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Isolation and characterization of colonic tissue-bound antibodies from patients with idiopathic ulcerative colitis

(inflammatory bowel disease/elution technique/immunofluorescence/autoimmunity)

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ABSTRACT **To determine if specific anticolon antibodies bound to colonic mucosa occur in ulcerative colitis, we obtained surgical specimens of colon from five patients with ulcerative colitis, one patient with diverticulitis, and three control subjects with carcinoma. Two specimens of ileum and cecum were also obtained from patients with Crohn ileocolitis. Tissue was homogenized and washed and bound Ig was eluted by citrate buffer, pH 3.2. Concentrated eluates of all specimens from patients with ulcerative colitis reacted with antisera to κ and γ and not with antisera to α and μ chains. Corresponding eluates from all other specimens did not react with these antisera, but did react with antialbumin. The presence of IgG in ulcerative colitis eluates was also determined by immunoelectrophoresis, immunocoprecipitation, and affinity chromatography with antisera against human IgG.**

Indirect immunofluorescence and uptake of radiolabeled antibody demonstrated antigenic sites in diseased colonic epithelium of biopsy specimens obtained from six additional patients with ulcerative colitis and three patients with idiopathic proctitis, but not in patients with Crohn disease, nonspecific diarrhea, and bacillary dysentery and control subjects. Although the role of colitis colon-bound antibody in the pathogenesis of ulcerative colitis is unclear, local antibody–antigen complexes may initiate colonic epithelial cytolysis by various immunologically mediated mechanisms.

Several immunological abnormalities have been described in patients with idiopathic ulcerative colitis. Their serum contains heterogeneous antibodies that react with colon from newborn and adults, germ-free rats, and human intestinal and gastric mucous cells (1–9). They also react with *Escherichia coli* 014 antigen. Circulating immune complexes and activated monocytes have been found in the blood of patients with active ulcerative colitis (10–11). Recent studies have further demonstrated a deficient mucosal secretory IgA system which may compromise the mucosal defenses of the host (12–14). This study reports isolation of disease-specific antibody bound to colonic mucosa in patients with ulcerative colitis.

MATERIALS AND METHODS

Antibody isolation

Colon specimens were obtained at surgery in five patients with ulcerative colitis, one patient with diverticulitis, and three patients with colon carcinoma. From the last group, only uninvolved segments of colon were used. Terminal ileum and cecum were also obtained from two patients with Crohn ileocolitis. Specimens were cleaned of luminal contents, serosal fat, and mesentery within half an hour of surgery and stored at −80°C.

Extraction of Antibody Bound to Colon. Acid elution (15–16) was performed with thawed colon or ileal specimens, dissected into small pieces (1–2 cm) containing the full thickness of the bowel wall. Tissue fragments were suspended in 10 vol of phosphate-buffered saline (10 mM sodium phosphate, pH 7.2), homogenized in an ice-jacketed blender (Virtis Co. Inc., NY) for 3 min, and centrifuged at 2000 × *g* for 30 min. Washing was continued 10–15 times until concentrates of washes (500- to 1000-fold) did not contain Ig, as determined by immunodiffusion and immunoelectrophoresis.

The washed tissue was suspended in 10 vol of 20 mM sodium citrate (pH 3.2), stirred for 2 hr at 37°C, and centrifuged at 2000 × *g* for 30 min. The supernatant was neutralized with 0.1 M NaOH and dialyzed for 24 hr against five to six changes of phosphate-buffered saline. The colon eluate was concentrated 60- to 100-fold by positive pressure filtration through an Amicon Diaflow membrane 10 PM 10. The concentration of protein was estimated by the method of Lowry *et al.* (17). Eluates (3–10 ml) were stored at −80°C.

Antibody Characterization. The colon eluate was tested by Ouchterlony immunodiffusion and immunoelectrophoresis with antisera against κ and λ light chains; Fab and Fc fragments of human IgG, γ, μ, and α chains; free secretory component; human IgE; albumin; β_1C; and C_1q. The antisera against human immunoglobulin were obtained from Cappel Laboratory, Downington, PA. Antiserum to human free secretory component was prepared by us (12). Monospecificity of all the antisera was demonstrated by immunoelectrophoresis. The colon eluates were also studied by acrylamide gel electrophoresis in sodium dodecyl sulfate (5%).

