Protease Digestion of Colonic Mucin

EVIDENCE FOR THE EXISTENCE OF TWO IMMUNOCHEMICALLY DISTINCT MUCINS*

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Colonic mucin was prepared by phenol-water partition extraction of the colonic mucosa, followed by ethanol precipitation of the water-soluble material, molecular sieve chromatography, and reductive sodium dodecyl sulfate-disc gel electrophoresis in an agarose-polyacrylamide gradient gel. Although this material was observed to be relatively homogeneous by physical criteria, it was shown to contain at least two components by immunodiffusion. Molecular sieve, ion exchange, adsorption, lectin affinity, and electrophoretic techniques failed to separate the two components. However, by protease digestion employing either 1% w/w pronase or papain, followed by molecular sieve chromatography, we were able to separate at least the portions of the molecules containing the immunodeterminants. Physicochemical and immunologic properties of both components were characteristic of mucins. Mucin A (pronase digest fraction 1 from molecular sieve chromatography) was enriched in threonine, proline, and sialic acid, while mucin B (pronase digest fraction 3 from molecular sieve chromatography) was enriched in serine, alanine, and fucose. Mucin A and mucin B were shown to be immunologically distinct. The evidence suggests that the protease-digested mucin fragments originate from two different mucins, one a sialomucin, the other a fucosamucin; however, the alternative explanation, that these two mucin fragments may originate from a single mucin molecule, cannot be ruled out at present.

Mucosal surfaces are covered by a film of mucus, of which the major constituents (aside from water and ions) are high molecular weight glycoproteins. Investigations of mucin-type glycoproteins have been largely restricted to ovarian cyst blood group mucins (1) and various gastric (2) and submaxillary mucins (3) of animal origin. These glycoproteins are capable of forming viscous solutions and so function, at least in part, to protect and lubricate the surface. In addition, the carbohydrate moieties of the glycoproteins have distinctive sugar sequences which act as highly specific immunologic determinants. Aside from studies of human gastric mucin (4), little is known about mucins derived from normal human tissues.

We have reported that a human colonic mucin can be isolated from the large bowel epithelium by phenol-water partition extraction, followed by ethanol precipitation and molecular sieve chromatography (5). A chemical and physical characterization of this material proved to be typical of mucins in general. The peptide core was rich in threonine and proline, and the carbohydrate chains were composed of sialic acid, fucose, galactose, glucosamine, and galactosamine. This mucin has been shown to have a determinant specific for the colon (6), and may thus prove useful in the immunodiagnosis of colonic neoplasia (7). Additionally, it has been implicated as a potential autoantigen in inflammatory bowel disease (8). Recent evidence has indicated the existence of at least two immunologically distinct mucins produced by the colon, one of which contains the organ-specific determinant, while the other cross-reacts with mucins of other organs. The current study was undertaken to examine the nature of these two mucins.

EXPERIMENTAL PROCEDURES

Preparation of Mucin—Isolation of human colonic mucin was performed on individual tissue samples by the procedure previously developed in our laboratory (9). Briefly, this consisted of a phenol-water partition extraction of the washed colonic mucosa followed by precipitation of the water-soluble material in 85% ethanol. This crude mucin fraction was chromatographed on Sepharose 4B (Pharmacia) and the void volume mucin material was collected. Further purification was performed as described under "Results."

Sonification—A Heat Systems-Ultrasonics, Inc., model W185 Sonifier equipped with a macrotip was employed in this procedure. Mucin, at a concentration of 2 mg/ml in 0.1 M ammonium bicarbonate containing 0.5 M sodium chloride, was placed in an ice bath and subjected to four 90-second bursts of 40 watts, with 2-min cooling periods between each burst.

Reduction and Alkylation—The procedure reported by Miller and Metzger was employed (9). Mucin samples (5 mg) were dissolved in 0.2 M Tris-HCl, pH 8.6, containing 5 M guanidine. To this was added an estimated 600 mol/mol excess of dithiothreitol. The mixture was allowed to react for 2 h at room temperature under a nitrogen atmosphere. An amount of iodoacetamide (twice recrystallized) equal to 2.2 mol/mol of dithiothreitol was added and the mixture was permitted to react for 30 min in the dark at room temperature. The sample was then dialyzed extensively against deionized water and lyophilized.

