Deglycosylation of mucin from LS174T colon cancer cells by hydrogen fluoride treatment

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Mucin from xenografts of LS174T human colon cancer cells was treated with anhydrous HF for 1 h at 0° C to give a product (HFA) with over 80 % of the glucosamine and hexose removed, but retaining some galactosamine, and for 3 h at room temperature to give a product (HFB) devoid of carbohydrate. Rabbit antibodies against HFA bound to HFA much more than to HFB, and bound to native mucin to an intermediate extent. Antibodies to HFB bound to HFB more than to HFA, and did not bind to native mucin. Both HFA and native mucin bound a number of lectins, but HFB did not. By SDS/polyacrylamide-gel electrophoresis and size-exclusion h.p.l.c., native mucin and HFA are of apparent molecular mass greater than 400 kDa, whereas HFB is heterogeneous and of low molecular mass. On Western blots, antibody to HFA detected both high-molecular-mass mucin and a 90 kDa protein in homogenates of LS174T cells. Antibody to HFB detected a major 70 kDa band as well as higher-molecular-mass species. In tissue sections of normal colon and colon cancers, antibody to HFA showed both cytoplasmic and extracellular staining, whereas antibody to HFB generally stained only cytoplasmic antigens. These results indicate that anti-HFB antibody is specific for apo-mucin, whereas anti-HFA antibody is specific for GalNAc-apo-mucin.

INTRODUCTION

Mucins, high-molecular-mass carbohydrate-rich glycoproteins with *O*-linked carbohydrates, have until recently been refractory to most classical techniques of protein characterization. Partial or complete removal of carbohydrates, by glycosidase digestion [1,2], by anhydrous-HF solvolysis [3–6] and by trifluoromethanesulphonic acid treatment [6–11], from a number of mucins has been reported. Carbohydrate-free or carbohydrate-depleted mucins have been used to generate antibodies that react with mucin precursors [4,6] and with mucin cDNA clones [5,12]. In order to deglycosylate human colon-cancer mucin, we have used the glycoprotein purified from nude mouse xenografts of the LS174T cell line. Of the available techniques for deglycosylation, we have chosen anhydrous-HF solvolysis. In the present work, we have monitored the extent of deglycosylation of LS174T-cell mucin treated under mild and harsh conditions, and have examined the reactivities of completely and partially deglycosylated mucins with rabbit antibodies.

Although the biosynthesis of mucin carbohydrates is relatively well characterized in a number of systems, little is known about the processing of the protein portion of mucin. Since mucin carbohydrate is added to the protein in a post-translational sequential manner [13], apo-mucin devoid of carbohydrate and GalNAc-mucin with monosaccharide linked directly to protein should be intermediates in the synthesis of fully glycosylated mucin. Therefore we have used the antibodies generated against

deglycosylated colon-cancer mucin to attempt to detect mucin precursors in colon cancer cells and in normal colon.

MATERIALS AND METHODS

Materials

LS174T-colon-cancer mucin was purified from nude mouse xenografts by gel filtration and CsCl-density-gradient centrifugation. Details of mucin purification are being published separately [14]. *Patella vulgaris* α -N-acetylgalactosaminidase was purchased from V-Labs, Covington, LA, U.S.A. ¹²⁵I-labelled Protein A, lectins and molecular-mass standards were prepared by the chloramine-T method [15].

HF solvolysis

Thoroughly dried mucin samples were treated in a closed system [3] with anhydrous HF without anisole, but containing 10 % (v/v) anhydrous methanol, for 1 h at 0 °C (to give HFA) or for 3 h at room temperature (to give HFB). Reactions were quenched in ice-cold water, dialysed for 4–6 days at 4 °C against water, then freeze-dried.

