Immunohistochemical Localization of Carbonic Anhydrase I and II in Eccrine Sweat Glands From Control Subjects and Patients With Cystic Fibrosis

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Isozymes of carbonic anhydrase (CA) were localized immunohistochemically by the immunoglobulin-peroxidase bridge technique on fixed paraffin sections of human eccrine sweat glands. Low-activity CA I was identified in the cytoplasm of the myoepithelial cells in the secretory coil and in the luminal and basal cells of both the coiled and straight segments of the duct. High-activity CA II was found in the cytoplasm of clear cells of the secretory coil. Although evidence has suggested that CA activity is altered in cystic fibrosis (CF), the present immunnohistochemical comparison of CF sweat glands revealed a distribution of and, semiquantitatively, a prevalence of CA isozymes identical to those of normal sweat glands. Abnormal enzyme activity cannot be ruled out, however, on the basis of immunocytochemical staining which depends solely on the antigenic properties of CA. (Am J Pathol 1983, 112: 250–257)

CARBONIC ANHYDRASE (CA) (EC 4.2.1.1), which catalyzes the reaction $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$, is thought to regulate a variety of cellular functions such as CO_2 exchange, pH balance, secretion of ions, calcification, and photosynthesis.[1–3] Biochemical analyses have revealed the ubiquitous distribution of the enzyme in mammalian tissues.[1,3–6] CA has been localized histochemically by the cobalt–bicarbonate methods of Haussler[7] and Hansson,[8] which are modifications of the manganese-based method of Kurata.[9] The enzyme has also been visualized by a radioautographic method using a labeled inhibitor.[10,11] Localization of CA by these techniques have been augmented by the immunohistochemical approach, which in addition to its specificity and sensitivity, has the advantage of identifying individual isozymes of CA.[12–20] A survey of rodent[17] and human[20] tissues using the immunoglobulin–peroxidase bridge method evidenced the distribution of CA principally in epithelial cells that generally could be classified as cells involved in fluid and ion transport rather than in the secretion of macromolecules.

The human eccrine sweat gland, an organ devoted principally to fluid and ion transport,[21] carries out several functions—namely, pH regulation[22,23] NH_3 secretion,[24] and K^+ [25] and HCO_3^- [21–23] transport—that have been related to carbonic anhydrase activity in other organs.[1–3] Identifying which isozymes of CA are present and localizing the enzyme to a particular cell type or segment of the sweat gland would contribute to understanding how and where these functions occur in the sweat gland. CA activity has been visualized histochemically in the sweat gland by the CoS precipitation technique,[26,27] but the localizations were not identical. Also, physiologic studies employing an inhibitor of CA activity implicate a role for CA in sweat secretion[28,29] and in the generation of transepithelial pH gradients.[22,23] Questions as to the reliability and specificity of these methods[31–34] make it necessary to localize CA by dependable and specific immunohistochemical methods.

In cystic fibrosis (CF), a fatal genetic disease that alters the function of ion-transporting epithelia,[25,35–38] Na^+, Cl^-, and K^+ concentrations are elevated in the

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sweat.[25] CF sweat glands also fail to establish a transepithelial pH gradient.[22,23] It has been suggested that CA activity may be altered in CF, because acetazolamide reduces the K^+ concentration in CF sweat to normal levels[30] and induces a CF-like abnormality in normal sweat glands by inhibiting the formation of a transepithelial pH gradient.[22,23] An immunohistochemical comparison of normal and CF sweat glands could detect a CF-dependent change in the distribution or relative abundance of CA isozymes.

In this report we describe the immunohistochemical localization of CA I and CA II isozymes in eccrine sweat glands from normal and CF patients. High activity CA II was localized exclusively in the clear cells of the secretory coil. Low activity CA I was identified in both cell types of the coiled (proximal) and straight duct and in the myoepithelial cells of the secretory coil. No difference in the distribution or intensity of immunostaining for either isozyme was observed between normal and CF sweat glands. These results suggest that abnormalities in fluid or ion transport in CF sweat glands cannot be simply explained as an alteration in the type of isozyme present or in the distribution of CA.

