Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 3425–3429, May 1987
Medical Sciences

Development of a monoclonal antibody specifically reactive to gastrointestinal goblet cells

(colon/mucus/M_r 40,000 protein/ulcerative colitis)

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Contributed by A. B. Novikoff, January 27, 1987

ABSTRACT **A mouse monoclonal antibody ($7E_6A_5$) of IgG isotype, reacting specifically with mucin-producing goblet cells of the human gastrointestinal tract, has been developed. $7E_6A_5$ reacts by an ELISA with colonic protein eluted from a DEAE column. A screening by immunoperoxidase assay of 76 specimens from 19 different human tissues showed that the immunoreactivity of $7E_6A_5$ was confined exclusively in the globules of goblet cells in the colon, the appendix, and the small intestine. Nongoblet small and large intestinal epithelial cells did not react. Immunoelectron microscopy demonstrated the reactivity with mucin droplets in a homogeneous granular pattern inside the globules of goblet cells. Mucus-secreting cells from remaining parts of the gastrointestinal tract and other mucus-secreting organs such as respiratory, genitourinary tracts, salivary and mammary glands did not show any reactivity to $7E_6A_5$. These findings indicate that the antigen recognized by $7E_6A_5$ is shared by the goblet cells of both the small and large intestines and is unique to them. The monoclonal antibody may be useful in the study of function of mucus-secreting goblet cells and may represent an important tool in the evaluation of diseases such as ulcerative colitis, colon cancer, and intestinal metaplasia in gastric mucosa that are associated with quantitative changes in goblet cell numbers or with qualitative differences in mucin secretion.**

Mucus-secreting cells can be observed in different human tissues, such as in the genitourinary, bronchial, and gastrointestinal tracts. However, goblet cells are mucus-secreting cells uniquely distributed along human small and large bowel. In the small intestine, they are interspersed among the more numerous columnar absorptive cells, but in the colon they represent <90% of crypt epithelial cells (1). Their most important known function is secretion of mucin, which provides a lubricating action for the passage of luminal content and a barrier to physical, bacterial, or viral aggression (2–4). The importance of normal mucin secretion seems to be indicated by the association between qualitative and quantitative changes in the goblet cell population and mucin production, and some colonic diseases such as ulcerative colitis (5, 6) and colon cancer (7).

In an earlier paper, we reported the presence of a human colon-specific M_r 40,000 protein, which is recognized by an IgG antibody present only in patients with ulcerative colitis (8). We recently developed monoclonal antibodies that enabled us to localize the M_r 40,000 protein to the plasma membrane of colonic epithelial cells (9). Here we describe the development of another monoclonal antibody that reacts with the semi-purified M_r 40,000 protein and cross-reacts with the mucin contained in globules of goblet cells from both human small and large intestine, but not with other mucus-secreting cells in the remaining parts of the gastrointestinal tract and other nongastrointestinal organs.

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MATERIALS AND METHODS

Preparation of Colon-Extracted Proteins. Normal segments of colonic specimens from patients who underwent colectomy for colon cancer were freed of serosal fat, minced, and homogenized in phosphate-buffered saline (PBS) (10 mM sodium phosphate/0.15 M sodium chloride, pH 7.5) containing 2 mM EDTA, 0.025 M NaN_3, and 2 mM phenylmethylsulfonyl fluoride, centrifuged sequentially at 4000 × g for 30 min, and at 10,000 × g for 60 min at 4°C. The supernatant was then subjected to DEAE ion-exchange chromatography. The proteins eluted with 0.35 M KCl contained an enriched amount of the M_r 40,000 protein(s) along with other proteins (8). This extract was used to inject the mice for the development of hybridomas.

