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#### Research Article

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## Composition of Human Colonic Mucin

#### SELECTIVE ALTERATION IN INFLAMMATORY BOWEL DISEASE

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ABSTRACT Human colonic mucin has been isolated from mucosal scrapings of fresh surgical specimens of normal controls as well as patients with Crohn's colitis and ulcerative colitis. Following sonication and ultracentrifugation, mucin fractions were separated from other soluble colonic glycoproteins by Sepharose 4B chromatography. After nuclease digestion, cesium chloride gradient centrifugation of the excluded material yielded colonic mucin with an average buoyant density of 1.52 g/ml. Subsequent chromatography of the apparently homogeneous colonic mucin on DEAEcellulose revealed the presence of at least six distinct mucin species (mucin I-VI). Each mucin species was found to have a distinctive hexose, hexosamine, sialic acid, and sulfate content as well as blood group substance activities. Mucin from five patients with Crohn's colitis was found to represent a mixture of at least six discrete species comparable to those isolated from normal colonic specimens. However, in mucin from eight patients with ulcerative colitis there was a marked and selective reduction of one component mucin subclass, designated species IV. Normal mucin and mucin from patients with Crohn's disease contained 48±17 and  $42\pm12$  mg of species IV/g, while mucin from patients with ulcerative colitis had  $5\pm3$  mg/g solubilized glycoprotein. The selective absence of species IV was found in preparations from both sigmoid (n = 7) and ascending (n = 4) colon and could not be accounted for by an overall decrease in total mucin content. The selective reduction of species IV was also found in mucin isolated from relatively noninflamed colonic mucosa of patients with ulcerative colitis. The carbohydrate composition and blood group activities of the remaining five mucin species were similar to their normal counterparts. Based on the results to date, there appears to be an underlying selective decrease of one colonic mucin subclass in ulcerative colitis.

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#### INTRODUCTION

Despite extensive studies over the last several decades, little is known about fundamental causes underlying ulcerative colitis and Crohn's colitis. Past investigative efforts have concentrated on the possible contributions of infectious agents or alterations in immunologic defenses. Relatively little attention has been directed toward the determination of structural defects of the bowel itself that might ultimately lead to the development of these idiopathic inflammatory conditions. Thus, comparatively little detailed information is available on the composition and structure of the colonic mucosa (1-3).

Certainly one important component of the normal colonic surface that serves to maintain its integrity is the dense layer of glycoprotein that coats the colonic surface (4). This coat appears to include both intrinsic colonic surface membrane-bound glycoproteins and high-molecular weight mucin glycoproteins secreted by goblet cells (5-7). Mucin glycoproteins are capable of forming a viscoelastic gel which, in addition to lubrication of the colonic surface to permit passage of luminal contents, may play an important role in protecting the colonic epithelium from chemical and physical injury as well as resisting the invasion of potential pathogens. Knowledge of the normal composition and structure of these potentially important compounds is incomplete. Marshall and Allen (8) isolated mucin from porcine colon and found that it was composed of a single, apparently homogeneous largemolecular weight species (8). Similarly, Murty et al. (9) and Slomiany et al. (10) describe a single species of colonic mucin in rats. The latter workers have demonstrated that this substance contains a wide range of oligosaccharide side chains of varying lengths. However, other studies have suggested that colonic mucin may contain more than one distinct species. Inoue and Yosizawa (11) found that apparently homogeneous pig colonic mucin contained four chromatographically

distinct subfractions, all of which were sulfated. More recently, LaMont and Ventola (12) found that rat colonic mucin could be fractionated into two major components, in addition to several other lesser constituents (12). Results of other studies have also suggested that human colonic mucin may contain more than one distinct species (13-14).

Several reports indicate that alterations in mucin content may be associated with various diseases including inflammatory bowel disease (15-17). However, despite the long-standing awareness that goblet cell mucin depletion is a frequent histologic hallmark of ulcerative colitis, more detailed information is limited (18-19). Histochemical studies have suggested that sulfomucins are more decreased than sialomucins (20). Ehsanullah et al. (21) using histochemical methods found an absolute excess of sialomucins in ulcerative colitis, which was not found in patients with nonspecific proctitis. Interestingly, they found persistence of the abnormal histochemical staining pattern during periods of remission in patients with residual dysplasia. These observations are supported by the work of Boland and co-workers (22) who studied glycoconjugate staining in rectal biopsies of patients with ulcerative colitis using fluoresceinated lectins. These investigators found a marked reduction in binding of Dolichos biflorus and soybean agglutinin lectins. Conversely, they noted an increase in peanut agglutinin binding, which appeared to identify a subpopulation at risk for the subsequent development of dysplasia.

The nature of the actual composition or structural changes underlying these altered lectin and histochemical staining properties remain unclear. Teague et al. (23) and Fraser et al. (24) have analyzed total monosaccharide content of mucin-containing fractions from biopsy and colectomy specimens and observed a substantial increase in mannose-containing fractions in patients with ulcerative colitis. Mannose is not typically an integral constituent of mucin class glycoproteins. They suggested that mannose-containing glycoproteins may alter the viscoelastic properties of the mucin glycoproteins.

MacDermott et al. (25) and Neutra et al. (26) have shown that cultured colonic mucosa is able to synthesize and secrete glycoproteins but little is known about alterations in this mechanism(s) in inflammatory bowel disease. In earlier studies, Kim and Isaacs (27) found no significant change in several glycosyltransferase activities in colonic mucosa of patients with granulomatous or ulcerative colitis.

The current studies were undertaken to characterize the content of colonic mucin glycoproteins more directly. We describe the isolation and partial characterization of mucin from normal human colon as well as patients with ulcerative colitis and Crohn's disease.