Materials and Methods

Tissue

Specimens of skin were obtained from scheduled surgery on 6 control and 4 cystic fibrosis patients (Table 1). A portion of each specimen was fixed in each of the following solutions for 2–5 hours at 23 C: 4% paraformaldehyde in 2% calcium acetate; Bouin's fluid; Carnoy's fluid; and a solution of 6% $HgCl_2$ in 1.3% sodium acetate. After fixation the tissues were dehydrated and embedded in Paraplast.

Preparation of Antiserums

CA I and CA II antigens were isolated by affinity chromatography of human hemolysates on sulfonamide-bound CM-Sephadex columns.[39] Purity was established by starch gel electrophoresis.[17] Antiserum directed against each isozyme was prepared in rabbits as previously described.[40] Specificity of the antisera was tested by the double immunodiffusion method of Ouchterlony.[41] Antiserum against CA I immunoprecipitated CA I but did not cross-react with CA II. Antiserum against CA II immunoprecipitated CA II and cross-reacted slightly with CA I. The isozymes of carbonic anhydrase are often distinguished by capital letter suffixes. However, the designations of CA I and CA II are preferred and employed here in place of CA-B and CA-C for reasons given previously.[42]

Immunohistochemistry

Rehydrated sections 5 μ thick were stained with the immunoglobulin–peroxidase bridge sequence[43] to localize CA I and CA II isozymes. Sections were pretreated for 10 minutes with 3% H_2O_2 and then sequentially incubated in the following: 1:40 normal goat serum, 1:40 to 1:500 rabbit antiserum to either CA I or CA II, 1:80 goat antiserum to rabbit IgG, 1:80 rabbit antiserum to horseradish peroxidase (Cappel Laboratories, Cochranville, Pa), and 0.5 mg/dl horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo). Antigenic sites were visualized by incubating sections for 10 minutes in the diaminobenzidine–H_2O_2 substrate medium for peroxidase[44] prior to dehydration and mounting in Permount. Except where noted, each step was carried out for 30 minutes at 23 C, and the solutions were diluted with phosphate-buffered saline (PBS), pH 7.2.

For control experiments each of the following were substituted for the primary antiserum at equivalent dilutions: normal rabbit serum; rabbit antiserum to CA I preincubated with 1 mg/ml CA I; rabbit antiserum to CA II preincubated either with 1 mg/ml CA II or with both CA I and CA II. These preincubations with antigens were carried out with undiluted antiserum for 18 hours at 4 C.

The intensity, distribution, and sensitivity of immunostaining by the immunoglobulin–peroxidase bridge sequence were compared with results obtained by the peroxidase-antiperoxidase (PAP) technique[45] and the avidin-biotin complex (ABC) method[46] (Vector Laboratories, Burlingame, Calif) using the same immunoreagents, concentrations, and incubation times where appropriate.

Results

Effect of Fixative

Tissues fixed in Carnoy's fluid demonstrated the strongest immunostaining with the lowest nonspecific background staining. Although morphologic features were better preserved with formalin or Bouin's fixative, the antigenicity of CA I and CA II in the tissue was completely masked. Specific immunostaining was evident in sections from B-4 fixed tissue, but nonspecific background coloration was unacceptably high.

Immunostaining of Normal Sweat Glands

Strong immunostaining for CA I was observed in the cytoplasm of luminal cells of the duct (Figures 1 and 2b, e). Specific staining appeared most intense in

Table 1 – Data on 4 Patients With Cystic Fibrosis

CF patient	Age/Race/Sex	Complications	Medication
1	15 years/W/F	Bronchiectasis/hypersplenism	Pancrease
2	17 years/W/F	Chronic obstructive lung disease/pneumonitis	Antibiotics/pancrease
3	21 years/W/M	Congestive heart failure/bronchiectasis/ bronchopneumonia	Steroids/bronchodilators/pancrease
4	5 weeks/W/M	Meconium ileus	–

the basolateral cytoplasm. Variable, light staining was frequently observed outlining the periphery of the basal duct cells (Figure 2e). Staining intensity and distribution appeared similar for both coiled and straight segments of the duct. In the secretory coil only the myoepithelial cells stained for CA I isozyme (Figures 1 and 2b, d). Intense staining occurred when rabbit antiserum to CA I was incubated at dilutions between 1:40 and 1:200. Specific staining was faint but distinguishable at 1:500 dilution of primary antiserum. Optimum ratios of specific to nonspecific staining were achieved with rabbit antiserum at dilutions from 1:100 to 1:200.