Monoclonal Antibody Production. Six-week-old BALB/c mice were immunized with an emulsion of 100 μg of highly enriched M_r 40,000 protein (1 mg/ml) in an equal volume of complete Freund's adjuvant given subcutaneously over the neck, in the footpad, and intraperitoneally. The mice were given 40 μg of highly purified M_r 40,000 protein intravenously 1 day prior to fusion. The splenic lymphocytes were mixed with cells of the nonsecretor BALB/c-derived myeloma line (NSO) in the mid-logarithmic phase of growth in a spleen to myeloma cell ratio of 8:1. Fusion was performed with 50% polyethylene glycol (M_r 4000; Merck, Darmstadt, Federal Republic of Germany) using the standard technique (10). Screening for antibodies was performed in an ELISA and positive hybrids were cloned in soft agar. Cells were injected intraperitoneally into 2,6,10,14-tetramethylpentadecane (Pristane; Aldrich)-primed BALB/c mice for the production of ascitic fluid.

Isotype Analysis. For determination of the immunoglobulin class, the plates were coated with rabbit anti-mouse isotype-specific immunoglobulin (Zymed Laboratories, Burlingame, CA) using a 1:1000 dilution. After overnight incubation, the culture supernatant was added to the wells and the ELISA was developed with rabbit anti-mouse immunoglobulin alkaline phosphatase conjugate (Zymed Laboratories) *p*-nitrophenyl phosphate (Sigma) as substrate.

ELISA. ELISA plates were coated with 50 μl of the colon extract enriched with M_r 40,000 protein at different concentrations (1.5–50 μg/ml) in carbonate buffer (pH 9.3) overnight at 37°C. Nonspecific binding was blocked with 100 μl of 0.15 M PBS (pH 7.4) per well with 2% bovine serum albumin for 1 hr at 37°C. Culture supernatant and a control unrelated monoclonal supernatant were incubated with different antigen concentrations for 3 hr at room temperature. After washings with PBS/0.05% Tween 20, a 1:500 dilution of alkaline phosphatase-conjugated rabbit anti-mouse IgG (Zymed Laboratories) was added (50 μl per well) for 1 hr at 37°C. After extensive washings with PBS/0.05% Tween 20, the substrate (disodium *p*-nitrophenyl phosphate in diethanolamine buffer, pH 9.8) was added. The plates were read at 405

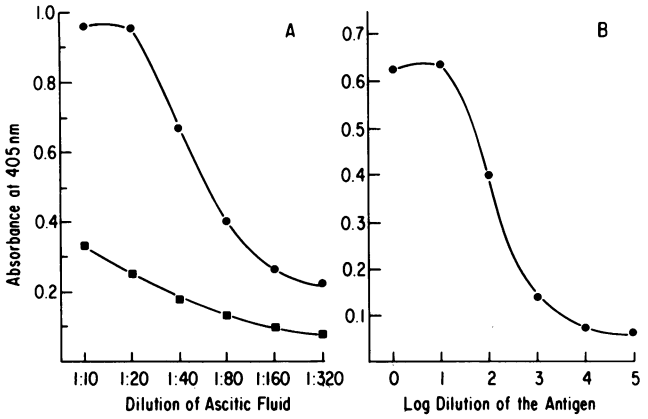

FIG. 1. Evaluation of the specificity of immunoreactivity of $7E_6A_5$ to colonic protein eluted from a DEAE column by 0.35 M KCl. (*A*) Different dilutions of $7E_6A_5$ ascitic fluid were tested against the protein at 25 μg/ml. $7E_6A_5$ (●) could be titrated out at around 1:60 dilution. An unrelated monoclonal antibody (■) of the same immunoglobulin isotype (4T001) did not show any reactivity. (*B*) $7E_6A_5$ at 1:20 dilution was tested against different concentrations of the coated proteins (1.5–50 μg/ml). The decrease in OD readings paralleled the decrease of coated antigen concentration.

Table 1. Human tissues examined for immunoreactivity against the monoclonal antibody $7E_6A_5$ by the immunoperoxidase method

Tissue	Number of tissue specimens	Number showing immunoperoxidase reactivity
Colon	16	16
Appendix	4	4
Oral mucosa	1	0
Esophagus	2	0
Stomach	7	0
Duodenum	3	3
Jejunum	3	3
Ileum	3	3
Liver	13	0
Biliary tract	4	0
Pancreas	2	0
Nasal mucosa	2	0
Lung/bronchus	4	0
Kidney and urinary tract	2	0
Urethra	2	0
Cervix	2	0
Salivary gland	1	0
Mammary gland	1	0
Skin	4	0

nm at 3 and 16 hr in a Titertek Multiskan ELISA reader (Flow Laboratories).