#### METHODS

Colonic specimens. Specimens of human sigmoid colon, each  $\sim 5 \times 12$  cm, were obtained from nine patients undergoing elective colonic resection for either diverticular disease (eight patients) or recurrent, benign sigmoid volvulus (one patient). Specimens of normal human ascending colon were also obtained from two patients with diverticulosis. Histological examination in the Pathology Department at this hospital confirmed the absence of inflammation or other mucosal abnormalities; these samples were considered "normal controls." Specimens of human colon were also obtained from eight patients with ulcerative colitis and five patients with Crohn's disease. Portions of both ascending and sigmoid colon were obtained in four patients with ulcerative colitis. A brief summary of the salient features of each inflammatory disease patient's clinical status is presented in Table I. In each instance histologic examination confirmed the clinical impression. All specimens of sigmoid colon demonstrated active inflammatory disease; two specimens of ascending colon also demonstrated gross involvement, while two specimens of ascending colon were relatively spared.

Specimens were obtained in the operating room within 10 min after interruption of the vascular supply and placed on ice. All isolation and purification procedures were carried out separately on material from individual specimens. These studies were approved by the Human Studies Committee of the Massachusetts General Hospital.

Isolation, purification, and fractionation of colonic mucin. Surgical specimens were washed in ice-cold phosphatebuffered saline (PBS) containing 2 mM phenylmethyl sulfonic fluoride, gentamycin  $(15 \,\mu g/m)$ , and penicillin (10,000U/ml) in order to remove any adherent fecal material. Colonic mucosal scrapings were obtained in 25 ml buffer after removal of the mucosa by sharp dissection, as previously described (12). The resulting suspension of mucosal scrapings was sonicated in 8, 15-s bursts with intermediate cooling using a Branson S-75 sonifier (No. 6 power setting, Branson Sonic Power Co., Danbury, CT). After ultracentrifugation at 105,000 g for 60 min, the supernatant containing soluble glycoprotein material was dialyzed against deionized water  $(0^\circ - 4^\circ C)$  and lyophilized.

Lyophilized material (120-245 mg) was subsequently dissolved in 2.5-5.0 ml 0.01 M Tris-HCl, pH 8.0 and applied to a 2.5  $\times$  100-cm column of Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in the application buffer. Column elution was carried out at a rate of  $\sim$ 18 ml/ h, collecting 4.5-ml fractions and monitored for neutral hexose (see below) and protein (continuous A<sub>280</sub>). Hexose-containing fractions (see below) in the excluded volume were pooled, dialyzed exhaustively against water (0°-4°C), and lyophilized. This material was rechromatographed on Sepharose 4B using the same conditions as before and the excluded carbohydrate-containing material (termed crude colonic mucin) was dialyzed and lyophilized before further purification steps.

Crude colonic mucin isolated from the Sepharose 4B excluded volume was subsequently digested with bovine DNase I and bovine RNase III (Sigma Chemical Co., St. Louis, MO) by the method of Marshall and Allen (8) to permit removal of nucleic acid contaminants. After overnight digestion (14 h) at room temperature in 5.0 ml PBS, pH 7.4, containing 1 mM MgSO<sub>4</sub>, the digest mixture was centrifuged at 15,000 g for 30 min to remove the resultant flocculent precipitate and the supernatant then dialyzed exhaustively against PBS (0°-4°C). Subsequently the volume of the dialyzed material was adjusted to 25.0 ml with PBS and 13.48

|            | Diagnosis          | Patient<br>No. | Age/<br>Sex | Disease<br>duration | Tissue studied              | Pathologic findings                                                                                                  | Blood<br>group |
|------------|--------------------|----------------|-------------|---------------------|-----------------------------|----------------------------------------------------------------------------------------------------------------------|----------------|
| <b>A</b> . | Ulcerative colitis | 1              | 35/M        | 7 yr                | Sigmoid and ascending colon | Diffuse ulcerative colitis; acute and chronic inflammation                                                           | 0-             |
|            |                    | 2              | 9/M         | 9 mo                | Ascending colon             | Ulcerative colitis, acute and chronic inflammation                                                                   | <b>A</b> +     |
|            |                    | 3              | 39/F        | 9 yr                | Sigmoid and ascending colon | Ulcerative colitis; ascending colon grossly<br>normal (crypt abscesses at sample<br>margin)                          | 0+             |
|            |                    | 4              | 49/M        | 12 mo               | Sigmoid                     | Ulcerative colitis; multiple pseudopolyps<br>and focal ulceration                                                    | <b>A</b> +     |
|            |                    | 5              | 22/M        | 5 yr                | Sigmoid                     | Ulcerative colitis; diffuse congestion,<br>edema with focal ulceration                                               | 0-             |
|            |                    | 6              | 42/F        | 2 yr                | Sigmoid and ascending colon | Extensive active ulcerative colitis; relative<br>sparing of ascending colon (crypt<br>abscess only at sample margin) | 0+             |
|            |                    | 7              | 59/F        | 5 yr                | Sigmoid                     | Ulcerative colitis; multiple superficial ulcers                                                                      | 0+             |
|            |                    | 8              | 59/M        | 3 yr                | Sigmoid                     | Active ulcerative colitis; multiple superficial ulcers                                                               | 0+             |
| B.         | Crohn's<br>colitis | 1              | 16/M        | 3 yr                | Sigmoid •                   | Focal erythema, chronic inflammation,<br>thickend and stenotic ileum                                                 | 0+             |
|            |                    | 2              | 31/F        | 7 yr                | Sigmoid •                   | Mucosal atrophy, submucosal fibrosis,<br>isolated ulcers                                                             | 0-             |
|            |                    | 3              | 45/F        | 12 yr               | Sigmoid                     | Acute and chronic inflammation with noncaseating granulomata                                                         | 0+             |
|            |                    | 4•             | 48/M        | 8 yr                | Sigmoid •                   | Multiple fissures and linear ulcers, chronic submucosal inflammation                                                 | 0+             |
|            |                    | 5              | 43/F        | 2 yr                | Sigmoid                     | Chronic inflammation with granuloma formation                                                                        | 0+             |