Antibody generation

New Zealand White rabbits received three or four subcutaneous injections of HFA (190–360 μ g of protein total) or HFB (70–150 μ g of protein total) in Freund's adjuvant at 3-week intervals. Starting 2 weeks after the

Abbreviations used: HFA and HFB, HF-treated mucins as defined in the text; PBS, phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4); BSA, bovine serum albumin.

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final injection, serum was collected at 2-week intervals, heated for 30 min at 56 °C, and used in e.i.s.a. assays and immunoblots. Three different rabbits were used to prepare antisera to each antigen, and similar titres were obtained with each. Anti-HFA antibody gave half-maximal reactivity in e.i.s.a. assays at dilutions of 1000-fold to 2000-fold, and anti-HFB antibody gave half-maximal reactivity at dilutions of 500-fold to 1000-fold. In some cases, the rabbit antibodies were further purified on Protein A-agarose.

E.i.s.a.

Protein and glycoprotein antigens, in 0.05 ml of PBS, were adsorbed on 96-well micro-titre plates (from Costar) overnight at 4 °C, then blocked for 1 h at room temperature with 0.3 ml of 1 % BSA/PBS. Rabbit antisera or purified IgG fractions, diluted 50–2000-fold in 0.5 % BSA/PBS, were incubated with the block plates for 1 h at room temperature, then peroxidase-labelled goat anti-(rabbit IgG) antibody (Zymed, South San Francisco, CA, U.S.A.), diluted 1000-fold in 0.5 % BSA/PBS, was added and the mixture was incubated for a further 1 h at room temperature. At each step the plates were washed three to five times with PBS. For lectin binding the primary antibody was omitted and peroxidase-labelled lectins (from Sigma Chemical Co.), at 0.001–0.01 mg/ml in 0.5 % PBS/BSA, were used instead of the secondary antibody. Bound peroxidase was detected with 0.1 ml of 0.03 % H_2O_2 /1 mM-2,2-azinodi-(3-ethylbenzothiazoline)-sulphonate/0.1 M-citrate buffer, pH 4.0, per well after 30 min reaction at room temperature, the plates were analysed on an e.i.s.a. reader at 414 nm.

H.p.l.c.

Size-exclusion analysis was performed on a column (0.75 cm × 30 cm) of Spherogel TSK 3000SW (Altex). Elution was at room temperature with PBS at a flow rate of 0.5 ml/min with a Beckman model 450 h.p.l.c. apparatus, monitored at 214 nm and 280 nm with Beckman model 160 and 163 detectors in series.

Amino acid analysis

Amino acid compositions were determined after hydrolysis for 24 h in an N_2 atmosphere in 6 M-HCl at 105 °C [16]. Quantification was by external standards, without correction for hydrolytic loss of hexosamines. Cystine, methionine and tryptophan were not quantified.

Alkaline-borohydride treatment

HFA (240 µg of protein) was incubated for 48 h at 50 °C in 0.4 ml of 0.05 M-NaOH/1 M- NaB^3H_4 (10 Ci/mol). After cooling on ice, the sample was neutralized with 10 % (v/v) acetic acid in methanol, then per-evaporated from methanol/acetic acid (200:1, v/v). The sample was desalted by gel filtration in water on a 0.9 cm × 56 cm column of Sephadex G-15, then applied to a 0.46 cm × 25 cm quaternary amine h.p.l.c. column (SAX; 5 µm particle size; from J. T. Baker). The unbound fraction was applied to two 0.4 cm × 30 cm amine-phase h.p.l.c. columns (AX-10; from Varian) equilibrated with 85 % (v/v) acetonitrile in water. After isocratic elution for 10 min at 0.5 ml/min, the acetonitrile content was decreased at 0.25 %/min. Standard *N*-acetyl[1- ^3H]galactosaminitol was prepared by direct reduction of *N*-acetylgalactosamine with NaB^3H_4 , followed by gel filtration on Sephadex G-15.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed with the discontinuous buffer system of Laemmli [17], with a 3 % polyacrylamide stacking gel and a 7 % polyacrylamide separating gel. A 10 µg portion of protein, with or without 2-mercaptoethanol, was applied in each lane. After electrophoresis, proteins were transferred to nitrocellulose paper electrophoretically [18]. The nitrocellulose sheet was saturated with 2 % BSA/PBS, then incubated with rabbit antibodies, then ^{125}I -Protein A. Alternatively, the nitrocellulose sheets were incubated in a solution (10^6 c.p.m./ml) of ^{125}I -labelled peanut agglutinin, wheat-germ agglutinin or *Helix pomatia* agglutinin. After washing, the sheets were subjected to autoradiography.