Protease Digestion—Mucin (5 mg/ml) was treated with 1% (w/w) of either trypsin ( Worthington) or pronase (Calbiochem) in 0.02 M Tris-HCl, pH 8.0, containing 0.01 M calcium chloride. The trypsin digestion mixture was incubated at 37 °C for 24 h under a tulene atmosphere. The pronase digestion mixture was incubated under identical conditions except that the temperature was 50 °C. Papain digestion was carried out by the addition of 1% (w/w) enzyme (Sigma) to mucin (5 mg/ml) in 0.1 M sodium phosphate, pH 5.95, containing 0.02 M cysteine HCl and 0.005 M Na₂EDTA. The mixture was incubated in the same way for trypsin. The protease-digested samples were passed through a column (0.9 x 120 cm) of Sepharose 4B which had been equilibrated and eluted with 0.1 M ammonium bicarbonate containing 0.5 M sodium chloride.

Analytical Procedures—Procedures for chemical analysis have been reported (10). A Beckman model 121 analyzer was utilized for

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the quantitation of amino acids. Samples were hydrolyzed for 6 and 24 h at 110 °C in 4.5 N HCl under a nitrogen atmosphere. Sugars were measured by gas chromatography of the alditol acetates. Sialic acid, after release from the mucin by hydrolysis with 0.1 N HCl at 80 °C for 1 h was determined by the thioarbiturate assay as described by Warren (11). The anthrone reaction was carried out as described by Mokrasch (12). Anthrone was for 1 h was determined by the thiobarbiturate assay as described by Warren (11). The anthrone reaction was carried out as described by Weber and Osborn (16). Due to the high molecular weights of the materials being electrophoresed, no stacking or sample gels were employed. Prior to electrophoresis, samples were dialyzed against several changes of distilled water and lyophilized. Each gel received 100 μg (dry weight) of sample. Immunodiffusion, immunoelectrophoresis, and immunoperoxidase (indirect) techniques have been reported (6, 7). The rabbit anti-mucin antiserum used in these procedures was prepared and adsorbed with human type AB red blood cells as previously described (6). Absorption of the antiserum with the isolated mucin A or B was performed as follows. To 0.1 ml of antiserum was added 0.1 ml of a 2 mg/ml solution of the mucin. The mixture was incubated at 37 °C for 2 h and then at 4 °C overnight. Before use, the antiserum was centrifuged at 20,000 × g for 10 min.

RESULTS

Mucin was prepared by the procedure previously described (5) and analyzed by SDS-1% gel electrophoresis on a 7.5% polyacrylamide gel. A single band excluded from the gel was observed when the gel was stained by the PAS method for carbohydrate. When stained with Coomassie blue, a major band excluded from the gel, as well as several faint bands throughout the gel, were noted. Immunodiffusion of the mucin sample with its homologous antiserum gave two precipitin arcs as shown in Fig. 1. Further purification of the mucin was attempted and included the following procedures: 1) molecular sieve chromatography on Sepharose 4B and 2B employing the elution solvents: (a) 0.1 M ammonium bicarbonate, containing 0.5 M sodium chloride and 0.1% SDS, (b) 0.1 M ammonium bicarbonate, containing 0.2 M sodium chloride, 0.1% SDS, and 5 mM dithiothreitol, (c) 0.5 M ammonium isothiocyanate, or (d) 5 M guanidine hydrochloride; 2) ion exchange chromatography on DEAE and ECTEOLA cellulose employing sodium chloride gradients in (a) 0.01 M HCl, (b) 0.05 M Tris-HCl, pH 8.0, or (c) 0.01 M ammonium hydroxide; with and without Triton X-100; and (3) hydroxylapatite chromatography with sodium phosphate step gradients containing (a) 0.1% SDS, (b) 0.1% SDS and 5 mM dithiothreitol, (c) 0.1% SDS and 3 mM ammonium isothiocyanate, or (d) 3 M urea. None of these procedures by itself, or in combination, was effective in removing all material stained by Coomassie blue in SDS-disc gel electrophoresis.