Radioiodination of Colitis Colon-Bound Antibody (CCA) and Normal Human IgG. CCA was iodinated (18) in a reaction mixture to which $Na^{125}I$ (0.5 mCi; New England Nuclear), CCA (50 μg), chloramine T (0.125 mg), sodium metabisulfite (0.3 mg), and potassium iodide (1 mg) were sequentially added at 10- to 15-sec intervals. ^{125}I-Labeled protein was separated from unreacted $Na^{125}I$ on a cation exchange (IRA 400) column (4 ml) and washed with 1 M Tris buffer (pH 7.5) and water, followed by bovine albumin (1 mg/ml). Normal human IgG of identical concentration was iodinated and served as a control. About 95% of counts in each preparation were precipitated by 10% trichloroacetic acid.

Specificity of CCA

The specificity was determined by immunofluorescent and radioactive binding studies with rectal and colon biopsy specimens from six additional patients with ulcerative colitis, three patients with idiopathic proctitis (19), two patients with Crohn disease, two with nonspecific diarrhea, one with bacillary dysentery, and three normal subjects. In patients with idiopathic

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Abbreviations: FITC, fluorescein isothiocyanate; CCA, colitis colon-bound antibody.

proctitis, biopsy specimens were obtained at colonoscopy from the diseased rectum and from uninvolved descending and transverse colon. Normal ileal specimens were obtained from two patients undergoing right hemicolectomy for carcinoma colon. Endoscopic gastric and duodenal specimens and percutaneous liver biopsy specimens (two each) were obtained from four patients (duodenal ulcer, two patients; chronic persistent hepatitis, two patients) without inflammatory bowel disease. Tissue specimens were stored at −80°C for a maximum of 2 months. Informed consent was obtained from all patients.

Immunofluorescent Studies of Tissue Specificity of CCA. Rabbit anti-human IgG conjugated with fluorescein isothiocyanate (FITC) was obtained from Behring Diagnostic, American Hoechst Corp., NJ. FITC-conjugated rabbit Fab'2 antisera against human IgG and κ and λ light chains were obtained from Warren Strober, National Cancer Institute, Bethesda, MD. Chromatographically purified human IgG was obtained from Cappel Laboratory, Downington, PA. The specificity of control IgG was tested by polyacrylamide gel electrophoresis and by immunoelectrophoresis. Antibody against three different CCA preparations was raised in rabbits and tested by immunodiffusion and immunoelectrophoresis. Antibody against three different CCA preparations was raised in rabbits and tested by immunodiffusion and immunoelectrophoresis.

Indirect immunofluorescence was performed on cryostat sections (4 μm) of tissues (12), with colon eluate as the first layer followed by staining with fluoresceinated rabbit anti-human IgG or fluoresceinated rabbit Fab'2 antisera against human IgG and κ and λ chains. The dilutions of conjugates that yielded maximal sensitivity with minimal background were selected by indirect immunofluorescence of large and small intestine and gastric and duodenal mucosa. Autofluorescence and nonspecific fluorescence were determined on parallel sections. Additional control experiments were performed by staining parallel sections initially with normal human IgG instead of CCA, followed by the fluoresceinated antisera. Sections were also stained with normal colon acid eluate followed by FITC-conjugated anti-IgG. Further specificity studies were performed by using the supernatant obtained from preincubation and precipitation of CCA with rabbit anti-CCA.