 TABLE I

 Summary of Inflammatory Bowel Disease Patient Data

• Also had ileal involvement.

g CsCl added; the sample was then distributed into nitrocellulose tubes for centrifugation. Ultracentrifugation was carried out in a Beckman L-50 preparative centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) using a Beckman SW41 rotor at 36,000 rpm for 48 h. After centrifugation, eight sequential 1.5-ml aliquots were placed into preweighed test tubes. After determination of density of the individual fractions, each was dialyzed exhaustively against water (0°-4°C) and then assayed for neutral hexose, protein, and nucleic acid as described below. Fractions with a density >1.48 (fractions 6-8) were pooled and represented "isolated pure human colonic mucin."

Fractionation of human colonic mucin. Purified, isolated human colonic mucin was lyophilized and resuspended in 1.0-2.0 ml 0.01 M Tris-HCl pH 8.0. The sample was applied to a  $1.5 \times 100$ -cm column of DEAE-cellulose (Whatman Chemical Separation Inc., Clifton, NJ) equilibrated in the application buffer. The column was eluted over 24 h with a discontinuous gradient of 50 mM increments in NaCl concentration (0–0.25 M) in 0.01 M Tris HCl, pH 8.0, followed by 5.0 M NaCl in the same buffer, at a rate of 15 ml/h in 3.75-ml fractions. Continuous  $A_{280}$  monitoring was maintained during column elution and individual fractions were assayed for hexose using 0.3-ml aliquots as described below. Fractions within each hexose-containing peak were pooled, the individual peaks exhaustively dialyzed against water (0°-4°C) and then lyophilized. Individual peaks were rechromatographed on DEAE cellulose under identical conditions before further characterization.

Analytical procedures. Neutral hexose was measured on column elutions by the phenol- $H_2SO_4$  method (28). Protein was monitored by measuring absorbance at 280 nm or by

the Lowry method (29), while nucleic acid was measured by absorbance at 260 nm or by the method of Chen et al. (30). Uronic acid was determined by the method of Bitter and Muir (31) and sulfate content assessed by a modification of the method of Spencer (32), following hydrolysis in 25% formic acid at 110°C for 18 h. Carbohydrate composition was determined on trimethylsilyl derivatives prepared by modification of published methods (33) after addition of inositol as internal standard. Analysis was carried out on a Varian 6000 gas chromatograph (Varian Associates, Inc., Palo Alto, CA) linked to a Hewlett Packard 3300A recording integrator (Hewlett-Packard Co., Palo Alto, CA). Aliquots of each sample were analyzed in 10-ft columns of 3% OV-17 (Supelco, Inc., Bellefonte, PA) and 3% SE-30 (Supelco, Inc.) using conditions previously described (33). Sialic acid content determinations were corroborated by the thiobarbituric acid method following hydrolysis of samples in 0.1 M H<sub>2</sub>SO<sub>4</sub> at 80°C for 1 h (34).

Blood group substance activity. Fresh type A, B, and O human erythrocytes were washed several times in 0.154 M NaCl, pH 7.4 with the volume adjusted to yield a 2% (vol/ vol) suspension. Anti-A, anti-B sera, or Ulex europus lectin (blood group H specific) were diluted in PBS, pH 7.4, to yield a concentration just sufficient for 50  $\mu$ l to agglutinate the appropriate specific erythrocyte suspension at room temperature after 30 min. Purified mucin fractions were suspended in PBS. Mucin samples or buffer (50  $\mu$ l) were added to 50- $\mu$ l erythrocyte suspensions. After 30-min incubation at room temperature, 50  $\mu$ l anti-A, anti-B sera, Ulex europus lectin, or buffer was added. Hemagglutination was assessed after 30 min of additional incubation at room temperature and expressed as the concentration of purified fraction necessary to inhibit agglutination.

#### RESULTS

Human colonic mucin was purified after solubilization by sequential Sepharose 4B chromatography, CsCl gradient ultracentrifugation and DEAE-cellulose chromatography. To obtain human colonic mucin most closely resembling native species, specimens of human sigmoid colon were obtained immediately following resection. Soluble glycoprotein material was obtained from isolated mucosa of individual specimens through a sequential process of scraping, sonication, and ultracentrifugation, yielding 180±38 mg lyophilized material/g wet wt of mucosa (termed "soluble glycoprotein"). This complex mixture was fractionated on Sepharose 4B to separate large-molecular weight fractions (excluded volume) containing mucin glycoprotein from other solubilized components (Fig. 1 A), with a calculated recovery of protein and carbohydrate of 82 and 87%, respectively. Approximately 85-90% of the material found in the excluded volume was again recovered in this position following rechromatography on Sepharose 4B for an overall yield in this peak of  $95\pm 22 \text{ mg/g}$  wet wt (Fig. 1 B).

