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Monoclonal antibodies as probes for unique antigens in secretory cells of mixed exocrine organs

(mucous glycoproteins/trachea/exocrine glands/secretion)

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ABSTRACT **In the past, it has been difficult to identify the secretory product and control mechanisms associated with individual cell types making up mixed exocrine organs. This report establishes the feasibility of using immunological methods to characterize both the biochemical constituents and regulatory mechanisms associated with secretory cells in the trachea. Monoclonal antibodies directed against components of tracheal mucus were produced by immunizing mice with dialyzed, desiccated secretions harvested from tracheal organ culture. An immunofluorescence assay revealed that of the total 337 hybridomas screened, 100 produced antibodies recognizing goblet cell granules; 64, gland cell granules; and 3, antigen confined to the ciliated apical surface of the epithelium. The tracheal goblet cell antibody described in this report was strongly cross-reactive with intestinal goblet cells, as well as with a subpopulation of submandibular gland cells, but not with cells of Brunner's glands or the ciliated cell apical membrane. The serous cell antibody was not cross-reactive with goblet, Brunner's gland, or submandibular cells, or the ciliated cell apical membrane. The antibody directed against the apical membrane of ciliated cells did not cross-react with gland or goblet cells or the apical membrane of epithelial cells in the duodenum. Monoclonal antibodies, therefore, represent probes by which products unique to specific cells or parts of cells in the trachea can be distinguished. The antibodies, when used in enzyme immunoassays, can be used to quantitatively monitor secretion by individual cell types under a variety of physiological and pathological conditions. They also provide the means for purification and characterization of cell-specific products by immunoaffinity chromatography.**

The understanding of the regulation of secretion from exocrine organs, such as the pancreas and parotid gland, has been facilitated by the availability of biochemical assays for the secretory product of these glands (i.e., amylase). Using amylase assays, the influence of neurotransmitters, Ca^{2+} and cyclic nucleotides on secretory activity has been assessed (1–3). Other exocrine organs do not contain well-characterized secretory products and, in addition, may be cellularly heterogeneous. Studies of cellular secretory mechanisms in these organs have been handicapped by the lack of biochemical markers for individual cell types. Among such organs are the stomach, duodenum, and trachea.

In the trachea (Fig. 1), goblet, gland, and ciliated epithelial cells generate a mixture of macromolecules, which constitutes ≈5% by weight of the complex tracheal secretion known as "mucus." The composition of mucous glycoproteins has been partially described (4–9). Properties of the secretion including volume (10), sulfur content (11, 12), total protein (10), and molecular weight (13) can be altered by various forms of autonomic nervous system stimulation. *A priori* considerations suggest that these variations may arise by differential effects of autonomic neurotransmitters on serous, mucous, goblet, and ciliated cells. However, it has been difficult to directly analyze either the factors controlling secretion or the secretions themselves on a cell-specific basis, because neither cell-specific markers nor homogeneous isolated cell preparations have yet been developed. (See, however, ref. 14). The purpose of this study was to obtain specific markers for serous, mucous, goblet, and ciliated cell products. We approached this by generating a family of monoclonal antibodies directed against discrete antigenic determinants present in the desiccated secretion. We report here the distribution of these antigens in the trachea, duodenum, and submandibular gland of cat, ferret, and sheep. A preliminary report of some of these findings has been published (15).

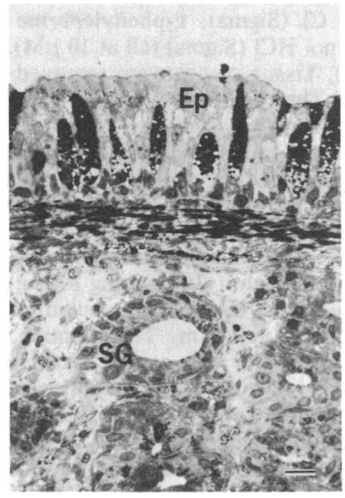

FIG. 1. Transverse section through cat trachea showing relationship of goblet cell-containing epithelium to gland-containing submucosa. Ep, epithelium; SG, submucosal glands. Epon section, 0.5 μm, toluidine blue. (Bar = 10 μm.)