For Western blots of the supernatant fractions from cultured colon-cancer cells, parental LS174T cells, the high-mucin variants HM-3 and HM-7 and the low-mucin variant LM-12 were maintained in culture, harvested, homogenized and centrifuged to yield 100 000 *g* supernatants as previously described [19]. Portions corresponding to 100 µg of protein were subjected to electrophoresis as above.

Immunohistochemical staining

Normal colonic tissues were obtained from 13 immediate autopsies of kidney donors without any evidence of colonic disease, as described in a previous report [20]. Fifteen colon cancer tissues were obtained at the time of surgical resection and included four well-differentiated, four moderately differentiated, five poorly differentiated and two mucinous carcinomas. All tissues were fixed in 10 % formalin, embedded in paraffin and cut into 5 µm serial sections.

The streptavidin-peroxidase technique of immunohistochemistry was performed as previously described [21], but modified for a rabbit polyclonal primary antibody. Briefly, after deparaffinization and rehydration, slides were treated with 3 % (v/v) H_2O_2 in methanol for 30 min. After three washes in PBS, 5 % (v/v) normal goat serum was applied for 30 min and then blotted off. Rabbit antiserum was then incubated for 90 min, with the following working dilutions made up in 1 % goat serum: anti-(native LS174T-cell mucin) antibody 1:1000; anti-HFA antibody 1:500; anti-HFB antibody 1:200. After three washes in PBS, biotinylated goat anti-(rabbit IgG) antibody (10 µg/ml) was incubated for 30 min and slides were again washed with PBS. Then, streptavidin-peroxidase conjugate (10 µg/ml) was applied for 30 min, followed by three washes in PBS. Finally, amino-ethylcarbazole was used as substrate for 15 min, and slides were counterstained with haematoxylin, dehydrated and mounted.

RESULTS AND DISCUSSION

The colon-cancer mucin isolated from nude mouse xenografts of the LS174T cell line was treated with anhydrous HF under relatively mild conditions, 1 h at 0 °C, to give HFA, and under more severe conditions, 3 h at room temperature, to give HFB. By amino acid analysis, the total protein yield, after HF treatment and extensive dialysis, was 78–86 % for HFA, but only 32–49 % for HFB. The content of threonine, serine and proline was over 50 % in native mucin, in HFA, and in

two out of three preparations of HFB (Table 1). HFB had over 97% of glucosamine and galactosamine removed. For HFA, 81% of the glucosamine but only 28% of the initial galactosamine was removed. The

Table 1. Amino acid analyses of HF-treated LS174T-cell mucin

Three preparations of native LS174T-cell mucin, of HFA and of HFB were hydrolysed and analysed for amino acid composition. Results are expressed as means $\pm$ S.D. Hexosamines are not corrected for hydrolytic losses and are not included in the summation of total amino acid residues.