Mucin in the Sepharose 4B excluded peak was further purified by CsCl density centrifugation. However, pilot studies demonstrated that the  $\sim 10\%$  (wt/wt) nucleic acid contamination of this material, found on preliminary compositional analysis could not be separated from carbohydrate-containing components during or after CsCl gradient centrifugation (Fig. 2 A). To obviate this problem, the Sepharose 4B-excluded material was first subjected to combined DNase and RNase digestion, as suggested by Marshall and Allen (8) who encountered similar difficulties with pig colonic mucin. Assay of nucleic acid content following nuclease digestion and dialysis confirmed the elimination of this contaminant (Table II). After nuclease digestion, mucin purification was achieved by subsequent CsCl gradient centrifugation. Mucin fractions were found in fractions of density >1.48 g/ml as previously described (8), with an average density of 1.52 g/ml (Fig. 2 B). Overall recovery of material from CsCl gradients was found to average 84% with a final yield of  $63\pm12$  mg mucin/g mucosal wet wt or  $300\pm25$ mg/g soluble glycoprotein.

To assess the heterogeneity of purified mucin, material recovered after CsCl gradient centrifugation was applied to a DEAE-cellulose column, and developed with a discontinuous NaCl gradient. As demonstrated in Fig. 3, the purified mucin was found to elute as six discrete carbohydrate-containing peaks. In addition, material eluting at high salt concentrations appeared to contain two incompletely separated components. These components, not separable by the sequence of Sepharose 4B chromatography, density centrifugation, and ion exchange chromatography, were pooled in further studies and termed fraction VI. Each of the six peaks was rechromatographed on DEAE-cellulose under identical conditions. The individual peaks were each recovered in positions identical to their respective recoveries from initial DEAE-cellulose chromatography (data not shown). The presence of these six discrete species (I-VI) was observed in each of nine mucin preparations isolated from individual surgical specimens. Mucin species II, III, IV, and VI eluting at intermediate and higher concentrations of NaCl were most abundant, representing collectively  $\sim 75\%$  of total mucin recovered; recovery ranged from 89 mg (mucin III) to 15 mg (mucin I)/g soluble glycoproteins as detailed in Table III.

Mucin fractions I-VI were further studied in order to determine whether they represented compositionally distinct mucin species. The results of compositional analysis of mucins I-VI are detailed in Table II and compared with the unfractionated material. Neither the unfractionated mucin nor the individual peaks recovered after DEAE-cellulose chromatography contained measurable uronic acid indicating the absence of significant contamination by glycosaminoglycan. As expected, both unfractionated and fractionated mucin were found to contain predominantly carbohydrate (5-7). Although protein was present in



FIGURE 1 Sepharose 4B column chromatography of solubilized human colonic glycoproteins. Lyophilized glycoproteins (120-245 mg) were applied to column ( $2.5 \times 80$  cm) of Sepharose 4B equilibrated in 0.01 M Tris HCl, pH 8.0, and the column developed at a rate of 15 ml/h, collecting 3.75-ml fraction under continuous A<sub>280</sub> monitoring. Hexose was monitored on individual fractions by the phenol-H<sub>2</sub>SO<sub>4</sub> method (23). Material in the excluded volume (Vo) was dialyzed exhaustively against deionized H<sub>2</sub>O and lyophilized. (A) Chromatography of crude glycoprotein solubilized from human colonic mucosal scrapings (B) Repeat chromatography of material in excluded volume (Vo) of A. Material in excluded volume of B was termed "crude colonic mucin" and subjected to further purification.

substantially lower relative amounts than carbohydrate, mucin I-VI varied in percentage of protein content from 16.9 to 46.5%. Mucin species IV and V contained the highest sulfate content (4.6 and 6.1% dry wt, respectively), while other fractions had markedly lower sulfate content. Mucin IV appeared to represent the major sulfomucin species present.

Carbohydrate composition was assessed by gas chromatography and chemical assay. All fractions contained substantial amounts of galactosamine, galactose, and glucosamine, typically found in mucin glycoproteins (35). Mucin fractions II-VI contained substantial amounts of sialic acid and lesser concentrations of fucose. In general, the later eluting main peaks contained progressively greater amounts of sialic acid. However, mucin VI, which eluted only with highest salt concentration, had lesser amounts of sialic acid. Mannose was not detected in four of six species (mucin III-VI), suggesting the absence of any contaminating nonmucin glycoprotein in these fractions. Minimal amounts of mannose were detected in mucin I and II indicating the potential presence of either small concentrations of nonmucin contaminants or, alternatively, the presence of true mixed glycoproteins containing both Oglycosidic and N-glycosidic (high mannose or complex type) oligosaccharides. In addition, preliminary examination of amino acid composition analysis suggest the presence of distinctive core proteins.<sup>1</sup>

<sup>&</sup>lt;sup>1</sup> Podolsky, D. K., H. Keutmann, and K. J. Isselbacher. Manuscript in preparation.



FIGURE 2 CsCl equilibrium centrifugation of human crude colonic mucin. Lyophilized human crude colonic mucin (43-85 mg) isolated by double-Sepharose 4B column chromatography (as in Fig. 1) was centrifuged 36,000 rpm for 48 h in 38% (wt/vol) CsCl gradient. (A) Before nuclease digestion. (B) After nuclease digestion (see Methods section for details). After centrifugation, 1.5-ml fractions were collected in preweighed test tubes for density determination. Density after centrifugation ranged from 1.36 g/ml (fraction 1) to 1.56 g/ml (fraction 8). Subsequently, fractions were exhaustively dialyzed against deionized water and monitored for nucleic acid (A<sub>260</sub>), protein (A<sub>280</sub>), and hexose [phenol-H<sub>2</sub>SO<sub>4</sub> method, A<sub>492</sub> (23)].