Intense immunostaining for CA II was observed in the cytoplasm of clear cells in the secretory coil (Figures 1, 3a, and 4). The predominantly basal position, pyramidal shape, and intermittent location of the immunostained cells served to identify them as clear cells (Figure 4). No staining was noted in the dark cells situated more toward the lumen, or in the peripheral myoepithelial cells of the secretory coil, or in the cells of the duct. Primary antiserum diluted from 1:40 to 1:150 produced intense immunostaining in tissue sections. Specific staining was faint but distinguishable from background staining when 1:400 rabbit antiserum was used. Optimal ratios of specific to nonspecific staining occurred with

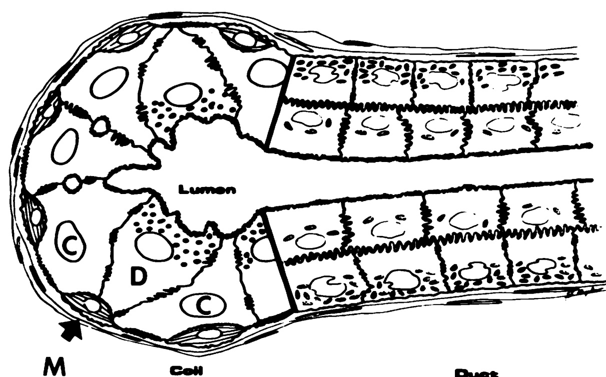

Figure 1 – Diagram of the human eccrine sweat gland. The secretory coil, which elaborates an isotonic precursor fluid, consists of a pseudostratified layer of clear cells (*C*) and dark cells (*D*) surrounded by a discontinuous network of myoepithelial cells (*M*). The duct, which reabsorbs Na^+ and Cl^- to yield a hypotonic sweat, is formed by a double layer of cuboidal cells.

rabbit antiserum at dilutions between 1:100 and 1:150.

For control experiments in which normal rabbit serum was substituted for the primary antiserum in the immunostaining procedure, staining of the sweat gland was very faint for both the secretory coil and the duct (Figure 2a). Sweat glands closest to the surface of the tissue blocks frequently displayed the lowest background staining with the best specific immunostaining. Preabsorption of rabbit anti-CA I with CA I antigen largely blocked immunostaining of cells of the duct and myoepithelial cells. Occasionally, however, some residual staining was observed at the lateral border of the luminal cells in the duct (Figure 2c). Preabsorption of rabbit and anti-CA II with either CA II antigen or with both isozymes reduced staining of the clear cells to slightly above background levels (Figure 3b). Preincubation of rabbit anti-CA I with CA II antigen or of rabbit anti-CA II with CA I antigen had no effect on the intensity or distribution of subsequent immunostaining.

PAP[45] and ABC[46] immunostaining procedures were compared with immunoglobulin-peroxidase bridge method[43] for localizing CA II in tissue sections. The same reagents, concentrations, and incubation times were used for identical steps in these procedures. All three methods produced comparable staining intensity, specificity, and sensitivity. Nonspecific staining was, however, slightly reduced in sections treated by the ABC procedure.

Immunostaining of CF Sweat Glands

Tissue sections containing sweat glands obtained from 4 CF patients were immunostained for CA I and CA II according to procedures outlined for normal sweat glands. There was no apparent difference, compared with normal sweat glands in the distribution, intensity, or specificity of staining for either isozyme.