Amino acid or amino sugar	Composition (mol of residue/100 mol of total amino acid residues)		
	Native mucin	HFA	HFB
Asx	3.3 $\pm$ 0.3	3.5 $\pm$ 0.3	4.6 $\pm$ 1.4
Thr	30.4 $\pm$ 2.9	27.0 $\pm$ 0.7	24.6 $\pm$ 6.4
Ser	13.3 $\pm$ 1.3	14.1 $\pm$ 1.0	13.2 $\pm$ 1.3
Glx	4.9 $\pm$ 0.9	5.9 $\pm$ 1.0	7.9 $\pm$ 2.4
Pro	14.5 $\pm$ 1.2	14.8 $\pm$ 4.0	11.9 $\pm$ 3.6
Gly	5.6 $\pm$ 0.4	6.6 $\pm$ 1.3	8.3 $\pm$ 2.7
Ala	5.7 $\pm$ 0.2	6.0 $\pm$ 0.2	5.9 $\pm$ 1.0
Val	3.6 $\pm$ 0.9	4.4 $\pm$ 0.5	4.2 $\pm$ 1.1
Ile	2.9 $\pm$ 0.2	2.8 $\pm$ 0.1	3.2 $\pm$ 0.1
Leu	4.2 $\pm$ 0.4	4.2 $\pm$ 1.0	5.0 $\pm$ 1.7
Tyr	0.9 $\pm$ 0.2	0.9 $\pm$ 0.2	0.9 $\pm$ 0.5
Phe	1.6 $\pm$ 0.7	1.5 $\pm$ 0.3	1.9 $\pm$ 0.3
His	3.6 $\pm$ 0.6	3.2 $\pm$ 0.3	2.7 $\pm$ 0.8
Lys	2.6 $\pm$ 1.1	2.5 $\pm$ 0.5	3.1 $\pm$ 0.8
Arg	2.0 $\pm$ 0.5	2.3 $\pm$ 1.5	2.3 $\pm$ 1.6
GlcN	19.8 $\pm$ 3.0	3.8 $\pm$ 2.5	0.2 $\pm$ 0.1
GalN	27.8 $\pm$ 1.8	20.1 $\pm$ 3.8	0.7 $\pm$ 0.3

native mucin has approx. 75% carbohydrate, with fucose, sialic acid, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine in the proportions 0.4:1.5:1.0:0.9:1.4. Since the average oligosaccharide in native LS174T-cell mucin has 1.4 galactosamine residues [14], the residual galactosamine in HFA corresponds to one residue per original oligosaccharide. By the phenol/H₂SO₄ assay [22], at least 90% of the neutral sugar (galactose and fucose) in HFA has also been removed.

Polyclonal antibodies to HFA, HFB and native LS174T-cell mucin were prepared in rabbits and examined for cross-reaction by e.l.i.s.a. (Fig. 1). Antibody against native mucin showed little reactivity with HFA or HFB (Fig. 1a). Antibody against HFA bound best to HFA, but showed cross-reaction with native LS174T-cell mucin and with HFB (Fig. 1b). Antibody prepared against HFB showed some cross-reactivity with HFA, but did not bind at all to the native mucin (Fig. 1c).

Binding of peroxidase-labelled lectins to HFA, HFB and native mucin was also examined (Fig. 2). *Ricinus communis* agglutinin, specific for β -linked galactose [23], bound to native LS174T-cell mucin more than to HFA, and did not bind to HFB at all. Peanut agglutinin, specific for Gal β 3GalNAc [23], on the other hand, bound to HFA more than to native mucin. The most likely explanation is that peripheral β -linked galactose residues, for example in Gal β 4GlcNAc, are removed in HFA, and cryptic Gal β 3GalNAc disaccharides are exposed. Binding of wheat-germ agglutinin (Fig. 2b) is greatly increased in HFA relative to native LS174T-cell mucin, although HFA has 5-fold less *N*-acetylglucosamine. Both *N*-acetylgalactosamine-specific lectins bound to a greater extent to HFA than to native mucin (Fig. 2c). HFA bound *Vicia villosa* B4 agglutinin more than *Helix pomatia* agglutinin, but the reverse was found for native LS174T-cell mucin. This is likely to result from the greater specificity of *Vicia villosa* agglutinin for the Tn

Fig. 1. Immunological cross-reactivity of HFA, HFB and native mucin

Native LS174T-cell mucin ($\square$), HFA ($\circ$) and HFB ($\triangle$) were adsorbed on polystyrene micro-titre dishes and assayed for the binding of rabbit antibodies. Each point represents the median for triplicate wells. (a) Antibody prepared against native LS174T-cell xenograft mucin; IgG fraction used at a 200-fold dilution relative to serum. (b) Antibody prepared against HFA; IgG fraction used at a 2000-fold dilution relative to serum. (c) Antiserum prepared against HFB; used at a 2000-fold dilution.

