one basal and one superficial cell and that a superficial cell divides to form two superficial cells (Blenkinsopp, 1967). Thus, the cell renewal system has a component of cell proliferation with minimal differentiation, and a component of maturation or differentiation in which some cells divide and others become terminally differentiated (Boren and Paradise, 1978). Since there is a pattern in cell renewal it is probable that the arrangement of cells in this epithelium is not random.

The secretory activity of the surface epithelium is regulated by factors that include short-term control over cell synthesis and discharge and long-term modulation of the balance of cell types within the population (Reid et al., 1983). Short-term cholinergic stimulation does not have a direct effect on goblet cells

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(Florey et al., 1932) but it enhances respiratory tract fluid production by increasing ion flux (Widdicombe and Welsh, 1980) and causes a powerful dose-dependent increase in mucociliary waves (Hybbinette and Mercke, 1982). In the rabbit, which lacks submucosal glands, pilocarpine causes changes in the pH of tracheal mucus which are susceptible to block by atropine (Gatto, 1985). Pilocarpine also causes mucous granules from goblet cells to be extruded and converted into surface mucus faster (Horstmann et al., 1975), but it has not been shown that this is a direct action of the drug.

Long-term cholinergic stimulation with 12 daily injections of pilocarpine causes an increase in the volume of intracellular secretion and number of goblet cells of all types (Sturgess and Reid, 1973), as well as an increase in the activity and mitotic index of all histochemical types of goblet cell in the rat trachea (Bolduc and Reid, 1978). A preliminary study of the rabbit trachea under comparable conditions showed an increase in the number of acidic mucus-secreting cells of the surface epithelium (Gatto and Amberger, 1979). The mechanisms suspected of modulating shifts in cell populations have been reviewed (Reid et al., 1983) and should justify to some extent the arrangement of the cells in a given treatment condition.

The objective of this study was to determine the pattern of cell distribution along the tracheal surface epithelium, in an effort to unveil spatial relationships of essence to mucociliary function. Further, such relationships could contribute indirectly to the current understanding of cell renewal and modulation in this tissue. Near-neighbor and grouping patterns in control rats were compared with those in randomized models to uncover biases in cell arrangement. Comparisons with animals subjected to long-term administration of pilocarpine were conducted to determine changes in cell arrangement associated with shifts in the cell population. Within the pilocarpine animals, comparisons with the randomized were made to ascertain changes in arrangement associated only with variations in the numbers of cells of each type. Comparisons with animals receiving only atropine were made to determine if cholinergic agents were normal modulators of the cell population constituting this epithelium.

MATERIALS AND METHODS

Drug Treatment

Adult female Wistar rats (180–250 g) were maintained for 1 month in isolation cages (Germfree Laboratories, Inc.) with free access to Purina rat chow and water. During the last 12 days of that period, the animals received one daily intraperitoneal injection as follows: six rats received 80 mg/kg pilocarpine nitrate (Sigma), another six rats received 5 mg/kg atropine sulfate (Sigma), and an additional six rats served as control and received 1.0 ml saline.

Histological Examination

On the day following the last injection, the animals were anesthetized with 50 mg/kg sodium pentobarbital and their tracheas were excised and fixed in 10% phosphate-buffered formalin. The midtracheas were embedded in Paraplast; serial transverse sections made at 7 μm were mounted on sequentially numbered slides. There were 12–15 sections per slide, and 40 slides were made from each animal. The even-numbered slides were stained for mucus with Alcian blue at pH 2.6 and periodic acid–Schiff and counterstained with Weigert's iron hematoxylin to enhance cytological detail. Odd-numbered slides were sometimes pretreated with diastase and compared to adjacent slides to rule out the possibility of mistaking glycogen for mucus. Five cell types were recognized in the surface epithelium by using phase contrast: Ciliated, periodic-acid-Schiff-positive (PAS+), Alcian blue–positive (AB+), nonstaining, and basal. Occasional lymphocytes were discerned from basal cells and were not included in the cell counts.