Discussion

Establishing the presence of carbonic anhydrase (CA) in the human eccrine sweat gland is an essential

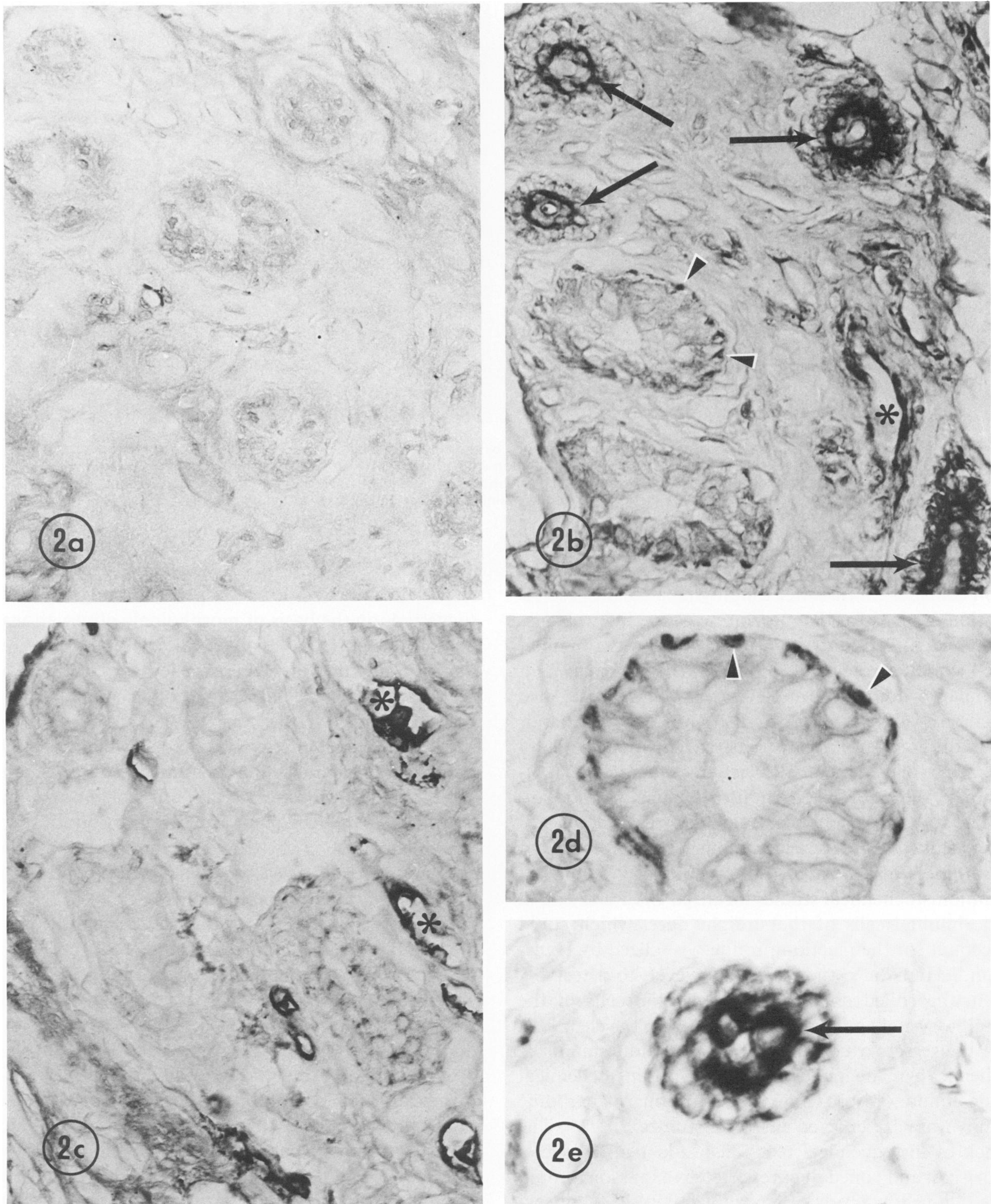

Figure 2—Serial sections of Carnoy-fixed sweat gland immunostained by the immunoglobulin-peroxidase bridge procedure,[43] with a 1:100 dilution of normal rabbit serum (**a**); rabbit anti-CA I (**b**, **d**, **e**); rabbit anti-CA I preabsorbed with 1 mg/ml purified CA I antigen (**c**). Intense immunostaining for CA I was observed in the basolateral cytoplasm of the luminal cells of both the coiled and straight segments of the duct (**b**, **e**: *arrows*) and in the myoepithelial cells of the secretory coil (**b**, **d**: *arrowheads*). Less intense immunostaining was frequently seen outlining the periphery of the basal cells of the duct (**e**). Preabsorption of undiluted antiserum