Immunocytochemical Experiments. Preliminary experiments were performed to evaluate the effect of several fixation procedures on antigen preservation. Since formalin fixation did not affect the antigen and gave the best structural preservation, formalin-fixed paraffin-embedded tissues from surgical or endoscopy samples were used. Sections (5 μm) were deparafinized, rehydrated, and incubated for 30 min in 0.1 M PBS (pH 7.4) containing 0.3% H_2O_2 to block endogenous peroxidase activity. Sections were washed, incubated for 2 hr with diluted (1:50) normal horse serum to block nonspecific binding, incubated overnight with the monoclonal antibody and then with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) for 90 min, washed in PBS, and incubated in avidin biotin peroxidase complex (Vector Laboratories) for 90 min. The reaction was revealed by 3,3'-diaminobenzidine hydrochloride (1 mg/ml) in 0.1 M Tris·HCl, pH 7.6/0.02% H_2O_2 for 10 min in the dark. Slides were washed, dehydrated, and mounted. All incubations were performed at room temperature except for the overnight incubation that was done at 4°C. Other slides from the same tissue block were deparafinized, rehydrated, and stained with hematoxylin and eosin for routine histological analysis.

Immunoelectron Microscopy. Endoscopic colon biopsy specimens were fixed for 3 hr in 4% paraformaldehyde and washed in 0.1 M phosphate buffer, pH 7.4/7.5% sucrose. Sections (20–30 μm) were cut in a freezing microtome, washed in PBS with 7.5% sucrose, and successively incubated according to the floating sections technique with PBS and 0.3% H_2O_2 for 30 min, normal horse serum (1:50) for 2 hr, $7E_6A_5$ and control monoclonal antibody overnight, biotinylated horse anti-mouse IgG for 2 hr, avidin biotin peroxidase complex for 2 hr and 3,3'-diaminobenzidine hydrochloride for 15 min. Between each incubation, extensive washings in cold PBS with 7.5% sucrose were done. Some of the sections were examined by light microscopy after mounting in glycerol gelatin (Sigma). Other sections were processed for electron microscopy as described (11). Epon sections (500–800 Å thick) were cut and examined with and without lead staining using an electron microscope.

RESULTS

A monoclonal antibody ($7E_6A_5$) was developed reactive with human colonic protein(s) eluted from a DEAE column.