METHODS

Preparation of Immunogen. The extrathoracic region of tracheas was removed from sheep anesthetized with sodium pentobarbital (40 mg/kg). Tracheas were rinsed (4 times) in 500 ml of sterile phosphate-buffered saline (P_i/NaCl), and loose connective tissue was removed by sterile dissection. Tracheas were divided to fit compactly into beakers containing 100 ml of fluid. Tracheal pieces were equilibrated for 30 min in a beaker containing Ham's F-12 nutrient medium, maintained at 37°C, and bubbled with 95% O_2/5% CO_2 in a Dubnoff's metabolic shaker. The tissue pieces were then transferred to a second beaker containing 100 ml of the same medium and the autonomomimetic agonists carbamyl-β-

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Abbreviation: P_i/NaCl, phosphate-buffered saline.

Table 1. Representation of major antibody types in hybridomas obtained from two fusions

Staining site	Positive culture wells,* no.
Submucosal gland cells only	24
Epithelial goblet cells only	60
Submucosal gland cells and epithelial goblet cells	40
Epithelial ciliated apical membranes	3
Total no. of positive wells	127

*337 wells screened.

methylcholine Cl (Sigma), L-phenylephrine HCl (Sigma), and isoproterenol HCl (Sigma) (all at 10 μM), and gentamycin (1 unit/ml). Tissue pieces were removed from the solution after 24 hr. The solution was dialyzed at 4°C (Spectropor tubing; M_r cutoff, ≈13,000 daltons) against 25 vol of P_i/NaCl for 2 hr and concentrated overnight by centrifugal evaporation (Speed Vac). An aliquot of the desiccated material was assayed for protein by the method of Lowry (16), and the remainder was stored at −80°C prior to use.

BALB/c mice were injected intraperitoneally with desiccated secretions (a quantity containing 100 μg of protein) dissolved in 100 μl of sterile P_i/NaCl emulsified with 100 μl of Freund's complete adjuvant. The immune response was stimulated 3 weeks later with an i.p. injection, and 5 weeks later with an i.v. injection of the immunogen.

Fusion. Three days after the i.v. injection, spleens were removed from immunized mice under sterile conditions and homogenized between frosted glass slides. Homogenates were filtered, suspended in P_i/NaCl, and fused with azaguanine-resistant SP2/0 myeloma cells using polyethylene glycol. The pellet containing fused cells was resuspended and plated into 96-well plates containing thymocyte feeder cells, DME H21, 20% fetal calf serum, and hypoxanthine/aminopterin/thymidine.

Immunofluorescence. Tissue sections for screening hybridoma supernatants were obtained by fixing pieces of trachea, intestine, and salivary gland in 0.1 M PO_4 buffer/4% paraformaldehyde, pH 7.4 (2 hr, 4°C). Tissue pieces were cryoprotected by an 18-hr incubation in 30% sucrose/0.1 M PO_4 buffer, pH 7.4. Tissue pieces were then frozen in embedding molds containing OCT compound (Miles). Sections (5 μm thick) were made using a Bright cryostat and melted onto gelatinized glass slides. Slides were briefly rinsed in P_i/NaCl to remove OCT. Hybridoma supernatant was diluted 1:1 with P_i/NaCl containing 2% normal goat serum/0.6% Triton X-100. The diluted supernatant was applied to the sections for 2 hr at room temperature. Sections were rinsed in P_i/NaCl containing 1% normal goat serum/0.3% Triton X-100. They were then incubated with goat anti-mouse IgG-fluorescein isothiocyanate for 30 min at room temperature. Then, they were rinsed in P_i/NaCl and covered with glycerin/P_i/NaCl (3:1) and glass coverslips. Slides were viewed in a Zeiss fluorescence microscope.