One section was randomly chosen from each even-numbered slide and a portion of tracheal wall other than pars membranacea was randomly selected at the microscope. An eyepiece micrometer was used at 1,000× to identify a continuous 100 μm length of epithelium from which a cell count was obtained. The cells were identified and recorded according to their order of occurrence from one end of the epithelial length to the other; thus, each cell count yielded a tally as well as the sequence in which the cells were arranged along the plane of the section. Twenty cell counts were made per animal, 120 per treatment condition.

Analysis of Cell Counts

The cell counts were recorded by computer and analyzed according to cell type for near-neighbor and grouping occurrences by using programs written by the author. Near-neighbor analyses per cell count compared adjoining cells and tallied the frequency with which the cell types occurred next to each other. Cell groups were defined as two or more adjoining cells of the same type, but groups extending beyond the edge of the epithelial length were disregarded. The groups were tallied per count according to cell type and number of cells. In addition each cell count was randomized: that is, within the count each cell was assigned an ordinal number derived from a random table and relocated accordingly. Three randomized versions of all counts were generated by repeating this process. The randomized counts were analyzed for near-neighbor and grouping patterns and were compared to each other and to their nonrandomized counterparts. Average values were expressed as means ± standard deviation of the sample. Differences in mean values were examined with one-way ANOVA or with Student's *t*-test. Significance was determined at the .05 level.

RESULTS

Cell Numbers

The average number of cells reaching the lumen per 100 μm of surface epithelium showed no significant differences between treatment conditions and was 18.8 ± 1.7 in the controls, 18.8 ± 2.2 in the pilocarpine group, and 19.0 ± 2.1 in the atropine group. However, when basal cells were included in the analysis (Table 1), the total cell number per 100 μm of epithelium was significantly greater in the pilocarpine group.

Both drug treatments were followed by a significant decrease in the number of ciliated cells and a significant increase in the number of PAS+ cells (Table 1).

TABLE 1. Number of cells per 100 μm of surface epithelium (values are mean ± SD; N = 120)

	Control	Pilocarpine	Atropine
Ciliated	10.2 ± 2.8[1]	6.3 ± 3.2[1]	9.0 ± 3.0[1]
PAS+	8.2 ± 2.6[1]	11.9 ± 3.0[1]	9.5 ± 2.7[1]
AB+	0.2 ± 0.6	0.2 ± 0.5	0.3 ± 0.5
Nonstaining	0.2 ± 0.4	0.4 ± 0.6[1]	0.3 ± 0.4
Basal	3.2 ± 1.6	5.6 ± 1.8[1]	3.4 ± 1.7
Total cell No.	22.0 ± 2.3	24.4 ± 3.0[1]	22.3 ± 2.7

[1]Significantly different from corresponding value in the other two treatment conditions.

TABLE 2. Frequency of the most common near-neighbor occurrences per 100 μm of surface epithelium (values are mean ± SD; N = 120)

Adjacent cells	Control	Pilocarpine	Atropine
Ciliated—ciliated	7.47 ± 3.54[1]	2.95 ± 2.90[1]	5.91 ± 3.70[1]
PAS+—PAS+	4.54 ± 2.99[1]	8.40 ± 3.88[1]	5.58 ± 3.21[1]
PAS+—ciliated	6.30 ± 2.41	4.77 ± 2.61[1]	6.91 ± 2.78
Basal—PAS+	3.42 ± 2.03	7.13 ± 3.07[1]	3.65 ± 2.05
Basal—ciliated	2.73 ± 1.86	3.02 ± 2.33	2.64 ± 2.01
Basal—basal	0.08 ± 0.40	0.60 ± 1.15[1]	0.24 ± 0.70

[1]Significantly different from corresponding value in the other two treatment conditions.