Fig. 2. Lectin binding of HFA, HFB and native mucin

Native LS174T-cell mucin ($\square$ and $\blacksquare$), HFA ($\circ$ and $\bullet$) and HFB ($\triangle$ and $\blacktriangle$) were adsorbed on polystyrene micro-titre dishes and assayed for the binding of peroxidase-labelled lectins. Each point represents the median for triplicate wells. (a) Peanut agglutinin ($\blacksquare$, $\bullet$ and $\blacktriangle$) at $10\ \mu\text{g/ml}$ and *Ricinus communis* agglutinin ($\square$, $\circ$ and $\triangle$) at $1\ \mu\text{g/ml}$. (b) Wheat-germ agglutinin ($\square$, $\circ$ and $\triangle$) at $1\ \mu\text{g/ml}$. (c) *Helix pomatia* agglutinin ($\blacksquare$, $\bullet$ and $\blacktriangle$) at $10\ \mu\text{g/ml}$ and *Vicia villosa* B4 agglutinin ($\square$, $\circ$ and $\triangle$) at $10\ \mu\text{g/ml}$.

Fig. 3. Size-exclusion h.p.l.c. of HFA, HFB and native mucin

Samples ($100\ \mu\text{g}$) of protein were chromatographed on Spherogel TSK 3000SW as described in the Materials and methods section. Arrows, left to right, show the elution positions of ferritin, BSA and trypsin and the total volume of the column. (a) Native LS174T-cell mucin. Samples ($2.5\ \mu\text{l}$) were assayed with $1\ \mu\text{g}$ of peroxidase-labelled *Ricinus communis* agglutinin/ml ($\square$). Other samples ($0.5\ \mu\text{l}$) were assayed with 200-fold-diluted antibody prepared against native mucin ($\blacksquare$). (b) HFA. Samples ($0.05\ \mu\text{l}$) were assayed with $10\ \mu\text{g}$ of *Helix pomatia* agglutinin/ml ($\circ$). Other samples ($0.5\ \mu\text{l}$) were assayed with 200-fold-diluted anti-HFA antibody ($\bullet$). (c) HFB. Samples ($50\ \mu\text{l}$) were assayed with 200-fold-diluted anti-HFB antibody ($\blacktriangle$).

Fig. 4. SDS/polyacrylamide-gel electrophoresis with antibody detection

Portions (10 μ g/lane) of HFA (lanes a, d and g), HFB (lanes b, e and h) and native mucin (lanes c, f and i) were subjected to electrophoresis, transferred to nitrocellulose and incubated with anti-HFA antibody (lanes a, b and c), anti-HFB antibody (lanes d, e and f) and antibody to native mucin (lanes g, h and i). The interface between stacking and separating gels is marked with an arrow. Positions of 125 I-labelled molecular-mass markers are shown at the right.

Fig. 5. SDS/polyacrylamide-gel electrophoresis with lectin detection

HFA (lanes a, d and g), HFB (lanes b, e and h) and native mucin (lanes c, f and i) were subjected to electrophoresis as in Fig. 4. Nitrocellulose blots were incubated with 125 I-labelled *Helix pomatia* agglutinin (lanes a–c), peanut agglutinin (lanes d–f) or wheat-germ agglutinin (lanes g–i). Positions of 125 I-labelled molecular-mass markers are shown at the right.