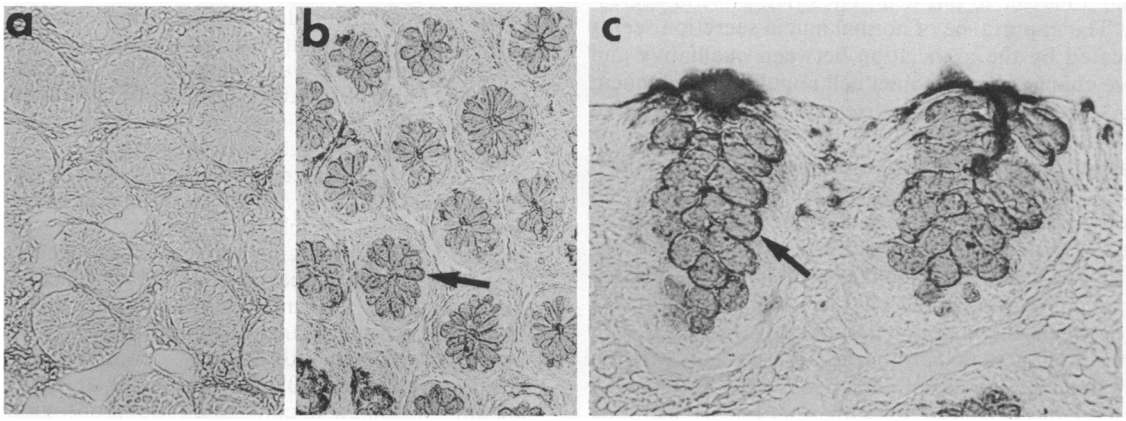

FIG. 2. Immunoreactivity of the monoclonal antibody $7E_6A_5$ against normal human colonic mucosa by the immunoperoxidase method. (*a*) Control monoclonal antibody (4T001). There is no reactivity. (×15.) (*b*) Immunoreactivity of $7E_6A_5$. The reactivity in the goblet cells is evident. (×15.) (*c*) Higher magnification of *b*. Localization in the globules of goblet cells is seen. No reactivity is observed at the basolateral areas of goblet cells and in the luminal epithelium. (×35.)

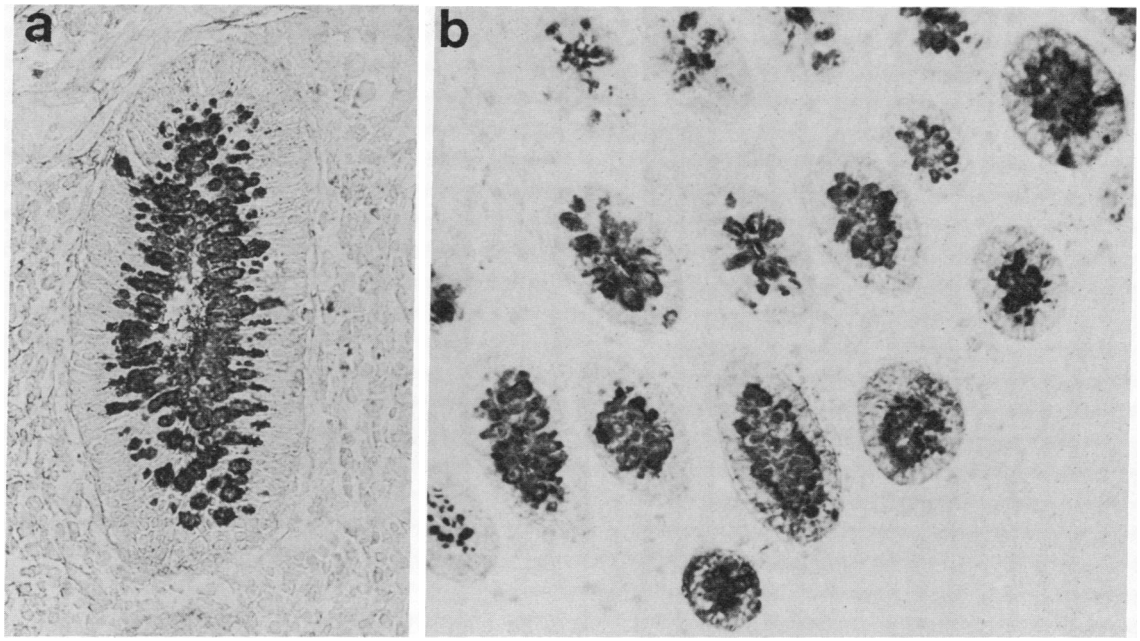

FIG. 3. Human appendix incubated with $7E_6A_5$: homogeneous immunoreactivity of globules of goblet cells is seen. (*a*) Longitudinal section (×35); (*b*) cross section (×25). The remaining parts of epithelial cells and lamina propria cells did not react.

Isotype analysis using double sandwich ELISA showed that $7E_6A_5$ belonged to the IgG_1 class. Fig. 1*A* shows the ELISA readings at different dilutions of the $7E_6A_5$ ascites compared to an unrelated ascitic fluid against the colonic protein(s). The titer of the $7E_6A_5$ ascitic fluid is at ≈1:50 dilution. Fig. 1*B* shows a 1:20 dilution of the monoclonal antibody when titrated against different concentrations of the coated antigen. The decrease in the OD reading paralleled the decrease of coated antigen concentrations.