Reductive SDS electrophoresis in a discontinuous agarose-polyacrylamide gradient did, however, prove effective. Disc gels were prepared with a 7.5% polyacrylamide plug (1 cm) at the bottom of the tube, a mid-section of 2% polyacrylamide (3 cm) and a top section of 1% agarose (1 cm). The mucin was unable to penetrate the 2% gel, while all other material had a high mobility through this gel and collected in the 7.5% gel. Mucin eluted from the agarose layer, when analyzed on 7.5% SDS-disc gels, gave a single excluded PAS-stained band. No staining was observed with Coomassie blue. The amino acid composition of this material was within experimental error, identical to the mucin fraction prior to electrophoresis. In immunodiffusion with the homologous antiserum, this mucin fraction gave two precipitin arcs. Of note at this point is that the data suggests the two precipitin bands observed in immunodiffusion represent two mucins. With reference to the immunodiffusion pattern (Fig. 1), we have designated the mucin closer to the antigen well as mucin A and the mucin further from the antigen well as mucin B.

Attempts to separate mucins A and B included those described above as well as lectin affinity chromatography employing Sepharose-bound concanavalin A, soybean agglutinin, wheat germ agglutinin, R. communis II agglutinin, soybean agglutinin (Vector Labs); concanavalin A (Pharmacia); and phytohemagglutinin E and Ulex europaeus lectins prepared in our laboratory (13, 14) and coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) (5 mg of protein/ml of gel) by the procedure of March et al. (15). Reductive sodium dodecyl sulfate-disc gel electrophoresis was performed in 7.5% polyacrylamide gels as described by Weber and Osborn (16). Due to the high molecular weights of the materials being electrophoresed, no stacking or sample gels were employed. Prior to electrophoresis, samples were dialyzed against several changes of distilled water and lyophilized. Each gel received 100 μg (dry weight) of sample. Immunodiffusion, immunoelectrophoresis, and immunoperoxidase (indirect) techniques have been reported (6, 7). The rabbit anti-mucin antiserum used in these procedures was prepared and adsorbed with human type AB red blood cells as previously described (6). Absorption of the antiserum with the isolated mucin A or B was performed as follows. To 0.1 ml of antiserum was added 0.1 ml of a 2 mg/ml solution of the mucin. The mixture was incubated at 37 °C for 2 h and then at 4 °C overnight. Before use, the antiserum was centrifuged at 20,000 × g for 10 min.

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SDS-disc gel electrophoresis (Fig. 3) of the pronase-digested fractions showed a clear separation of two components. Untreated material gave an excluded PAS-stained band; Pro 1 fraction gave a single tight PAS band slightly included in the
indicated that the Pro 1 fraction resembled the untreated material except for an enrichment in sialic acid. (Insufficient material precluded the determination of sialic acid content for the trypsin- and papain-digested fraction.) The Pro 3 and Pap 3 fractions were found to be enriched in fucose as compared to the other fractions.

A comparison of the two trypsin-digested fractions Try 1 and Try 2 indicated a similar qualitative and quantitative chemical composition. Both fractions had an increased content of serine, proline, and glycine as compared to the untreated material (Table I). Try 1 was also found to be enriched in threonine. A comparison of the amino acid compositions of the pronase and papain fractions was even more striking with the void volume fractions Pro 1 and Pap 1 substantially enriched in threonine and proline. Serine and alanine showed the reverse pattern and were concentrated in the Pro 2, Pro 3, Pap 2, and Pap 3 fractions. Carbohydrate analysis (Table II) indicated that the Pro 1 fraction resembled the untreated material except for an enrichment in sialic acid. (Insufficient material precluded the determination of sialic acid content for the trypsin- and papain-digested fraction.) The Pro 3 and Pap 3 fractions were found to be enriched in fucose as compared to the other fractions.