with 1 mg/ml CA I antigen reduced staining to background levels (**c**). Peroxidaselike activity of capillaries (**b**, **c**: *) resulted in nonspecific reaction product (**a**, **b**, **c**, ×400; **d**, ×830; **e**, ×900)

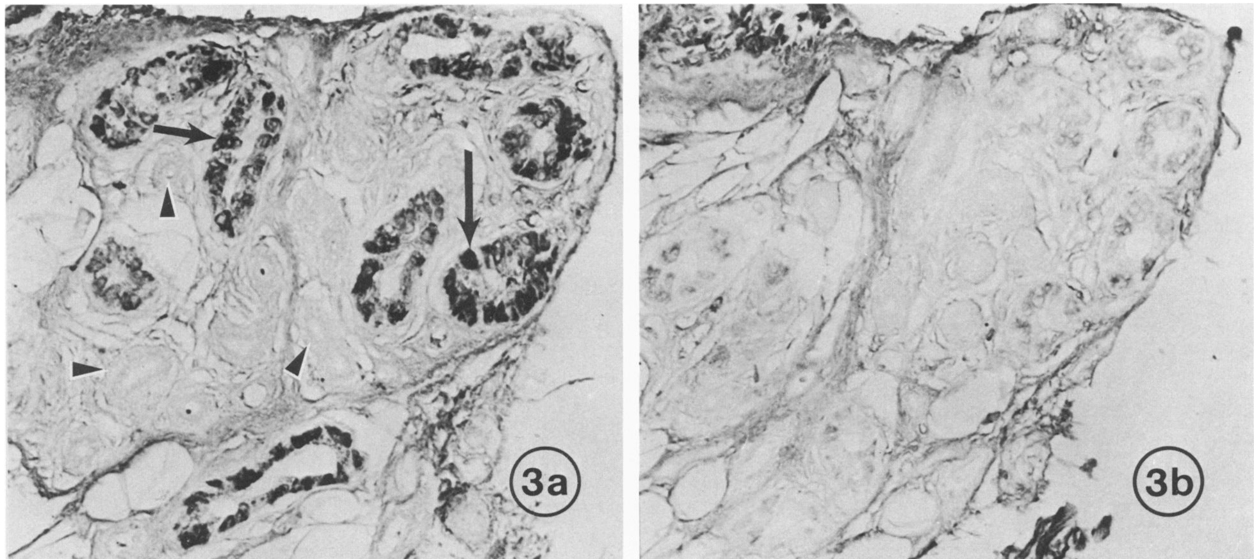

Figure 3—Serial sections of Carnoy-fixed sweat glands immunostained by the immunoglobulin-peroxidase bridge method[43] with a 1:100 dilution of rabbit-anti CA II (**a**) or rabbit anti-CA II preabsorbed prior to dilution with 1 mg/ml CA II antigen (**b**). Only the clear cells (**a**: *arrows*) of the secretory coil were immunostained specifically. No staining was evident in either cell type of the duct (**a**: *arrowheads*). Preabsorption of antiserum with only CA II antigen (**b**) or with both isozymes reduced staining of clear cells nearly to background levels. (×170)