To localize the immunoreactive protein in tissue by $7E_6A_5$, a total of 76 biopsy specimens from different organs were examined by the immunoperoxidase method as shown in Table 1. A positive reactivity was detected only in mucosal biopsy specimens from the colon, the appendix, and the small bowel. All other tissues were uniformly negative. Figs. 2–5 show the results of immunoperoxidase studies in different biopsy specimens from large and small intestine. The monoclonal antibody reacted exclusively with the goblet cells of the colon (Fig. 2), the appendix (Fig. 3), and the small intestine (Fig. 4). The reactivity was localized in the globules of goblet cells in the colonic mucosal glands (Figs. 2, 3, and 5) and in the goblet cells lining the colonic lumen, which were interspersed among the absorptive epithelial cells (Fig. 5). Nongoblet colonic epithelial cells lining the lumen did not stain (Fig. 5). Small intestinal epithelial cells (enterocytes) did not show any immunoreactivity (Fig. 4). The reaction with mucin droplets by $7E_6A_5$ showed a homogeneous pattern, as was further confirmed by immunoelectron microscopy (Fig. 6).

A duodenal polyp showed the same reactivity as normal duodenal mucosa—that is, the staining was localized to

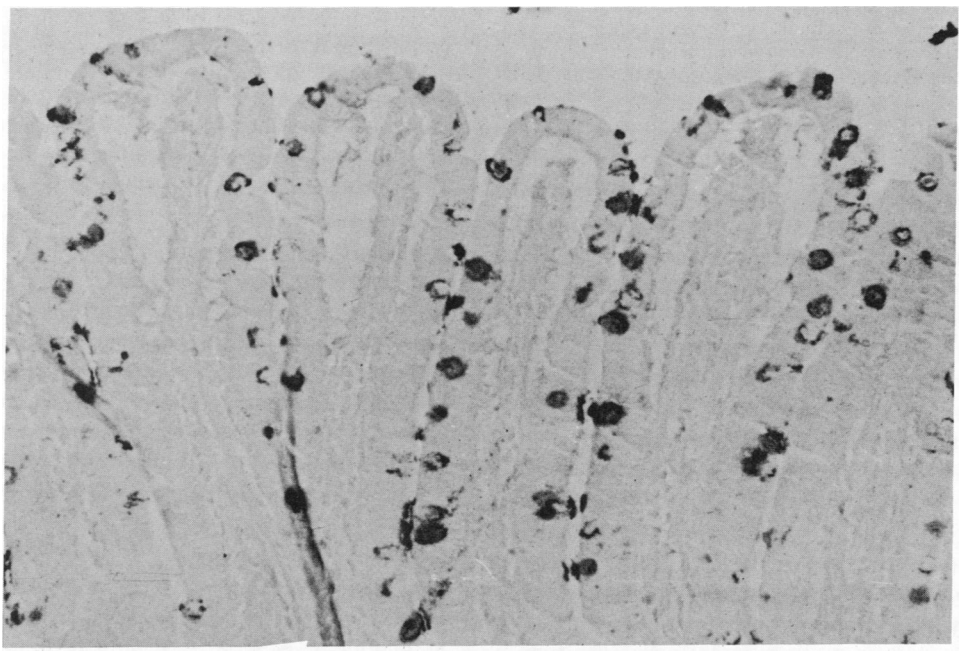

FIG. 4. Human small bowel mucosa showing immunoreactivity of goblet cells only by $7E_6A_5$. (×16.)