**Fig. 2.** Molecular sieve chromatography of protease-digested colonic mucin. The column of Sepharose 4B (1 x 120 cm) was equilibrated and eluted with 0.1 M ammonium bicarbonate containing 0.5 M sodium chloride and 0.025% sodium azide at a flow rate of 3 ml/h, at room temperature. Fractions (0.75 ml) were collected and examined for protein (A280) (●) and carbohydrate (anthrone assay for hexose, A540) (▲). Colonic mucin: A, untreated; B, treated with 1% (w/w) trypsin; C, treated with 1% (w/w) pronase; D, treated with 1% (w/w) papain.

**Fig. 3.** SDS-disc gel electrophoresis (7.5% polyacrylamide gels stained by PAS method for carbohydrate) of the pronase-digested colonic mucin fractions. 1, untreated mucin; 2, Pro-1 fraction; 3, Pro-2 fraction; 4, Pro-3 fraction. Arrow indicates the top of the disc gels.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Amino acid composition of the protease digest fractions</th>
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<tbody>
<tr>
<td></td>
<td>Lys</td>
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<td>Untreated</td>
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<td>Try 1</td>
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<td>Pap 1</td>
<td>5.13</td>
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<tr>
<td>Pap 2</td>
<td>2.94</td>
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<tr>
<td>Pap 3</td>
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<tr>
<th>Table II</th>
<th>Carbohydrate molar ratios</th>
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<td></td>
<td>Untreated</td>
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<tr>
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<td>Fucose</td>
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<td>Threonine</td>
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<tr>
<td>Serine</td>
<td>0.38</td>
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*ND* not determined.
Aliquots of the anti-mucin antiserum, absorbed with either mucin A (Pro 1) or mucin B (Pro 3), were examined by immunodiffusion. The antiserum absorbed with mucin A gave a precipitin reaction only with mucin B (Fig. 4A), while the antiserum absorbed with mucin B gave a precipitin reaction only with mucin A (Fig. 4B). The absorbed antiseras were coupled to Sepharose 4B for antibody affinity chromatography in an effort to separate the intact mucins, however, this attempt was not successful. The absorbed antiseras were then employed in the indirect immunoperoxidase assay with normal adult stomach, jejunum, ileum, colon, pancreas, bronchus, lung, heart, spleen, and kidney as substrates. Mucin B was present in all mucin-secreting organs while mucin A was restricted to the colonic epithelium.

## DISCUSSION

Previous reports from this laboratory (5, 10) have described the isolation of a colonic mucin which, by immunoelectrophoretic analysis, isoelectric focusing experiments, as well as behavior in molecular sieve chromatography, appeared to be relatively homogeneous. In the present study, however, the use of a new anticolononic mucin antiserum revealed that at least two components were present within the mucin fraction. Additionally, heterogeneity was evidenced by a number of faint Coomassie blue stained bands in disc gel electrophoresis. These bands may have represented minor amounts of contaminants or integral components of the mucin released under the reductive conditions employed. Efforts at further purification of the mucin by molecular sieve, ion exchange, and adsorption techniques failed to remove all of these materials, emphasizing their strong interaction with the mucin. Reductive SDS electrophoresis in an agarose-polyacrylamide gradient gel enabled us to prepare a mucin fraction which, by disc gel electrophoresis, as well as other procedures, appeared to be relatively homogeneous. This mucin fraction however, still gave two precipitin arcs in immunodiffusion, suggesting that it contained at least two immunologically distinct mucins.

Numerous investigators have observed immunochemical heterogeneity in mucin preparations which, by physical criteria, were thought to be relatively homogeneous. Tsuiki et al. (17) isolated BSM, which in free boundary electrophoresis at pH 7.4 and 10.3 gave a single sharp peak. Additionally, in ultracentrifugation, a single hypersharp peak was observed.