Mucin was subsequently purified from colon specimens of patients with active ulcerative colitis and Crohn's colitis. Overall recovery of material from CsCl gradients was found to be  $82\pm7\%$  with a final yield of  $215\pm32$  mg mucin/g soluble glycoprotein in specimens from patients with ulcerative colitis (n = 11) and  $287\pm43$  mg/g in patients with Crohn's colitis (n = 5)indicating a reduction in total mucin content recovered in patients with ulcerative colitis (P < 0.05).

Purified mucin isolated from patients with inflammatory bowel disease was also fractionated on DEAE- cellulose to assess the compositional heterogeneity. As demonstrated in a representative chromatographic profile depicted in Fig. 4, purified mucin from sigmoid colon mucosa of patients with ulcerative colitis appeared to contain only five of the six species found in normal mucin. The mucin from patients with ulcerative colitis was markedly deficient in the mucin species normally eluted in 200 mM NaCl (designated IV). The chromatographic mobility of the remaining species appeared identical to that observed in normal colonic mucin. The selective diminution or absence of mucin species IV was observed in each of seven separate sigmoid colon specimens. Results of quantitative determinations of recovery of the individual species are detailed in Table III. Although there was an overall decrease in total mucin content from mucosa of patients with ulcerative colitis, as noted above, the deficiency in species IV appeared to be selective, with a mean reduction of species IV recovery of >87%, in contrast to a more modest reduction of total mucin (27%) and of other, individual mucin species (0-36%).

In contrast to the results in patients with ulcerative colitis, mucin from patients with Crohn's colitis was found to contain six discrete mucin species similar to those detected in normal controls (Fig. 5). Quantitation of separate mucin species I-VI from five patients with Crohn's disease revealed similar amounts of each fraction when compared with normal controls (Table III).

Mucin was also isolated from four segments of proximal ascending colon in patients undergoing total colectomy for ulcerative colitis. In two specimens, the proximal colon was also grossly inflamed. However, in two patients there was relative sparing of the proximal bowel (gross disease began in midtransverse colon in one patient and hepatic flexure in the other), although areas adjacent to the specimens did have microscopic evidence of disease. Mucin preparation from these segments were found to be similar to those obtained from sigmoid colon specimens with gross colitis (Table IV). Again there was a marked and selective reduction in the mucin IV. It should be noted that this decrease was observed both in ascending colonic specimens without gross disease as well as in those more obviously involved by the inflammatory process. Mucin isolated from two specimens of normal control ascending colon revealed a mucin species pattern identical to that found in the normal sigmoid colon (data not shown). Thus, the decrease in species IV in relatively spared segments of ascending colon in patients with ulcerative colitis does not appear to be related to normal regional variation in colonic mucin composition.

The composition of mucin fractions I-VI from patients with ulcerative colitis was determined in order to assess the relationship to their counterparts isolated from normal colon (Table V). The marked decrease

|                      |                        |                | Mucin species§ |                |                |                |                |                |  |  |
|----------------------|------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|--|--|
| Chemical composition | Crude*<br>mucin        | Puret mucin    | 1              | II             | 111            | IV             | v              | VI             |  |  |
| Protein              | 38.9±3.8 <sup>  </sup> | 31.1±2.6       | 42.5±3.8       | 26.4±2.9       | 24.5±3.8       | 16.9±2.1       | 28.4±3.1       | 33.1±3.8       |  |  |
| Carbohydrate         |                        |                |                |                |                |                |                |                |  |  |
| Fucose               | $0.8 \pm 0.02$         | $3.2 \pm 0.4$  | $1.2 \pm 0.1$  | $3.8 \pm 0.3$  | $7.4 \pm 2.4$  | $8.1 \pm 2.2$  | 3.7±0.6        | $1.0 \pm 0.2$  |  |  |
| Mannose              | $4.6 \pm 1.2$          | <0.5±0.3       | <0.5±0.3       | $1.5 \pm 0.5$  | O¶             | 0              | 0              | 0              |  |  |
| Galactose            | 7.4±0.8                | $13.4 \pm 1.1$ | $2.3 \pm 0.1$  | $10.2 \pm$     | $14.6 \pm 2.3$ | $14.7 \pm 2.6$ | 13.3±2.8       | 14.1±2.9       |  |  |
| Glucose              | 2.4±0.8                | <0.5±0.2       | <0.5±0.3       | $1.0 \pm 0.3$  | <0.5±0.3       | 0              | 0              | 0              |  |  |
| Galactosamine        | $9.2 \pm 1.1$          | $14.2 \pm 2.1$ | 4.8±0.9        | $12.5 \pm 1.8$ | 18.4±3.4       | $15.4 \pm 2.8$ | 10.6±11.9      | $14.2 \pm 2.3$ |  |  |
| Glucosamine          | $12.8 \pm 2.1$         | $12.6 \pm 1.8$ | $1.3 \pm 0.1$  | $11.5 \pm 1.5$ | $16.5 \pm 2.8$ | $14.5 \pm 3.1$ | $14.5 \pm 3.2$ | 14.1±3.8       |  |  |
| Sialic acid          | 6.9±1.0                | 4.8±0.3        | $1.10 \pm 0.2$ | 7.1±0.8        | $7.8 \pm 1.1$  | $12.8 \pm 1.2$ | $13.8 \pm 2.1$ | 10.4±1.4       |  |  |
| Sulfate              | $1.9 \pm 0.1$          | $2.0 \pm 0.1$  | $1.5 \pm 0.2$  | $1.2 \pm 0.1$  | $1.5 \pm 0.3$  | 4.6±0.6        | 6.1±1.1        | 2.5±1.2        |  |  |
| Nucleic acid         | $11.2 \pm 2.8$         | 0              | 0              | 0              | 0              | 0              | 0              | <1.0           |  |  |

TABLE II Composition of Human Colonic Mucin

• Crude mucin recovered in excluded volume after double-Sepharose 4B chromatography (see text and Fig. 1 B).