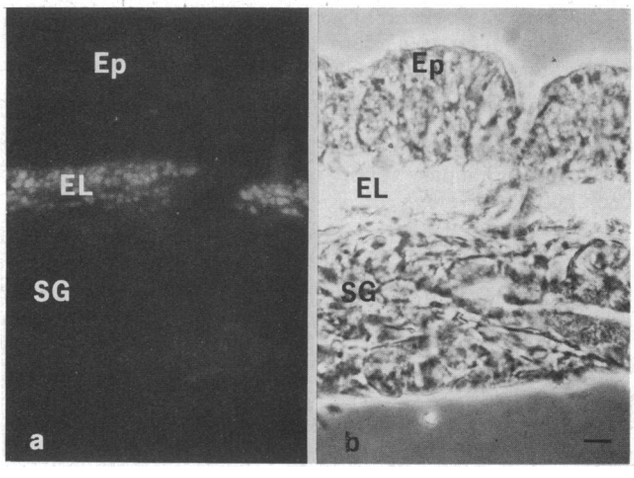

FIG. 2. (*a*) Control fluorescence micrograph showing results of substituting Sp2/0 myeloma supernatant for specific antibody-containing hybridoma supernatant in immunofluorescence protocol. The autofluorescence is related to the elastic lamina (EL) in cat trachea. Ep, epithelium; SG, submucosal glands. (*b*) Same field as in *a*. Phase optics. Elastic lamina is interrupted by a gland duct. (Bar = 10 μm.)

RESULTS

Two fusions yielded 337 cell lines. Supernatant from these cell lines contained three types of antibodies: those directed against constitutents of goblet cell granules, those directed against gland cell granules, and those directed against the ciliated epithelial membrane. Table 1 shows the frequency with which each type of antibody occurred. We describe below the cross-species and cross-tissue specificities for one antibody of each major type.

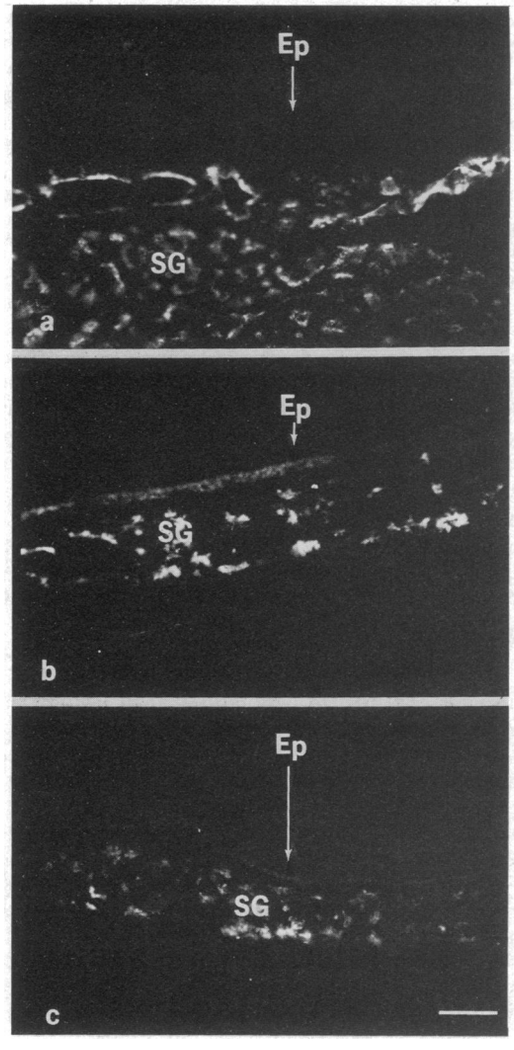

FIG. 3. Fluorescence micrograph showing staining pattern obtained using antibody 5C7 on sheep trachea (*a*), cat trachea (*b*), or ferret trachea (*c*). Glands are stained, epithelium is not stained. Abbreviations are as in Fig. 1. (Bar = 100 μm.)

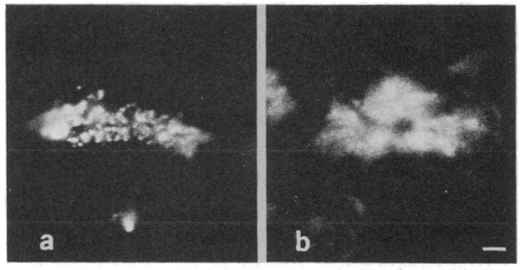

FIG. 4. (*a*) Fluorescence micrograph showing staining pattern obtained using antibody 5C7 on cat trachea. Small punctate deposition of reaction product suggests granules are serous type. (*b*) Using antibody 5C5 on cat trachea. Diffuse staining suggests granules are mucous type. (Bar = 10 μm.)