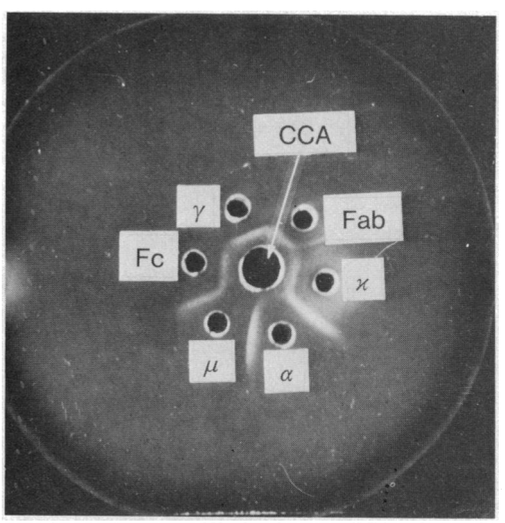

FIG. 1. Double immunodiffusion in agar: CCA-colitis colon eluate at the central well. Peripheral wells contain antisera to Fab and Fc fragments of human IgG and γ, κ, α, and μ chains. Precipitation lines between peripheral wells result from components of human Ig in commercial antisera.

Fluorescence Microscopy. A Zeiss microscope (Carl Zeiss Inc., NY) equipped with vertical illuminator HBO 200W mercury lamp, FITC exciter filter, FL-500 reflector, and 53 barrier filter was used. Photographs were taken with GAF color slide film ASA 500 (Gaf Corp., NY). Sections were examined with ×120–800 magnification to determine the presence and distribution of immunofluorescence without prior knowledge of the source of tissues.

Radioactive Antibody-Binding Studies with ^{125}I-Labeled CCA and Normal Human IgG. Cryostat sections (4 μm) of rectal and colonic mucosal biopsy specimens from diseased and normal tissues were placed on coverslips (24 × 30 mm). Coverslips containing the consecutive tissue sections were placed on either side of an adhesive tape 6 inches (15 cm) long. Each tape contained three to four pairs of coverslip with sections from the same tissue block. The whole tape was placed in 95% ethanol for fixation and washed in phosphate-buffered saline. Variable concentrations in 10 μl (0.01–0.2 μg) of ^{125}I-labeled CCA (one side of the tape) or ^{125}I-labeled normal human IgG (the other side of the tape) were placed on each tissue section and the entire tape was incubated in a moist chamber for 30 min followed by washing three times in phosphate-buffered saline. Similar experiments were simultaneously performed in multiple chambers. The same amount of protein was added to the tissues in each chamber. After it was washed, each coverslip was placed in a counting vial and radioactivity was measured in a gamma counter.

RESULTS

Acid Eluates. The CCA preparations (3–10 ml final volume) contained 0.8–1 mg of protein per ml, whereas acid eluates from three control, two Crohn disease, and one diverticulitis specimens contained less than 0.2 mg of protein per ml.

Serologic Studies for Characterization of Acid Eluates. In immunodiffusion and immunoelectrophoresis studies, five CCA preparations reacted with antisera against γ and κ chains and Fab and Fc fragments of IgG, and albumin and not against antisera to α and μ chains (Fig. 1). Concentrated (×100) acid eluates from the control, Crohn disease, and diverticulitis colonic specimens did not react with any of these antisera. None of the colon acid eluates, including CCA, reacted against antisera to λ chain, IgE, β_1C, C1q, and free secretory component.

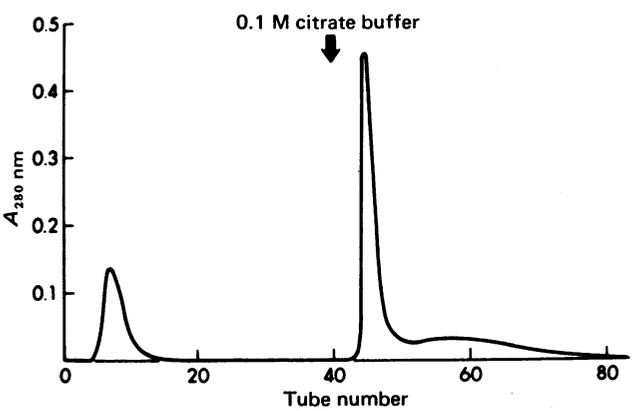

FIG. 2. Affinity chromatography of colitis colon eluate on AH-Sepharose 4-B column coupled with rabbit anti-human IgG antisera (IgG fraction). Sample was eluted with 40 ml of phosphate-buffered saline (pH 7.2), followed by 40 ml of 0.1 M citrate buffer (pH 3.2). Sixty-five percent of the colitis colon eluate was bound to the column and was recovered by citrate buffer. The phosphate-buffered saline eluate contained mainly albumin.