‡ Pure mucin recovered after CsCl equilibrium centrifugation following nuclease digestion and dialysis (see text and Fig. 2 B).

§ Individual mucin species recovered after DEAE-cellulose chromatography of pure mucin (see text and Fig. 3).

<sup>II</sup> Percent dry weight±SD.

¶0, not detectable.

in recovery of species IV precluded meaningful determinations of this fraction in ulcerative colitis. The remaining five mucin species were found to contain large amounts of bound carbohydrate with protein representing only 22.4-38.5% of total dry weight. Carbohydrate composition of the five species closely resembled that found in their normal counterparts (Table II). All contained substantial amounts of hexos-



FIGURE 3 DEAE-cellulose chromatography of pure human colonic mucin. Human mucin recovered from CsCl equilibrium centrifugation (>1.48 g/ml; fractions 6-8) was lyophilized and applied to DEAE-cellulose column ( $1.5 \times 100$  cm) equilibrated in 0.01 M Tris HCl, pH 8.0. Column was developed at a rate of 15 ml/h, collecting 3.75-ml fractions with a discontinuous NaCl gradient (from 0 to 0.25 M in 0.05 M increments) followed by elution with 5.0 M NaCl in application buffer under continuous A<sub>280</sub> monitoring. Total hexose in individual fractions was determined by phenol-H<sub>2</sub>SO<sub>4</sub> method at A<sub>492</sub> (23).

| -                  | -        |                 |       |                     |        |       |              |  |  |  |  |
|--------------------|----------|-----------------|-------|---------------------|--------|-------|--------------|--|--|--|--|
|                    |          | Mucin fractions |       |                     |        |       |              |  |  |  |  |
| Diagnosis          | I        | 11              | 111   | IV                  | v      | VI    | Total        |  |  |  |  |
|                    |          |                 | mg    | g solubilized prote | tin±SD |       |              |  |  |  |  |
| Normal*            |          |                 |       |                     |        |       |              |  |  |  |  |
| $(n = 9)\ddagger$  | $15\pm5$ | 68±15           | 89±12 | 48±14               | 22±9   | 71±16 | $300 \pm 25$ |  |  |  |  |
| Ulcerative colitis |          |                 |       |                     |        |       |              |  |  |  |  |
| (n = 8)            | 17±8     | 58±12           | 57±11 | 5±3§                | 16±10  | 75±16 | 215±25       |  |  |  |  |
| Crohn's colitis    |          |                 |       |                     |        |       |              |  |  |  |  |
| (n = 5)            | 19±10    | 72±14           | 79±18 | 32±12               | 14±5   | 70±9  | 287±37       |  |  |  |  |

 TABLE III

 Quantitation of Colonic Mucin Fractions Isolated by DEAE Cellulose Chromatography

\* Sigmoid colon resected for diverticulosis (without active diverticulitis at time of operation) or benign volvulus.

‡ Refers to number of patients.

P < 0.001.

amine with a predominance of galactosamine. It should be noted that each species appeared to contain the same absolute and relative amount of sialic acid and sulfate as their corresponding fractions isolated from normal colon. The constant relative composition of normal and ulcerative colitis fractions suggests that the reduction in species IV does not reflect altered composition of this component with resulting altered chromatographic properties such that it comigrates with one of the other mucin species.

To further examine the relationship between the normal mucin species and those isolated from patients with ulcerative colitis, blood group activity of each fraction was determined by hemagglutination inhibition (Table VI). Indeed, blood group activity of the corresponding fractions were remarkably similar. Mucin I had no detectable activity for any of the major determinants, while mucins II, III, and VI had activity against all tested antisera, although at different concentrations. In contrast, mucin V inhibited only substance H. Mucin IV isolated from normal sigmoid colon appeared to possess both group B and H determinants.

#### DISCUSSION

Mucin forms an integral part of the viscoelastic gel coating the surface mucosa and thus is potentially an



FIGURE 4 Heterogeneity of colonic mucin in ulcerative colitis. Purified sigmoid colon mucin isolated from a patient with ulcerative colitis (patient A5; Table I) was lyophilized and applied to DEAE-cellulose column (1.5  $\times$  100 cm) equilibrated in 0.01 M Tris HCl, pH 8.0. Column was developed at a rate of 15 ml/h collecting 3.75-ml fractions with a discontinuous NaCl gradient (0-0.250 M; 0.05 M increments) followed by elution with 5.0 M NaCl in application buffer under continuous A<sub>280</sub> monitoring. Total hexose in individual fractions was determined by phenol-H<sub>2</sub>SO<sub>4</sub> method at A<sub>492</sub>.



FIGURE 5 Heterogeneity of colonic mucin in Crohn's colitis. Colonic mucin purified from sigmoid colon of patient with Crohn's colitis (patient B3; Table I) was suspended in 1.5 ml 0.01 M Tris HCl, pH 8 and applied to DEAE-cellulose column (1.5  $\times$  100 cm). The column was developed with discontinuous ascending NaCl gradient as described in legend to Fig. 1; elution was monitored by A<sub>280</sub> absorbance for protein and phenol-H<sub>2</sub>SO<sub>4</sub> determination for hexose (A<sub>492</sub>).

essential factor in maintenance of normal colonic function and resistance to disease. However, our understanding of its role in health and disease has been limited by the lack of detailed information on composition and structure. In this study we have isolated human colonic mucin from fresh surgical specimens of both normal controls and patients with inflammatory bowel disease involving the colon.