These changes were most marked in the pilocarpine group, where there was also a significant increase in the number of basal cells. AB+ and nonstaining cells were rare. Within each treatment condition the frequency of occurrence of any cell type followed a normal distribution and did not differ significantly among animals.

Near-Neighbor Occurrences

Ciliated and PAS+ were the most common cell types and appeared next to each other with the greatest frequency (Table 2). The frequency of occurrence of basal cells next to ciliated cells was the only value in Table 2 that did not show change across treatments.

The differences in frequency of near-neighbor occurrences between the treatment conditions were caused by shifts in cell numbers and not by another type of treatment-related bias in the arrangement of cells along the epithelium. This was demonstrated in Table 3, where a randomized version of the cell counts showed significant differences between treatments that were similar to those for corresponding values in the nonrandomized counts presented in Table 2. The same occurred in each of the other two randomized versions of the counts.

Comparisons of near-neighbor occurrences showed that the randomized had significantly less basal cells occurring next to PAS+ cells and more basal cells next to each other, regardless of treatment condition. This was shown in Table 3, where the average for each near-neighbor occurrence was compared with the corresponding average from Table 2. One other significant difference presented in Table 3 was that, in the control, the average occurrence of ciliated next to PAS+ cells was greater in the randomized counts.

TABLE 3. Frequency of the most common near-neighbor occurrences per 100 μm of surface epithelium in a randomized version of the cell counts (values are mean ± SD;[1] N = 120)

Adjacent cells	Control	Pilocarpine	Atropine
Ciliated—ciliated	6.90 ± 3.46	2.88 ± 2.96	5.31 ± 3.61
PAS+—PAS+	4.69 ± 2.88	8.48 ± 3.66	5.99 ± 3.27
PAS+—ciliated	7.13 ± 2.18[2]	5.36 ± 2.29	7.12 ± 2.40
Basal—PAS+	2.44 ± 1.66[2]	5.52 ± 2.73[2]	2.73 ± 1.97[2]
Basal—ciliated	2.67 ± 1.64	2.96 ± 2.13	2.82 ± 1.97
Basal—basal	0.85 ± 1.31[2]	1.89 ± 1.83[2]	0.82 ± 1.28[2]

[1]Significance of differences between treatment conditions is same as those marked in Table 2.
[2]Significantly different from corresponding value in Table 2.

Cell Groups

The numbers of groups of cells of the same type did not differ significantly within each treatment and averaged 4 ± 1 per epithelial length in the control and atropine animals, while pilocarpine was followed by a significant decrease to 3 ± 1. The same occurred in the randomized counts, indicating that the decrease in number of groups in the pilocarpine animals was associated with shifts in cell numbers and not to a specific bias in the arrangement of the cells along the epithelium.

All the cell groups were either ciliated or PAS+, with the exception of two groups of AB+ cells. The number of groups of ciliated cells averaged 2 ± 1 per epithelial length in the control and atropine animals and showed a significant decrease to 1 ± 1 with pilocarpine. The average number of PAS+ groups was 2 ± 1 in all treatments, and due to variations in decimal places it was significantly greater with pilocarpine than in the control. Significant differences between treatments in the randomized counts paralleled those presented above, indicating that the occurrence of groups of cells of a given type was related to the total number of cells of that type.

A comparison of occurrence of cells within groups vs. outside of groups (Table 4) showed a bias toward grouping, as the number of cells found within groups was significantly greater than in the randomized counts for ciliated cells in the control and atropine animals, as well as for PAS+ cells in the animals receiving pilocarpine.

Cell groups were stratified by size according to number of cells, and each size within a treatment showed a normal frequency distribution. Approximately one-half of all the ciliated groups consisted of two cells, although some were as large as 20 cells in the control (Fig. 1a). The decrease in the number of ciliated cells that followed pilocarpine was reflected by a significantly lesser number of ciliated groups of all sizes (Table 5). The rise in PAS+ cells with pilocarpine was associated with an increased occurrence of larger PAS+ groups, as shown in Figure 1b, and this increase was significant (Table 5) for groups larger than three cells.