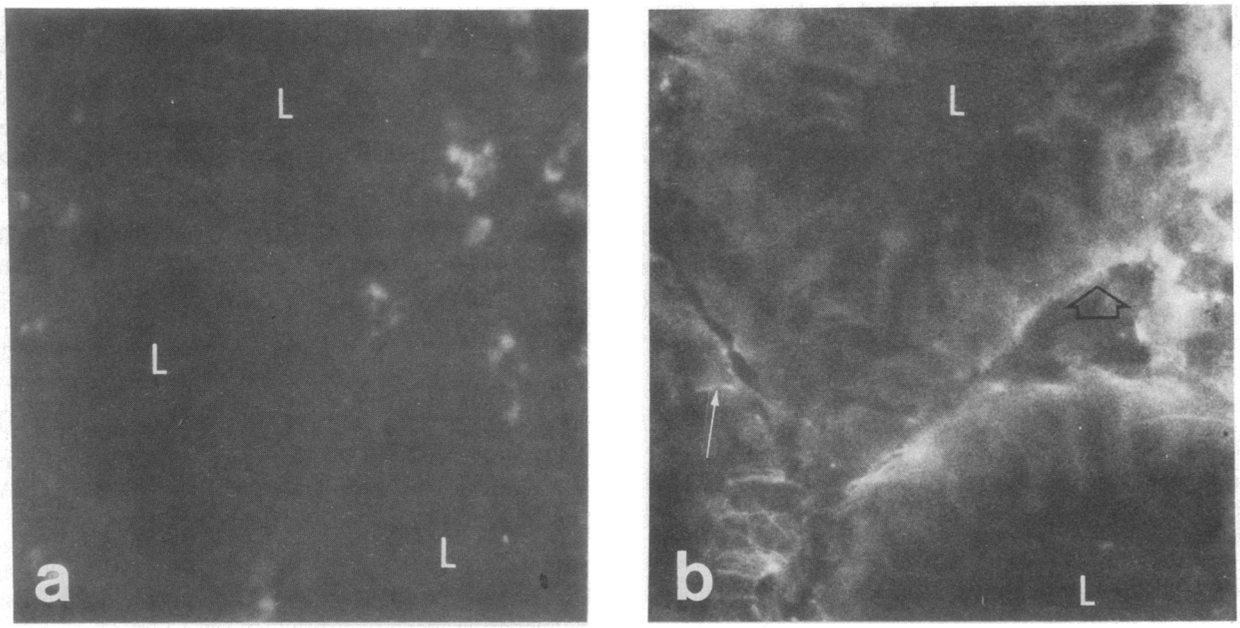

FIG. 3. Immunohistochemical localization of (*a*) normal human IgG and (*b*) colitis colon-bound antibody (CCA) in glandular epithelium from a section of an alcohol-fixed biopsy specimen from diseased colonic mucosa in a patient with ulcerative colitis by the indirect method. The first stain was normal human IgG or CCA followed by FITC-conjugated anti-human IgG antisera. Note that (*a*) normal human IgG does not stain the epithelial cells whereas (*b*) CCA staining is present along the basement membrane (arrowhead) and the periphery of the cells (arrow). L indicates lumen of the gland. (×400.)

Immunoprecipitation of ^{125}I-labeled CCA with antihuman IgG antisera (against both heavy and light chains) in the presence of 25–50 μg of human IgG, precipitated about 60–70% of the radioactivity, indicating that IgG is the major protein in the eluate. Acrylamide gel electrophoresis with sodium dodecyl sulfate (5%) of CCA eluates demonstrated small amounts of protein of molecular size identical to native IgG, and major fragments of about 40,000 and 80,000 daltons. The band at 80,000 daltons appears to be a dimer of 40,000-dalton disulfide-linked subunits since after reduction with mercaptoethanol, a single band of 40,000 daltons occurs. After chromatography on immunoabsorbant column of AH-Sepharose 4B (Pharmacia Chemicals) coupled with the IgG fraction of antisera to human IgG, 65% of CCA was bound and recovered after elution with 0.1 M sodium citrate buffer, pH 3.2 (Fig. 2). After neutralization and dialysis, the acid eluate from the immunoabsorbant column contained 40,000- and 80,000-dalton fragments in addition to the small amount of intact IgG. These protein fractions reacted with antisera against γ chain and against Fab and Fc fragments of IgG, but not with antisera to albumin and to α and μ chains. These proteins seem thus to be fragments of IgG that retain the light- and heavy-chain de-