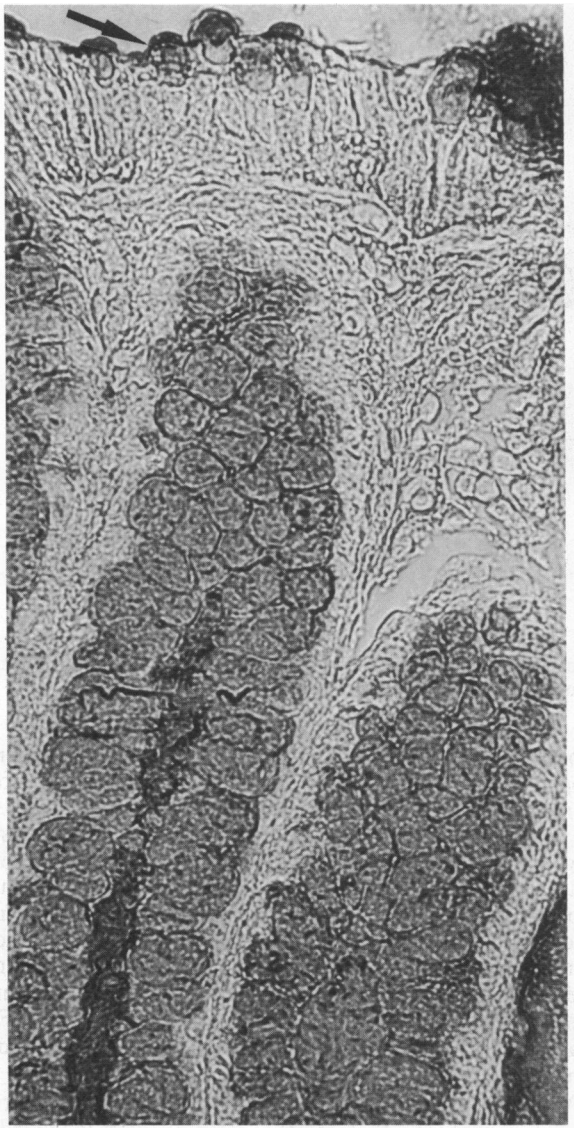

FIG. 5. Immunoreactivity of $7E_6A_5$ with human colonic mucosa. No reactivity of the luminal epithelium is observed, except for the goblet cells, interspersed among absorptive epithelial cells (arrow). Globules of goblet cells in the glands are stained. (×90.)

goblet cells only, while enterocytes were not stained (Fig. 7). More than 95% of goblet cells showed reactivity, with no apparent differences between cells of the upper and lower portion of the crypts and cells from different parts of the small and large bowel. Nongoblet cells and lamina propria cells did not show any reactivity. Other mucus-secreting cells from remaining parts of gastrointestinal tract, hepatobiliary, pancreatic, respiratory, and genitourinary epithelium did not react with $7E_6A_5$ (Table 1). Salivary and mammary glands also did not react.

DISCUSSION

Mucin, a high molecular weight glycoprotein, is the main component of mucus, a complex secretion of epithelial cells from the gastrointestinal, respiratory, and genitourinary tracts. It is not known yet whether the differences observed in the composition of mucin from different organs are due to the existence of distinct mucus protein cores identifying unique mucin molecules or to changes in the carbohydrate moieties caused by different forms and degrees of activity of glycosylating enzymes present in different cells. The functional significance of these structural differences remains to

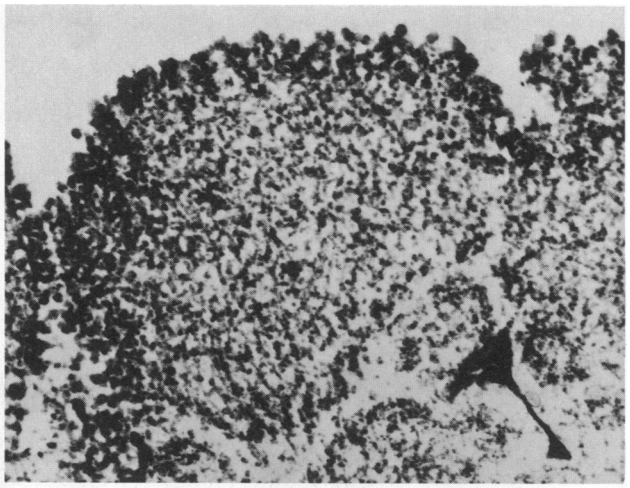

FIG. 6. Immunoelectron microscopic examination of human colonic mucosa with $7E_6A_5$. Note the reactivity in the mucus containing globule at the luminal aspect of the goblet cell. (×10,800.)