Horowitz et al. (18) examined the immunochemical homogeneity of this material and reported the presence of two immunologically distinct mucins by immunodiffusion and immunoprecipitation analyses. Subsequent studies by Tettamanti and Pigman (19) on the further purification of bovine submaxillary mucin demonstrated that hydroxylapatite adsorption could be used to separate a BSM-major and a BSM-minor. Each mucin was judged homogeneous by electrophoretic and ultracentrifugation techniques. However, in immunoelectrophoresis, BSM-major and BSM-minor each gave essentially the same pattern as the original preparation, two precipitin arcs. Similar results have been obtained in studies of ovine submaxillary mucin (20) and porcine submaxillary mucin (21).

Our attempts to separate the intact colonic mucins proved fruitless and indicated a great physicochemical similarity between them. This, in turn, prompted questions as to the possibility of one mucin being a degradation product or disulfide-linked subunit of the other. However, the data from sonification and reduction and alkylation experiments suggested that this was not the case. Additionally, it should be noted that the ability to detect, by immunodiffusion, both mucins in an extract of a well differentiated colon carcinoma grown as a serial transplant in nude mice argues against the possibility of one mucin being a bacterial component or a mucin washed into the colon from upper portions of the gastrointestinal tract.

Although the intact mucins were not separable, a differential susceptibility to proteolytic digestion permitted the separation of at least the portions of the mucins which carried the immunodeterminants. Digestion of the crude mucin fraction with either pronase or papain followed by molecular sieve chromatography produced high molecular weight carbohydrate containing fractions and a low molecular weight peptide fraction. Mucin A was found to be in the void volume fraction whereas mucin B was present in an included fraction (about 60% of bed volume). By specific absorption of the antiserum, these two mucin fractions were shown to be immunologically distinct. Additionally, it was demonstrated that mucin A contained the organ specific determinant previously described (6).

The carbohydrate moieties of colonic mucin were shown to be attached to the peptide core via an O-glycosidic linkage between galactosamine and threonine (5). In the present study, the molar ratios of galactosamine and threonine (and/or serine) suggested that all galactosamine residues were internal and linked to the protein core. Additionally, it should be noted that the crude mucin fraction did not exhibit blood group A activity arguing against terminal galactosamine residues. In mucin A, threonine occurred with a frequency of 1:2–3. If all threonines were glycosylated, the closely situated oligosaccharide chains would tend to inhibit proteolytic digestion of the protein core. This may be an important phenomenon in vivo, where the mucin comes in contact with host as well as bacterial proteases. On the other hand, in mucin B, the glycosyl-peptide linkage sites would be further apart, explaining the greater susceptibility of this mucin to proteolytic digestion. Other than the amino acids associated with the O-glycosidic type of glycosyl-peptide linkage (threonine, serine, and proline), the only amino acid found to be enriched in one mucin fraction as opposed to the other was alanine, almost twice as much in mucin B as in mucin A.

In an examination of the carbohydrate molar ratios, the sugars, fucose, and sialic acid (probably terminal sugars), exhibited an inverse relationship between the two mucins; mucin A was enriched in sialic acid while mucin B was 2D. V. Gold, D. S. Shohat, and F. Miller, unpublished data.
enriched in fucose. A reciprocal relation between fucose and sialic acid has been noted for many types of glycoproteins including duodenal mucus glycoproteins (22) and mucinous ovarian cyst glycoproteins (23). This may be of potential interest in the study of cystic fibrosis. Dische et al. (24) noted an increased fucose:sialic acid ratio of duodenal fluid glycoproteins of cystic fibrosis patients. Roelfs et al. (25) obtained similar results in their examination of the rectal mucus of cystic fibrosis patients. This may represent an imbalance in the concentrations of mucin A and mucin B within the mucus secretion, or a chemical alteration of one or the other of the mucins.

Finally, we must emphasize that although the protease-digested mucins have been shown to be immunochemically distinct, we cannot as yet determine whether these originated from two different mucins, one a sialomucin and the other a fucomucin, or, alternatively, were part of the same mucin molecule. Because of their high molecular weight, microheterogeneity, and viscoelastic properties, the technology available at present cannot be adapted to answer this question. Nevertheless, further study of the protease-digested fractions should prove useful in a determination of the relationship of the A and B mucin fractions and their functional roles in health and disease.

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REFERENCES