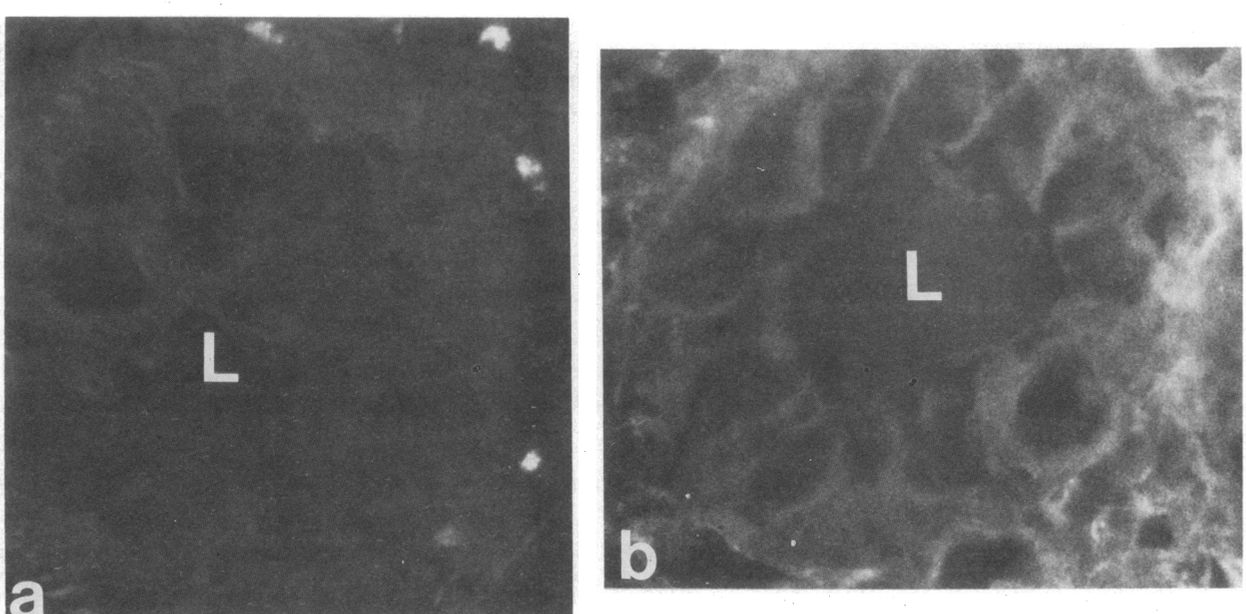

FIG. 4. Identical staining as in Fig. 3 of diseased colonic mucosa showing only one gland in higher magnification. Immunofluorescent staining of CCA is localized to basement membrane areas and cellular periphery (*b*) whereas no such staining is present for normal human IgG (*a*). L indicates lumen of the gland. (×800.)

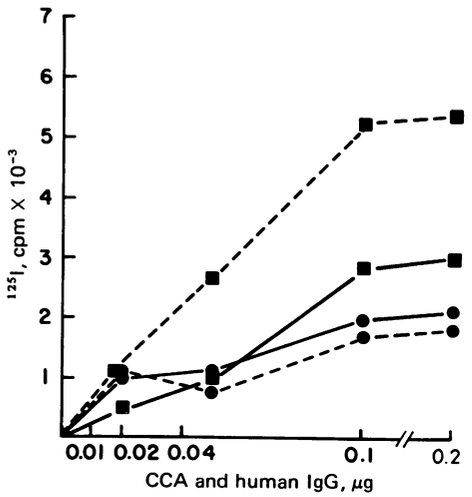

FIG. 5. Binding of ^{125}I-labeled CCA (colitis colon-bound antibody) and ^{125}I-labeled normal human IgG by diseased mucosa from a patient with ulcerative colitis and normal colonic mucosa. In diseased mucosa, ^{125}I-labeled CCA binding is 2.5-fold higher than that of ^{125}I-labeled normal human IgG. In normal mucosa, binding of ^{125}I-labeled CCA and ^{125}I-labeled human IgG is identical. ●, Normal mucosa; ■, diseased mucosa. (- - -) CCA; (—) normal human IgG.