Fig. 6. Western blot of cellular proteins from the high-mucin variant of LS174T cells

Samples (100 μ g) of proteins from the 100000 g supernatant of HM7 cells with (lanes b, d, f, h, j and l) or without boiling for 5 min (Heat) and with (lanes c, d, g, h, k and l) or without 2-mercaptoethanol (2ME) were electrophoresed in SDS/polyacrylamide gel, transferred to nitrocellulose and overlaid with anti-HFA antibody (lanes a–d), anti-HFB antibody (lanes e–h) and anti-(native mucin) antibody (lanes i–l). Positions of 125 I-labelled molecular-mass markers are shown at the right. The large arrowhead marks the interface between stacking and separating gels.

antigen, GalNAc α Thr/Ser [23]. *Helix pomatia* agglutinin may be binding to peripheral *N*-acetylgalactosamine residues in the native LS174T-cell mucin.

The HF-treated mucins and native LS174T-cell mucin were examined by size-exclusion chromatography on a column of Spherogel TSK-3000SW. Native mucin (Fig. 3a) was exclusively at the void volume, with an apparent molecular mass in excess of 400 kDa. Binding of *Ricinus communis* agglutinin and of rabbit antibody against the native mucin coincided with the A_{214} and A_{280} profiles. HFA was also at the void volume (Fig. 3b). Binding of *Helix pomatia* agglutinin and anti-HFA antibody paralleled the u.v.-absorbance profiles. In contrast, HFB was predominantly of low molecular mass and gave a much lower A_{214} peak. Binding of anti-HFB antibody was very weak and spread through the column, from void volume to included volume.

The behaviour of HFA on size-exclusion h.p.l.c. indicates that there was little if any peptide cleavage in the mild HF treatment. HFB, on the other hand, appears to have undergone extensive peptide cleavage. It should be noted that enzymically deglycosylated submaxillary apo-mucin, of 96.5 kDa molecular mass, has been reported to behave as a much larger molecule upon gel filtration in the absence of denaturants [1]. Thus the apparent sizes of HFA and HFB may be over-estimates. In order to investigate further the size and reactivity with lectins and antibodies of HFA and HFB, they were subjected to electrophoresis on SDS/polyacrylamide slab gels and Western blotting. Anti-HFA antibody bound to

Fig. 7. Immunohistochemical staining of normal colon and colon cancer

(a) Normal colonic mucosa stained with antibody to native mucin. Secreted mucin and an occasional goblet cell are strongly stained. Magnification $\times 50$. (b) Normal colonic mucosa stained with anti-HFA antibody. Although staining intensity is weaker, antigen is expressed in secretions, more numerous goblet cells (arrow), and cytoplasm of some cells. Magnification $\times 50$.

(c) Normal colonic mucosa stained with anti-HFB antibody. Only the supranuclear cytoplasm is positive (arrows). Goblet cells and secretions are negative. Magnification $\times 88$. (d) Poorly differentiated colon cancer stained with anti-HFB antibody. Antigen expression occurs in the cytoplasm. Magnification $\times 88$.

HFA (Fig. 4, lane a) and to native mucin (lane c), but not to HFB (lane b). Anti-HFB antibody bound most effectively to HFB (lane e), and little to HFA (lane d). Antibody to native mucin bound to the native mucin (lane i) and, to a small extent, to HFA. The apparent sizes of the three antigens are in accord with the results of size-exclusion h.p.l.c. HFA is near the top of the 7%-acrylamide separating gel, with an apparent molecular mass in excess of 400 kDa. HFB is a broad smear with no distinct bands apparent. The native LS174T-cell mucin has strong staining in the 3%-acrylamide stacking gel. It should be noted that 2-mercaptoethanol was omitted from the samples, because the immunoreactivity of native mucin was greatly decreased by reduction. HFA and HFB were little affected by 2-mercaptoethanol (results not shown). Radioiodinated lectins were also used for detection of nitrocellulose-immobilized HFA and native mucin (Fig. 5). As with the glycoproteins immobilized on polystyrene micro-titre dishes, HFA bound more *Helix pomatia* agglutinin, peanut agglutinin and wheat-germ agglutinin than did the native mucin. No lectin binding to HFB was detectable under the same conditions.