Controls. Control sections incubated with Sp2/0 rather than hybridoma supernatant showed no fluorescence in the epithelium or submucosa (Fig. 2 *a* and *b*). The elastic lamina below the epithelium was distinctly autofluorescent in cat trachea and less fluorescent in ferret and sheep.

Gland Cell Antibody. Antibody 5C7 stained a subpopulation of gland cells. Fig. 3 shows the staining pattern obtained with this antibody in sections of sheep, cat, and ferret trachea. In all cases, staining was confined to the submucosal glands. Epithelium, including goblet cells and ciliated apical membrane, did not stain. The specificity of antibody 5C7 for serous cells was indicated by the punctate nature of the stain (Fig. 4*a*). More diffuse granular staining, observed using antibodies such as 5C5 (Fig. 4*b*), was tentatively identified as being associated with mucous granules. Complete analysis of this antibody was precluded, because the 5C5 hybridoma stopped producing antibody early in the study. Paired immunofluorescence and phase contrast micrographs of sections stained with antibody 5C7 (Fig. 5) showed that the area occupied by mucous granules (arrows) did not stain. Cross-tissue comparison revealed no staining with 5C7 in intestinal goblet, Brunner's gland, or submandibular gland cells.

Ciliated Cell Surface Antibody. Antibody 1E3 stained the apical portion of the tracheal epithelial cells. Fig. 6 *a*, *c*, and *d*, shows the staining pattern observed in cat, ferret, and sheep trachea. In cat and ferret, staining was confined to the apical surface of ciliated cells and stopped abruptly in the gland duct (Fig. 6 *a* and *b*). In sheep trachea, the pattern was somewhat different. Patches of immunofluorescence were seen both on the apical surface and deeper in the epithelial cell layer (Fig. 6*d*). Cross-tissue comparison revealed no staining on the apical epithelial surface of duodenal epithelium, tracheal or intestinal goblet cells, Brunner's, or the submandibular gland. The localization of this antigen is consistent with its identification as part of the glycoconjugate-containing "surface layer" previously detected using histochemical methods (17).

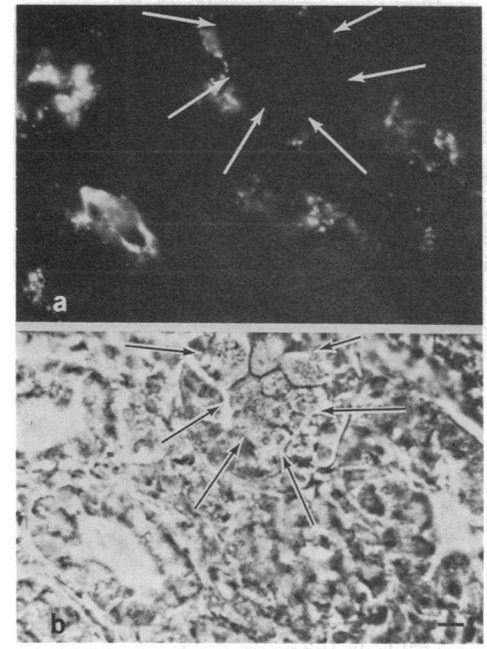

FIG. 5. (*a*) Fluorescence micrograph showing staining pattern obtained using antibody 5C7 (as in Fig. 4*a*). Focal plane was chosen to demonstrate overall distribution of reaction product. Reaction product is not present in area demarcated by arrows. (*b*) Same field as in *a*. Phase optics. Area corresponding to that in *a* (no fluorescence) contains mucous cells. (Bar = 10 μm.)