terminants. Fragmentation probably occurred during tissue extraction. This conclusion is further supported by the observation that antiserum that was raised in rabbits against three different CCAs reacted with all CCAs and normal human IgG by immunodiffusion and immunoelectrophoresis. An identical immunoabsorbant column coupled with the antisera to human IgA (IgG fraction) demonstrated that the IgG activity of CCA was exclusively present in the phosphate-buffered saline eluate and not in the acid eluate.

Immunofluorescent Studies. Indirect immunofluorescent studies with CCA followed by FITC-conjugated anti-human IgG revealed antigenic sites in the epithelium of biopsy specimens from diseased colonic mucosa of patients with ulcerative colitis or idiopathic proctitis, but not in control subjects or in patients with Crohn colitis, bacillary dysentery, or nonspecific diarrhea. Immunofluorescent staining did not occur with normal ileum, duodenum, stomach, or liver. A distinctive staining of the basement membrane area and periphery of individual colonic mucosal epithelial cells was detected in biopsy specimens in ulcerative colitis (Figs. 3*b* and 4*b*). Luminal contents are unstained. The proximal colonic mucosa in one patient with idiopathic proctitis showed staining with CCA similar to that in the diseased distal tissue. Normal colon acid eluate and control human IgG did not stain normal colonic or diseased epithelium (Figs. 3*a* and 4*a*).

Table 1. Radiolabeled binding studies of ^{125}I-labeled CCA and ^{125}I-labeled human IgG.

	^{125}I-labeled CCA/^{125}I-labeled human IgG
Ulcerative colitis and proctitis (n = 9)	1.9 ± 0.2 (range 1.4–2.8)
Control subjects (n = 8)	1.0 ± 0.04 (range 0.8–1.1)
Statistical analysis	t = 3.8 $P < 0.005$

Cryostat sections (4 μm) were used of diseased tissues from patients with ulcerative colitis and idiopathic proctitis compared to tissues (diseased and normal) from control subjects, including Crohn disease, nonspecific diarrhea, bacillary dysentery, and normal individuals. Results are expressed as the mean (±SEM) ratio of ^{125}I-labeled CCA to ^{125}I-labeled human IgG.

Radiolabeled Binding Studies. Fig. 5 shows binding of radiolabeled CCA and human IgG when incubated with variable concentrations of the proteins in the identical coverslip preparation of cryostat sections of colonic mucosa from a patient with ulcerative colitis and from a normal subject. A plateau was reached at 0.1 μg. Table 1 shows binding of ^{125}I-labeled CCA and ^{125}I-labeled human IgG by diseased tissues obtained from six patients with ulcerative colitis and three patients with idiopathic proctitis, and the tissues (both diseased and normal) from eight control subjects, including two patients with Crohn disease, two patients with nonspecific diarrhea, one patient with bacillary dysentery, and three normal individuals. The binding of ^{125}I-labeled CCA was significantly higher than the binding of ^{125}I-labeled human IgG ($P < 0.005$) in ulcerative colitis and idiopathic proctitis tissue.