step in understanding the mechanisms of fluid and ion transport in this site. Employing a cobalt precipitation method for demonstrating CA activity, Hansson[27] identified reaction product only in the luminal cells of the straight duct. In an earlier study using a similar procedure Braun-Falco and Rathjens[26] also demonstrated reaction product in the sweat gland, but the localization was generalized, and no gland segment or cell type with activity was defined. Although the metal-trapping histochemical method appears to be specific for carbonic anhydrase activity,[47] the frequency of artifacts diminishes the validity and usefulness of the cobalt salt technique.[32-34] Our immunohistochemical studies localized CA I isozyme in the luminal cells of the straight duct, which could account for the reaction product visualized by Hansson.[27] Hansson's study failed, however, to detect CA I in the coiled duct and myoepithelial cells of the secretory coil and high-activity CA II in the clear cells of the secretory coil that we observed immunohistochemically. The inconsistency between histochemical and immunohistochemical localizations of carbonic anhydrase is reported in other studies.[17,18,20,48] The lack of histochemical reaction product in the secretory coil and coiled duct reported by Hansson[27] could be due to low levels of enzyme activity in these segments of the sweat gland, the loss of enzyme activity during preparation of the tissue, or the less sensitive nature of the cobalt precipitation method.[45,47,49]

Identifying carbonic anhydrase in a cell type or segment of the sweat gland may help resolve speculation on the location of the enzyme's function with respect to fluid and ion transport. Montagna et al[50] suggested, on the basis of morphologic observations, that the clear cell is involved primarily in the secretion of fluid and ions, whereas the dark cell contributes macromolecules to the precursor sweat. At the ultrastructural level the clear cell has the appearance of a metabolically active cell involved fluid and ion transport, ie, abundant mitochondria and glycogen, elaborately folded basolateral plasma membrane, intercellular canaliculi.[51-53] The localization of carbonic anhydrase in the clear cell and the lack of the enzyme in the dark cell provide evidence supporting Montagna's original speculation.

Kaiser and Drack[23] found that the concentration of bicarbonate in sweat ranged from 2 to 10 mM and that the concentration increased with the sweat rate. The site and mechanism of sweat acidification are not known, but Kaiser and Drack[23] speculated that the duct acidifies the precursor sweat by reabsorbing HCO_3^- and/or exhanging H^+ for luminal Na^+. The presence of low activity CA I in the luminal cells of both the coiled and straight segments of the duct could conceivably be responsible for the regulation of sweat pH, but this role cannot be exclusively relegated to the duct because of the possible contribution of CA I and high activity CA II in the secretory coil. Knowing that CA exists in the sweat gland, however, does not clarify whether the cells are secreting or reabsorbing protons or bicarbonate ions. Some CA-rich cells such as the parietal cell[17,18] secrete pro-