be clarified. While the stomach mostly contains neutral mucin, the small intestine contains sialic acid-rich mucin and the colon contains both sialomucins and neutral mucins together with sulfomucins (8). Furthermore, a fair amount of cytochemical, biochemical, and immunological data seem to indicate that mucin composition differs between goblet cells from the right and the left colon and between goblet cells from the upper and the lower portion of the same crypt (11–16). From the study of the three major components of colonic mucins, 21 different oligosaccharide chains have been identified, some of them being shared by the three major colonic mucins, others being specific for a given mucin species (17). Immunofluorescence experiments with mouse monoclonal antibodies reacting with different mucin specificities have shown that many heterogeneous goblet cell populations can be recognized on the basis of their pattern of reactivity with the monoclonal antibodies (18). These findings indicate a broad heterogeneity of colonic mucins.

The monoclonal antibody $7E_6A_5$ reported here, however, reacts with all populations of goblet cells present in both small and large intestine. No differences have been observed in the staining pattern or in the cellular distribution of the specific immunoreactivity throughout the intestine. More than 95% of goblet cells in the duodenum, jejunum, and ileum, as well as in the right and left colon, are recognized by the monoclonal antibody with a diffuse and uniform staining of the globules. It was interesting that the goblet cells in the duodenal polyp were also stained with $7E_6A_5$. The reactivity of $7E_6A_5$ differs, on the basis of the staining pattern and/or the cellular and tissue distribution, from what has been observed by Podolsky *et al.* in the study of their anti-mucin monoclonal antibody library (18, 19). Our findings indicate that the antigenic determinant(s) recognized by the monoclonal antibody $7E_6A_5$ is shared by all goblet cells in the small and large intestine irrespective of their location in the crypts and luminal surface. However, no reactivity was observed when other mucus-secreting tissues (e.g., nasal mucosa, respiratory, and genitourinary tracts) were studied by immunoperoxidase techniques with this monoclonal antibody. The staining was also absent in the remaining parts of gastrointestinal tract, salivary and mammary glands, and in squamous epithelium such as esophagus and skin. This finding, together with the presence of the specific reactivity in all goblet cells of the gastrointestinal tract indicate that this monoclonal antibody recognizes an antigen that is common to all gastrointestinal goblet cells and unique to them.

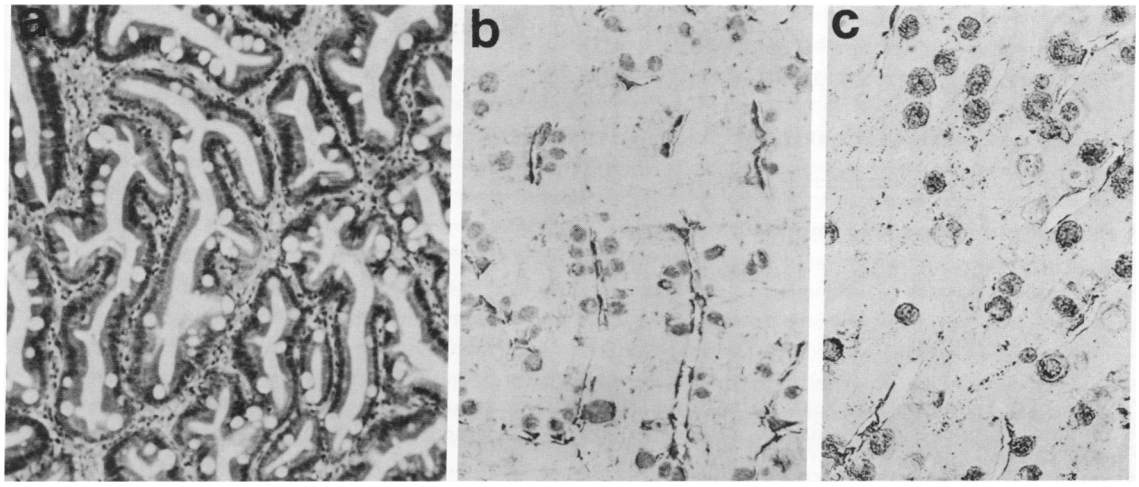

FIG. 7. Duodenal polyp. (*a*) Stained with hematoxylin and eosin (×15); (*b*) immunoperoxidase staining (×15); (*c*) immunoperoxidase staining (×25). All goblet cells are stained by $7E_6A_5$.