Cell groups were consistently of greater size in the non-randomized counts. The maximum number of cells in ciliated groups were 20 in the control, nine in pilocarpine, and ten in atropine; corresponding values in the randomized were 11, six, and ten. The largest

TABLE 4. Occurrence of cells within groups vs. outside of groups per 100μm of surface epithelium and comparison with a randomized version of the counts (values are mean ± SD; N = 120)

	Control		Pilocarpine		Atropine	
	In	Out	In	Out	In	Out
Ciliated	7 ± 4[1]	3 ± 2	3 ± 3	3 ± 2	6 ± 4[1]	3 ± 2
Cil., random	6 ± 3[2]	4 ± 2[2]	3 ± 3	3 ± 2	5 ± 4	4 ± 2[2]
PAS+	4 ± 3	4 ± 2	8 ± 4[1]	3 ± 3	5 ± 3[1]	4 ± 2
PAS+, random	4 ± 3	4 ± 2	7 ± 4[2]	5 ± 4[2]	5 ± 3	4 ± 3

[1]Significantly greater within groups than outside groups for this cell type and treatment condition. Also applies to the randomized.
[2]Significantly different from corresponding value in the non-randomized.

PAS+ groups had eight cells in the control, 16 in pilocarpine, and 12 in atropine; corresponding values in the randomized were seven, ten, and nine.

It was noted that the occurrence of large groups may have been somewhat underestimated as a consequence of the methodology followed in the identification of cell groups in the counts, since groups extending beyond the edge of the epithelial length could not be considered because of their unknown size. Given the average of 19 cells per epithelial length, it was possible that ciliated groups larger than the maximum reported above were not recognized as groups. For this same reason, the number of cells occurring outside groups may have been overestimated. The natural tendency of the cells in this tissue to occur in groups was, nonetheless, of statistical significance.

DISCUSSION

Ciliated cells were predominant in the control rats, as reported elsewhere (Andrews, 1974). Most of the mucus-secreting cells featured neutral glycoprotein and stained only with PAS, while some others also contained acid glycoprotein and stained with AB. There were fewer AB+ cells here than in other reports on rats (Hayashi et al., 1978; Jones et al., 1973; McCarthy and Reid, 1964) or wild-caught mice (Gatto and Aiello, 1981). This may have reflected differences in environmental conditions, since the number of AB+ surface epithelial cells is subject to change with exposure to irritants (Jones et al., 1973). Cells in the category of nonstaining were infrequent and their numbers were similar to those reported for brush cells in the mouse (Pack et al., 1981) and rat (Jeffery and Reid, 1975). There were no zonal differences in cell concentration, in agreement with a previous study (Gatto and Houck, 1985).

The number of basal cells in the control rats was similar to that found in the hamster trachea (Boren and Paradise, 1978; McDowell et al., 1983), although this was double the number reported for the mouse (Pack et al., 1981) and half that for the pathogen-free rat (Jeffery and Reid, 1975). Discrepancies among reports of basal cell numbers may be explained in part by the suggestion made elsewhere (McDowell et al., 1983) that basal cells may be systematically confused with the basilar portions of differentiated secretory cells.

Treatment was followed by histological changes that were statistically significant in spite of interanimal variability that might have been due to changes in hormone levels during the estrous cycle (Hayashi et al., 1978). The increase in the number of mucus-secreting cells that followed the chronic administration of pilocarpine was in general agreement with previous reports. However, there were no signs of cell hypertrophy as was reported in the rat (Sturgess and Reid, 1973), and the number of cells reaching the lumen per unit length of epithelium did not change significantly, as it did in the rabbit (Gatto and Amberger, 1979). Dissimilarities with previous reports may have been due to differences between cell populations; the increase presented here occurred exclusively among PAS+ cells, while in the cited studies there was an increase in the number of AB+ cells, even when conducted under germ-free conditions (Sturgess and Reid, 1973).