DISCUSSION

Each of five colonic specimens affected by ulcerative colitis contained tissue-bound antibody(ies) directed against one or several constituents of colonic mucosal epithelial cells. Similar antibody was not demonstrable in control specimens, Crohn ileocolitis, or diverticulitis. Patients with granulomatous colitis have not been studied. Antibody eluted from colitis colon belonged to the IgG class. Immunocoprecipitation and affinity chromatography indicated that IgG is the major protein in CCA. Fragmentation of CCA may result from proteolysis during extraction. The fragments retain light- and heavy-chain antigenic determinants. CCA labeled with ^{125}I bound preferentially to diseased mucosa due to ulcerative colitis but not to normal colonic mucosa. Indirect immunofluorescence with CCA demonstrated staining of colonic epithelial cells in the diseased colitis tissue. Direct immunofluorescence with fluoresceinated anti-human IgG did not reveal staining, probably because an insufficient amount of CCA was bound to colonic epithelium. By concentrating CCA and using a "sandwich" technique, we observed CCA binding to ulcerative colitis colon epithelium. This finding differs from the present control experiments and earlier findings, when normal human IgG was used and no staining of the epithelial cells was noted (12, 20, 21). Specificity for CCA is indicated by its ability to recognize colonic tissue from patients with ulcerative colitis and its lack of reactivity with diseased colonic tissue in Crohn disease, bacillary dysentery, and nonspecific diarrhea.

CCA differs in many respects from circulating anticolon antibodies reported by others (1–9). All the five CCA preparations contained IgG but neither IgA nor IgM. Circulating anticolon antibodies have been found in 15–20% of patients with ulcerative colitis (6, 9) and rarely in patients with Crohn disease or in healthy family members of patients with inflammatory bowel disease (22). In contrast to CCA, these heterogeneous antibodies contain λ and κ light chains, γ, μ, and α heavy chains, and, occasionally, secretory IgA (8). They react with sterile human newborn colon, adult normal or diseased colon (23), germ-free rat colon (4), and *E. coli* 014 (7, 24).

Immunofluorescent staining with CCA outlined the basement membrane and periphery of colonic epithelial cells only in diseased tissue. This phenomenon is probably related to destruction of the epithelial basement membrane, which is considered to be an early morphological sign of the disease (25). Similar studies with circulating antibodies showed staining of goblet cells, glandular luminal mucus (5, 26), and cytoplasm of colonic epithelial cells without staining of basement membrane or peripheral cellular areas (2, 6).

It is conceivable that bacterial antigens in colonic biopsy sections may theoretically react or crossreact with CCA. However, peripheral, rather than luminal, staining with CCA in diseased tissue and lack of staining of colonic biopsy specimens from normal subjects suggest that the antigen(s) to CCA is a constituent of colonic tissue and is unrelated to fecal bacteria. Furthermore, preliminary studies with *E. coli* 014, 0119:B14, and 075 (The American Type Culture Collection, Rockville, MD) do not reveal agglutination in the presence of CCA (unpublished experiments). Reaction of CCA with antigens from anerobic bacteria has not been investigated.

Results of the present studies permit formulation of experimentally testable hypothesis for a relation between CCA and the pathogenesis of ulcerative colitis. The local S–IgA system is deficient in histologically abnormal and normal colonic mucosa in patients with idiopathic proctitis (12), abnormal colonic mucosa of patients with ulcerative colitis (13), and in the saliva of patients with ulcerative colitis and their relatives (14). This deficiency may compromise host mucosal defenses. Since secretory IgA could not be available to bind and agglutinate putative antigen(s) such as food or bacteria, and prevent their penetration through the mucosal barrier (27, 28), such antigen(s) could thus associate with colonic epithelial cells. A response to this antigenic stimulus may enhance CCA production. This is in agreement with immunofluorescent investigations showing a 30-fold increment of IgG immunocytes in the lamina propria in ulcerative colitis (29). CCA may also combine with epithelial cell constituents, as suggested by staining and binding of CCA to the colonic mucosa of patients with ulcerative colitis and idiopathic proctitis. The reaction between IgG (CCA) and antigen may activate complement, release chemotactic factors, and result in a local reaction of the Arthus type. By the immunoperoxidase technique, IgG and β_1C have been demonstrated on the basement membrane of colonic epithelium in patients with active ulcerative colitis (30). Increased local synthesis of IgG may protect against infection (31) and, by complexing with antigen, assist in hepatic removal of circulating antigens (32). Circulating immune complexes found during active stages of ulcerative colitis contain IgG (10, 33). Whether they contain CCA is unknown. CCA, or CCA complexed to target organs, may lead to cytolysis by an antibody-mediated lymphocyte or monocyte-dependent cytotoxicity process (34–36).

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