Mucin isolated in these studies was devoid of detectable contamination by either nucleic acid- or uronic acid-containing substances, and thus appeared to be pure normal colonic mucin. Of particular importance was the finding that purified human colonic mucin is not homogeneous; rather it represents a mixture of at least six species separable by DEAE-cellulose chromatography (Fig. 3). Each of the six fractions had

 TABLE IV

 Colonic Mucin Fractions in Ulcerative Colitis

| Mucin fractions |                     |                                             |                                                                               |                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                 |
|-----------------|---------------------|---------------------------------------------|-------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1               | 11                  | 111                                         | IV                                                                            | v                                                                                                                                                                                                                                          | vi                                                                                                                                                                                                                                                                              |
|                 | mg/g sol            | ubilized g                                  | lycoprote                                                                     | ein (mean                                                                                                                                                                                                                                  | )                                                                                                                                                                                                                                                                               |
|                 |                     |                                             |                                                                               |                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                 |
| 17              | 58                  | 57                                          | 5                                                                             | 16                                                                                                                                                                                                                                         | 75                                                                                                                                                                                                                                                                              |
|                 |                     |                                             |                                                                               |                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                 |
|                 |                     |                                             |                                                                               |                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                 |
| 24              | 54                  | 51                                          | 5                                                                             | 24                                                                                                                                                                                                                                         | 70                                                                                                                                                                                                                                                                              |
|                 |                     |                                             |                                                                               |                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                 |
|                 |                     |                                             |                                                                               |                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                 |
| 21              | 52                  | 65                                          | 8                                                                             | 30                                                                                                                                                                                                                                         | 68                                                                                                                                                                                                                                                                              |
|                 | 1<br>17<br>24<br>21 | 1 11<br>mg/g sol<br>17 58<br>24 54<br>21 52 | Mucin 1<br>1 11 111<br>mg/g solubilized g<br>17 58 57<br>24 54 51<br>21 52 65 | Mucin fractions           I         II         III         IV           mg/g solubilized glycoprot.           17         58         57         5           24         54         51         5           21         52         65         8 | Mucin fractions           I         II         IV         V           mg/g solubilized glycoprotein (mean         17         58         57         5         16           24         54         51         5         24           21         52         65         8         30 |

a characteristic and distinctive protein, total carbohydrate and sulfate composition (Table II). Furthermore, each fraction had a unique amino acid<sup>1</sup> and carbohydrate composition varying specifically in hexosamine, galactose, and sialic acid. Inasmuch as the mucin species were defined by their mobility on an ion exchange resin (DEAE-cellulose), it is possible that the elution pattern might be due to a variation of the sialic acid content of otherwise similar molecules. However, as noted in Results, the order of elution could not be related simply to sialic acid (or sulfate) content. Furthermore, the molar ratios of the various sugar residues confirmed the distinctive composition of each mucin peak obtained by DEAE-cellulose chromatography (data not shown). However, it should be noted that the derived molar ratios almost certainly represented a mean of a range of different oligosaccharide side chains in each mucin species (10). The finding of a consistent composition in the species isolated from nine separate colonic specimens supports the validity of the distinctions in protein, carbohydrate, and sulfate content. Studies are currently in progress to determine actual oligosaccharide and peptide structure of the six individual mucin species. We are currently attempting to develop antisera specific to each of the mucin species I-VI.

Collectively, these data demonstrate the presence of a complex mixture of sulfate- and nonsulfate-containing mucins in the normal human colon. Gold, Shochat, and Miller (14) previously reported the detection of at least two immunologically distinct human colon mucins (14). The present studies confirm and extend

|               | Mucin fractions* |                |                |      |                |                |  |  |  |
|---------------|------------------|----------------|----------------|------|----------------|----------------|--|--|--|
| Composition   | 1                | 11             | 111            | īvţ  | v              | vi             |  |  |  |
|               |                  |                | % dry weigh    | t±SD |                |                |  |  |  |
| Protein       | 38.5±3.5         | 25.4±2.9       | $22.4 \pm 4.1$ |      | $23.5 \pm 2.8$ | 31.9±3.4       |  |  |  |
| Carbohydrate  |                  |                |                |      |                |                |  |  |  |
| Fucose        | $1.0 \pm 0.2$    | 4.1±0.4        | $3.2 \pm 1.8$  | _    | 3.8±0.8        | 2.6±0.6        |  |  |  |
| Mannose       | $1.2 \pm 0.4$    | $1.4 \pm 0.5$  | OŞ             |      | 0              | 0              |  |  |  |
| Galactose     | $3.8 \pm 0.8$    | $12.5 \pm 1.8$ | $15.1 \pm 2.4$ | _    | $13.7 \pm 2.9$ | $14.2 \pm 2.1$ |  |  |  |
| Glucose       | <0.5±0.2         | $1.0 \pm 0.3$  | 0.8±0.3        | _    | 0              | 0              |  |  |  |
| Galactosamine | $5.8 \pm 0.7$    | $12.1 \pm 1.6$ | 16.8±7.8       | _    | $11.2 \pm 1.8$ | $16.2 \pm 2.3$ |  |  |  |
| Glucosamine   | $2.8 \pm 0.4$    | $10.5 \pm 1.3$ | $15.4 \pm 2.2$ | _    | $14.8 \pm 2.9$ | $13.8 \pm 3.1$ |  |  |  |
| Sialic acid   | $1.5 \pm 0.3$    | 6.3±0.8        | 7.4±0.8        | —    | $14.1 \pm 2.4$ | $15.8 \pm 8.1$ |  |  |  |
| Sulfate       | $1.8 \pm 0.4$    | $1.5 \pm 0.2$  | 2.1±10.4       | _    | $3.9 \pm 2.1$  | 4.1±1.3        |  |  |  |
| Nucleic acid  | 0                | 0              | 0              | —    | 0              | <1.0           |  |  |  |
| Total         | 58.6             | 74.8           | 83.2           |      | 85.0           | 86.2           |  |  |  |

 TABLE V

 Protein and Carbohydrate Composition of Ulcerative Colitis Colonic Mucin Fractions

• Purified mucin fractions recovered after DEAE-cellulose chromatography of pure mucin.