The decrease in the number of ciliated cells with pilocarpine has not been reported before. It was no reason to suspect impairment of mucociliary function since ciliated cells are normally fewer in areas of the rat nose (Popp and Martin, 1984), and effective mucus transport activity can occur with 10% of the epithelium being ciliated (Battista et al., 1972).

The effects of long-term administration of atropine on the cell population of this epithelium are reported here for the first time. The only significant changes in cell numbers associated with atropine were an increase in secretory and a decrease in ciliated cells, albeit the pilocarpine effect was five times greater. These findings indicated that cholinergic agents were not normal modulators of this cell population, and it was concluded that the increase in secretory cell numbers associated with pilocarpine was secondary to its direct effect on mucociliary function.

It has been suggested that epithelial changes such as those following pilocarpine may involve the transformation of one type of secretory cell to another and metaplasia of nonsecretory cells to secretory cells, with or without increase in cell concentration by cell division (Reid et al., 1983). Ciliated cells arise from secretory cells and not from basal cells (Boren and Paradise, 1978), and this cytodifferentiation occurs independently of cell proliferation (Otani et al., 1986). Shifts between secretory cell types seemed unlikely because the cells that stained for mucus did not differ in size or in staining characteristics between treatment conditions. A shift of superficial cells from nonsecretory to secretory would involve nonstaining cells representing a component of proliferation with minimal differentiation, but the number of nonstaining cells was not sufficient to account for all the additional secretory cells following treatment. Given the current understanding of the mechanisms for development and maintenance of this epithelium, a decrease in the rate of ciliated cell differentiation emerged as the most suitable explanation for the substantial increase in PAS+ cells without change in the concentration of cells reaching the lumen.

Observations concerning near-neighbor patterns and grouping of the cells in this epithelium are reported here for the first time. The most common cell types appeared next to each other the most often, and variations in near-neighbor occurrences could be explained by the shifts in cell numbers that marked each treatment condition. However, comparisons with the

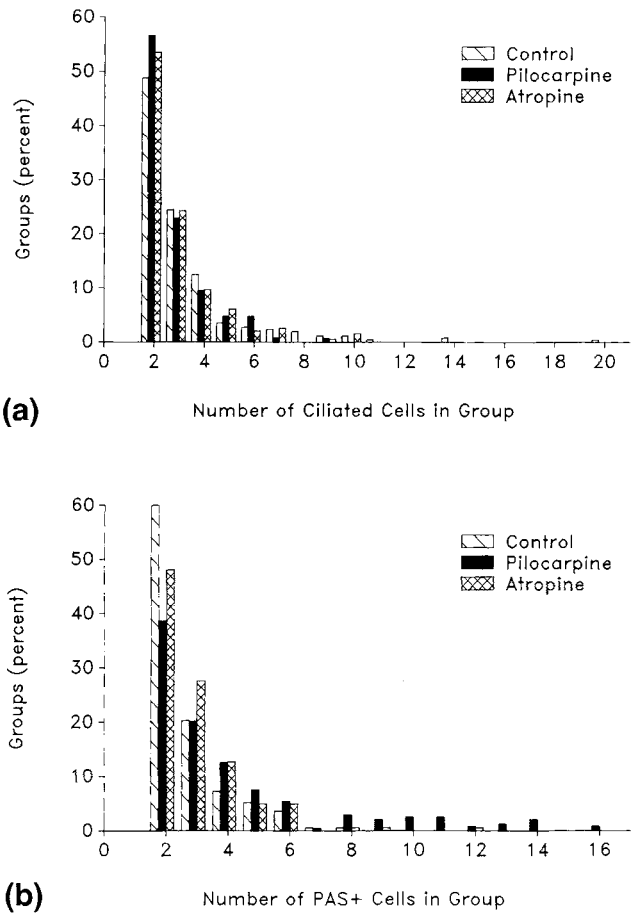

Fig. 1. **a:** Frequency distribution of groups of ciliated cells according to group size in the control (n = 258), pilocarpine (n = 127), and atropine (n = 198) animals. **b:** Frequency distribution of groups of PAS-positive cells according to group size in the control (n = 192), pilocarpine (n = 238), and atropine (n = 181) animals.