Goblet Cell Antibody. Antibody 3E9 stained epithelial goblet cells in cat trachea. Fig. 7 shows the staining pattern observed in cat trachea. When the distribution of goblet cells as

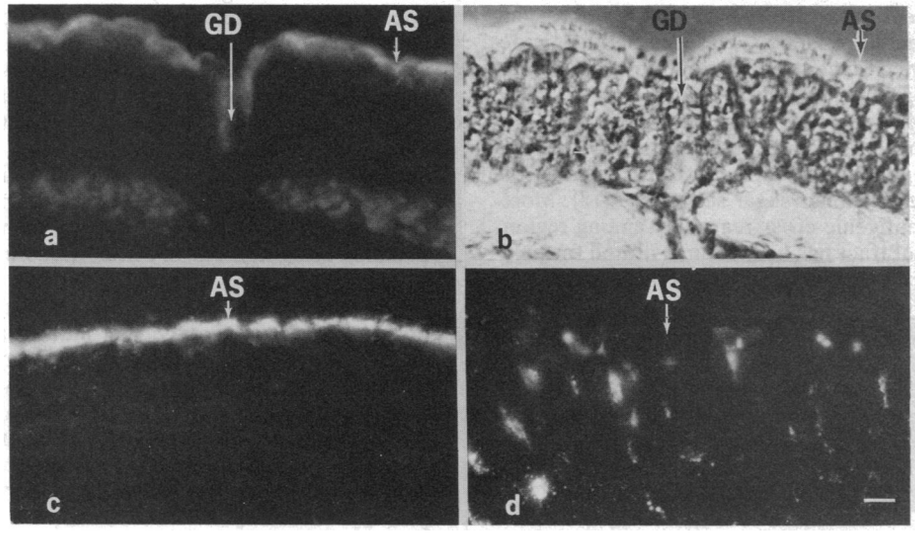

FIG. 6. (*a*) Fluorescence micrograph showing staining pattern obtained using antibody 1E3 on cat trachea. Apical surface (AS) of the epithelium is stained. Fluorescent reaction product follows the contour of the apical surface as it descends to form a gland duct (GD). Fluorescence stops abruptly, apparently at level of cilia (see *b*). (*b*) Same field as in *a*. Phase optics. (*c*) Fluorescence micrograph showing staining pattern obtained using antibody 1E3 on ferret trachea. (*d*) Same as in *c*. Sheep trachea. (Bar = 10 μm.)

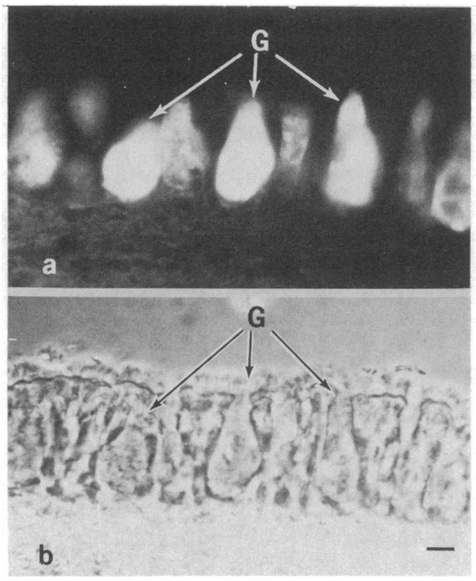

FIG. 7. (*a*) Fluorescence micrograph showing staining pattern obtained using antibody 3E9 on cat trachea. G, goblet cells. Some cells appear more faintly stained, due to their position out of the focal plane of the micrograph. (*b*) Same field as in *a*. Phase optics. (Bar = 10 μm).

seen by phase optics was compared with the distribution of immunofluorescent cells, it was clear that all goblet cells were antigen positive. Goblet cells are rare in ferret trachea, and no staining was seen in this species. Interestingly, in the sheep, the antibody did not stain goblet but, rather, stained gland cells. Cross-tissue comparison revealed positive staining of goblet cells in the duodenal epithelium (Fig. 8). Brunner's gland was devoid of immunofluorescence, but a small subpopulation of acinar cells stained in the submandibular gland.

The cross reactivity of each antibody across species and tissues is summarized in Table 2.