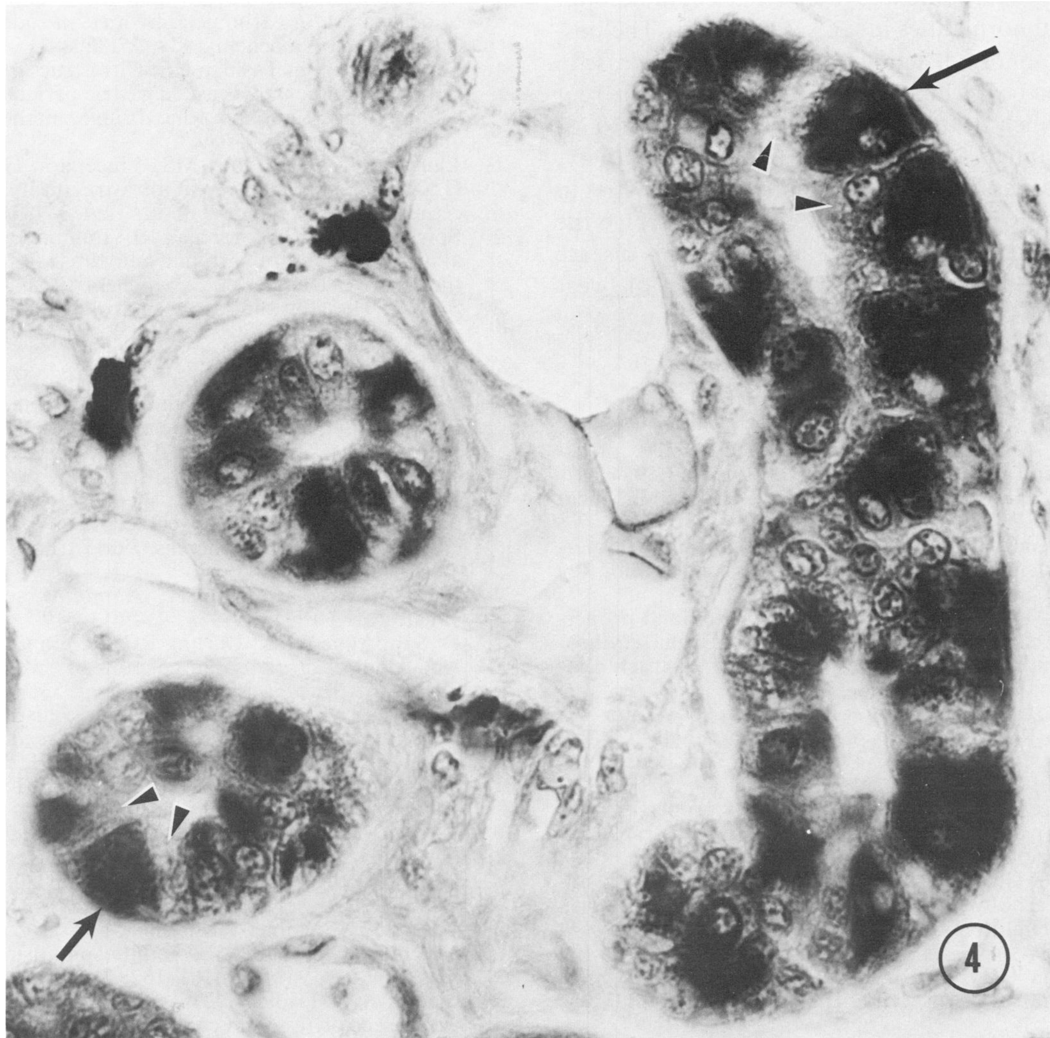

Figure 4—Higher magnification of the secretory coil displaying strong immunostaining in the cytoplasm of the clear cells (*arrows*) and a lack of staining of the dark cells (*arrowheads*). The predominantly basal position, pyramidal shape, and intermittent location of the immunostained cells served to identify them as clear cells. (×910)

tons; others, like the pancreatic duct cells,[20] secrete bicarbonate ions; whereas others, such as the superficial columnar cells of the colon,[20] carry out mainly resorptive activities. Presumably other factors determine the direction of movement and the kind of ion transport that is driven by cytoplasmic CA.

The human eccrine sweat gland is currently the most practical tissue for the *in vitro* investigation of defective fluid and ion transport in CF. Since no animal model has been developed for this disease, human tissues must be used for experimentation. Of all the organs affected by CF, the sweat gland is the most accessible. Unlike other tissues, the parenchyma is not damaged secondarily by the precipitation of macromolecular secretory products. In addition, secretory and resorptive functions of the sweat gland can be studied separately, because these activities occur in anatomically distinct regions. Finally, the sweat gland is primarily involved in fluid and ion transport, and therefore its study is not obscured by secretion of macromolecules.

In CF, the sweat glands exhibit two functional abnormalities that implicate a change in the activity of carbonic anhydrase in this disease. Unlike normal glands, which maintain a transepithelial pH gradient, CF sweat glands elaborate sweat at a constant pH near neutrality.[23] Also, in CF, where the sweat K^+ concentration is slightly elevated, compared with normal,[25] acetazolamide (a potent inhibitor of carbonic anhydrase activity) reduces K^+ levels in CF sweat to normal values but does not alter the concentration of K^+ in normal sweat.[30] Our studies did not detect any change in the distribution, the kind of isozyme, or at least semiquantitatively the number of

molecules of carbonic anhydrase that could account for these abnormalities in CF sweat glands. The possibility of abnormal activity of carbonic anhydrase in CF cannot be excluded on the basis of the present studies, which dealt with the antigenic properties of carbonic anhydrase and not its enzymatic activity. Biochemical assay of carbonic anhydrase activities in normal and CF sweat glands would help resolve the question of an altered enzyme activity in this disease but may be hampered by the limited number of sweat glands available.

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