TABLE 5. Number of cell groups per 100 μm of surface epithelium (values are mean ± SD; N = 120)

Group size	Control	Pilocarpine	Atropine
Groups of ciliated cells			
2 cells	1.1 ± 1.0	0.6 ± 0.8[1]	1.1 ± 1.0
3 cells	0.5 ± 0.7	0.2 ± 0.4[1]	0.5 ± 0.7
4 cells	0.3 ± 0.5	0.1 ± 0.3[2]	0.2 ± 0.4
All others	0.3 ± 0.5	0.1 ± 0.4[1]	0.3 ± 0.5
Groups of PAS+ cells			
2 cells	1.0 ± 1.0	0.8 ± 0.8	0.9 ± 0.9
3 cells	0.3 ± 0.5	0.4 ± 0.6	0.5 ± 0.7
4 cells	0.1 ± 0.3[1]	0.3 ± 0.5	0.2 ± 0.5
All others	0.2 ± 0.4	0.6 ± 0.6[1]	0.2 ± 0.4

[1]Significantly different from corresponding value in the other two treatment conditions.
[2]Significantly lower than corresponding value in the control.

randomized versions of the counts unveiled specific traits in the arrangement of cells along the epithelium, and interpretation of these findings offered support for some of the proposed mechanisms of cell renewal and response to treatment.

PAS+ secretory cells occurred next to basal cells significantly more often than in the randomized counts in all treatments, in agreement with the possibility that mucous cells arise directly from basal cell division (Boren and Paradise, 1978). Ciliated cells may originate directly from basal cells in response to irritants (Jeffery et al., 1982) but this could not be regarded as the main source of ciliated cells since the occurrence of basal next to ciliated cells was the same as in the randomized and did not vary with treatment condition.

Substantial amplification of cell numbers in this epithelium is believed to take place as secretory cells divide once or twice (Boren and Paradise, 1978). This explained the observation that PAS+ cells in the pilocarpine rats were found mostly within groups and that this occurred to a greater extent than in the randomized counts. The same was true of ciliated cells in the control, supporting the notion that clusters of sibling secretory cells differentiated to form groups of ciliated cells. Drug influence over mucociliary function would have repressed this last step, in agreement with the proposal that ciliogenesis may be inhibited by exogenous factors (Donnelly et al., 1982).

The relative amounts and sizes of ciliated vs. nonciliated patches in a respiratory airway are presented

here for the first time. The grouping of ciliated cells is in agreement with the report that cilia beat in waves that form and fade within regular patches covering up to eight cells (Sanderson and Sleigh, 1981). The largest nonciliated area was relatively small and amounted to 16 cells, although changes in airway length under normal conditions accentuate discontinuities in the ciliary cover (Gatto and Houck, 1988). It has been proposed that transport over nonciliated areas may be owed to the fibrous nature of mucus, with the secretion probably being pulled over these areas (Morgan et al., 1984).

It was concluded that the cells of the tracheal surface epithelium were not randomly distributed. Basal cells appeared to be immediately related to PAS+ cells but not to ciliated cells. A bias toward grouping indicated that the cell renewal mechanism involved the concurrent differentiation of clusters of sibling cells. Drug treatment caused a substantial increase in PAS+ cells without an increase in cell concentration, probably through a decrease in the rate of ciliated cell differentiation. The increase in the occurrence of larger groups of secretory cells that followed pilocarpine suggested that part of the shift in cell population involved cell division without differentiation through the basal cell pathway. Cholinergic agents were not the predominant modulators of this epithelium, and the histological changes that followed drug treatment appeared as secondary to influence over mucociliary function.

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