‡ Insufficient material available for evaluation.

§ 0, not detectable.

the suggestion that the human colon contains multiple mucin species. In rat and sheep colon, the presence of multiple mucin components has also been reported (11-12).

The importance of the heterogeneity of normal human colonic mucin is emphasized by our finding of an

TABLE VI Blood Group Specific Hemagglutination Inhibition Activity of Human Colonic Mucin Species

|                    |                  | Hemagglutination inhibition of human<br>erythrocytes types* |      |      |  |  |
|--------------------|------------------|-------------------------------------------------------------|------|------|--|--|
| Diagnosis          | Mucin<br>species | ۸                                                           | В    | н    |  |  |
| Normal             | I                | >100*                                                       | >100 | >100 |  |  |
|                    | II               | 5                                                           | 40   | 5    |  |  |
|                    | 111              | 12                                                          | 5    | 5    |  |  |
|                    | IV               | >100                                                        | 10   | 20   |  |  |
|                    | v                | >100                                                        | >100 | 20   |  |  |
|                    | VI               | 5                                                           | 40   | 40   |  |  |
| Ulcerative colitis | I                | >100                                                        | >100 | >100 |  |  |
|                    | II               | 10                                                          | 20   | 10   |  |  |
|                    | III              | 5                                                           | 5    | 20   |  |  |
|                    | v                | >100                                                        | >100 | 40   |  |  |
|                    | VI               | 20                                                          | 20   | 40   |  |  |

• Micrograms per milliliter of purified colonic mucin species necessary to inhibit hemagglutination of type-specific human erythrocytes by limiting dilution specific antisera (A and B) or lectin (H) at 30 min and room temperature. See Methods for details. altered mucin composition in the colon of patients with ulcerative colitis. Specifically, mucin from patients with ulcerative colitis was deficient in the component containing substantial amounts of both sulfate and sialic acid designated species IV, which normally comprises  $\sim 15\%$  of colonic mucin. Although we found an overall decrease in total colonic mucin, reductions in the amounts of the other five mucin fractions were significantly less than that observed for species IV. It is thus unlikely that the diminution in fraction IV was simply a reflection of the general mucin depletion, known to be a characteristic histologic feature of ulcerative colitis.

The basis for the deficiency of species IV in ulcerative colitis remains to be determined. The close similarity of the carbohydrate, sulfate, and protein composition of the remaining species to their normal counterparts suggests that the finding cannot be explained simply by comigration of fraction IV with one of the other species. Altered chromatographic migration of species IV would be expected to lead to an alteration in the composition of one of the other species. We are currently attempting to determine whether the decrease in mucin species IV reflects a defect in synthesis or inappropriate degradation.

It is noteworthy that the deficit of mucin species IV was not found in patients with Crohn's disease (Fig. 5 and Table III). Thus, while the present results suggest that the deficiency is more specifically related to ulcerative colitis, it is not possible to assess the absolute specificity of this association at this time. It will be important to examine the compositional heterogeneity of mucin in other types of colonic mucosal inflammation or injury such as in acute infectious, ischemic, and radiation colitis. Because colonic resection is rarely indicated in these other inflammatory disorders, it has not been possible to perform analyses in such patients using the methods described here. However, we have recently developed techniques utilizing methods of radiolabeling the oligosaccharide molecules that should permit determination of mucin composition on small punch biopsies of rectal or colonic mucosa.<sup>2</sup>

The relationship of the mucin fraction IV deficiency to disease activity in ulcerative colitis also needs further evaluation. As indicated in Table IV, in two colon specimens a similar mucin alteration was found in segments that appeared morphologically free of disease. If this is confirmed in additional studies, the absence of mucin species IV may indeed represent an underlying deficit in ulcerative colitis independent of the active inflammatory process. Since, as indicated above, we are now able to analyze mucin composition on small biopsy samples, a more detailed examination of the association of the deficiency in mucin species IV and disease activity is now feasible.

The role which a selective deficit of mucin species IV might play in the evolution of ulcerative colitis is unclear. If indeed the absence of mucin species IV preceeds the development of overt disease, this underlying defect in the mucin coat might make an individual susceptible to injury by an additional exogenous or endogenous agent. Thus, with such a concept, ulcerative colitis might require two factors for expression of disease activity, namely, a defect in a mucin glycoprotein that permits injury by a second factor. The absence of species IV might alter the viscoelastic properties of the mucus gel leading to disruption of the protective capacity of the mucin; alternatively absence of this glycoprotein might increase susceptibility to a second agent by exposing some receptor or other colonic surface membrane component. Injurious second factors might include a toxic substance in the lumen (dietary or bacterial product), an infectious agent, or even altered immunity. Finally, whether there might be a relationship between the alteration in mucin IV content and risk of malignant transformation in patients with long-standing ulcerative colitis also remains a subject for future study.

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