$7E_6A_5$ has been developed using an antigen the M_r 40,000 enriched preparation of colon extract. However, it is unknown whether $7E_6A_5$ reacts with the M_r 40,000 protein. In ELISA, $7E_6A_5$ reacts with the colon extract enriched with M_r 40,000 protein, but in immunotransblot experiments, the monoclonal antibody neither reacts with the M_r 40,000 protein nor with any of the other proteins present in the extract. This suggests that the immunoreactive protein in the colon extract recognized by $7E_6A_5$ is denatured by NaDodSO$_4$ treatment. The monoclonal antibody against M_r 40,000, $7E_{12}H_{12}$, however, reacts with the M_r 40,000 protein in immunotransblot (9). There is a clear difference in cellular reactivity of $7E_{12}H_{12}$ and $7E_6A_5$, as demonstrated by immunoperoxidase assay. $7E_{12}H_{12}$ specifically reacts with colonic epithelial cells but not small intestine, and the staining is localized along basolateral domains of the plasma membrane (9). In contrast, $7E_6A_5$ stains only the globules of the goblet cells present in both large and small intestine. Indeed, in the colonic mucosa, $7E_6A_5$ does not stain the absorptive epithelial cells lining the lumen (Fig. 5), whereas these cells are clearly stained by the anti-M_r 40,000 monoclonal antibody (9). Because of the specific staining of the mucus globules, it appears that $7E_6A_5$ may be directed against a glycopeptide or sugar residue specific to intestinal goblet cells. The enriched preparation of M_r 40,000 protein may contain this component as a contaminant or $7E_6A_5$ may be cross-reactive to the M_r 40,000 protein. The explanation for staining of the small intestinal goblet cells by $7E_6A_5$ may be due to the common glycopeptide or sugar residue shared by the goblet cells to which $7E_6A_5$ reacts but $7E_{12}H_{12}$ does not. The absence of M_r 40,000 protein from the small intestine is shown by immunotransblot using the natural antibody against M_r 40,000 protein eluted from ulcerative colitis colon mucosa (8) and by immunoperoxidase assay with $7E_{12}H_{12}$.

Further studies are needed to determine whether the observed reactivity of $7E_6A_5$ is due to an epitope present in the protein core or in the carbohydrate side chains of goblet cell mucins and if there is a relation to the M_r 40,000 protein associated with ulcerative colitis (8). The characterization of the reactive antigen(s) may clarify some of the structural and functional differences between goblet and absorptive cells in the colon and between mucin from different organs. In addition, clinically the monoclonal antibody can be applied in studies of colonic diseases associated with either quantitative changes in goblet cell numbers or with qualitative differences in mucin secretion, such as ulcerative colitis and colonic neoplasia. It may also be useful in the study of extracolonic conditions in which the appearance of metaplastic goblet cells may help identify a possible precancerous lesion, such as intestinal metaplasia in gastric mucosa, or demonstrate colonic metastases, particularly in the liver.

It is with deep sorrow that we announce the untimely death of our esteemed colleague, Dr. Alex B. Novikoff, on January 9, 1987. The investigation is supported in part by a research grant from the National Foundation for Ileitis and Colitis (K.M.D.), National Institute of Diabetes, Digestive and Kidney Diseases Research Grants AM-26403 (K.M.D.) and AM-32371 (B.D.), National Cancer Institute Research Grant CA-06576 (A.B.N.), and National Cancer Institute Career Award CA-14923 (A.B.N.) from the National Institutes of Health, Bethesda, MD. We acknowledge the help of Dr. Rachel Morecki, Professor of Pathology, for providing us with appropriate pathological specimens.

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