Table 2. Summary of regional staining

	Antibody		
	5C7	1E3	3E9
Trachea			
Cat			
Goblet cell	−	−	+
Gland cell	+	−	−
Ciliated cell	−	+	−
Sheep			
Goblet cell	−	−	−
Gland cell	+	−	+
Ciliated cell	−	+	−
Ferret			
Goblet cell	−	−	−
Gland cell	+	−	−
Ciliated cell	−	+	−
Duodenum			
Cat			
Brunner's gland cell	−	−	−
Goblet cell	−	−	+
Sheep			
Brunner's gland cell	−	−	−
Goblet cell	−	−	−
Ferret			
Brunner's gland cell	−	−	−
Goblet cell	−	−	−
Submandibular			
Cat	−	−	+
Ferret	−	−	−
Sheep	−	−	−

DISCUSSION

Our results show that complex secretions emanating from cellularly heterogeneous organs can be dissected immunologically by the use of monoclonal antibodies. Screening methods can be selected to identify classes of antigenic determinants having specific cellular distributions, physiologic properties, or distinguishing chemical characteristics. In the present study, we chose to screen for and study these antigens confined to single epithelial cell types in the trachea.

The analysis of epithelial glycoproteins has revealed the frequent appearance of similar sugar sequences (18). Moreover, considerable antigenic cross-reactivity among regions of the gastrointestinal tract has been demonstrated for polyclonal antibodies directed against intestinal mucins (19). Despite this, we have identified many monoclonal antibodies recognizing sequences confined to a single epithelial cell type. This may be due to the extremely narrow antigenic specificity of monoclonal as opposed to polyclonal antibodies, or to the distinctive biochemical profiles of individual tracheal cells. We interpret the observed cellular specificity as evidence against the possibility that the antibodies recognize carbohydrates responsible for blood group activity.

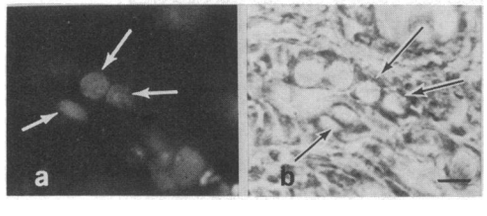

FIG. 8. (*a*) Fluorescence micrograph showing staining pattern obtained using antibody 3E9 on cat duodenum. Arrows indicate reaction product in goblet cells of villar epithelium. (*b*) Same field as in *a*. Phase optics. Arrows indicate goblet cells. (Bar = 10 μm.)

The importance of the present findings lies in the demonstrated feasibility of producing antibodies with specificity for the contents of individual epithelial cells. Those antibodies can now be used for characterizing both the biochemical constituents and regulatory mechanisms associated with glycoconjugate-producing cells in the trachea. Previous studies have yielded measurements of the efflux of tracheal fluid (10, 20) or radiolabeled glycoconjugates (e.g., see refs. 21 and 22) representing the net contribution of gland, goblet, and ciliated cells (23–25) or subpopulations of these cells (26). Conceivably, using these methods, drugs having an excitatory effect on one cell type and an inhibitory effect on another cell type could lead to measurements indistinguishable from spontaneous secretory baselines. That is, results obtained using non-cell-specific measures are difficult to interpret.

Previous work from this laboratory has focused on developing probes for the activity of individual cell types. These include stereological assessment of degranulation in identified cells (27), the use of an assay for lysozyme, a product that originates mainly in a single tracheal cell type (14), and the autoradiographic analysis of neurotransmitter receptor distribution on the various secretory cells (28). Immunochemical methods now offer the opportunity to link morphology, function, and biochemistry for each cell type.

Using monoclonal antibodies, it is clear that the cellular distribution of antigen is conserved across species for some (5C7 and 1E3), but not all (3E9), antigens. For physiological studies, the antibodies must, and do, maintain cellular speci-

ficity among individuals within a given species. Given that, it is possible to carry out parallel studies in which (*i*) the antibody is used to purify and subsequently characterize cell-specific antigens and (*ii*) the antibody can be used in immunoassays to quantitatively monitor the secretory activity of individual cell types. Knowledge of both control mechanisms and contents of the several tracheal secretory cells should provide better understanding of factors producing mucus of different physical properties in health and disease. A similar strategy should be useful in characterizing the control mechanisms and biochemical properties of individual cell types of other mixed exocrine organs.

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