HFA was digested with α -N-acetylgalactosaminidase in order to determine the extent to which anti-HFA antibody recognized α -linked N-acetylgalactosamine residues. After an extended incubation (8 days at 37 °C in 0.01 M-KHPO₄, pH 5.2, with 20 munits of *Patella vulgaris* α -N-acetylgalactosaminidase added at 0, 1 and 5 days), followed by dialysis, 43% of the total galactosamine, measured by amino acid analysis, had been removed. The binding of anti-HFA antibody and of *Helix pomatia* agglutinin was decreased about 3-fold (results not shown). In spite of the extensive removal of N-acetylgalactosamine, no increase in reactivity with anti-HFB antibody was observed (results not shown). The composition of HFA (Table 1) suggested that most of the carbohydrate was present as single N-acetylgalactosamine residues, O-glycosidically linked to protein. From the residual hexose and glucosamine in HFA there should be no more than 12% and 13% of Gal-GalNAc and GlcNAc-GalNAc oligosaccharides. HFA was treated with mild alkali in the presence of NaB³H₄ to release and radiolabel O-linked oligosaccharides. After desalting, the neutral oligosaccharide fraction was analysed by h.p.l.c. on an amine-phase column. The peak of ³H was co-eluted with a standard of N-acetyl-[1-³H]galactosaminitol (results not shown). Small peaks eluted before and after N-acetylgalactosaminitol were not further characterized. Taken together with the partial susceptibility of HFA to α -N-acetylgalactosaminidase, the release of N-acetylgalactosaminitol indicates that the bulk of the carbohydrate in HFA is present as GalNAc α Thr/Ser.

The cellular proteins from the high-mucin variant of LS174T cells [19] were examined by Western blotting for the molecules carrying antigenic determinants. Several combinations of sample treatments, either (a) with or without heat treatment for 5 min or (b) in the presence or absence of 2-mercaptoethanol, were utilized to examine the effect of heat or reduction on antigenic expression. Anti-HFA reacted with several bands in LS174T-cell cytosol, including a band of about 90 kDa and bands of higher molecular mass (Fig. 6). The 90 kDa band was insensitive to reduction, but the antigenicity of the higher-molecular-mass bands was abolished after reduction. The amount of high-molecular-mass antigen was greater

in the high-mucin variants HM3 and HM7 than in the parental cells or the low-mucin variant LM12 (results not shown). Anti-HFB antibody reacted with a major band of about 70 kDa as well as two faint bands at approx. 200 kDa and 400 kDa. The amount of 70 kDa peptide was similar in the variants, but only the high-mucin variants had large amounts of the 200–400 kDa antigen recognized by anti-HFB antibody. The antigenicities were not changed by heat or reduction. The antigen recognized by antibody against native mucin was located predominantly in a broad band at the top of the stacking gel, although there were also two distinct bands at approx. 60 kDa and 30 kDa. After reduction, reactivity with antibody to native mucin was greatly decreased. These results suggest that antigen determinants of anti-HFA and anti-(native mucin) antibodies were more dependent on protein structure or the conformation of mucin macromolecules. When membrane and soluble fractions of LS174T-cell homogenates were examined separately, the immunostaining profiles of membrane proteins were essentially the same as those with cytosol proteins (results not shown). The nature of the low-molecular-mass bands at 90 kDa, 70 kDa, 60 kDa and 30 kDa is unclear at present. They could be mucin precursors or their degradation products, or they could be non-mucinous proteins that cross-react with mucin.

A previous study, with antibody prepared against deglycosylated gastric mucin to immunoprecipitate metabolically labelled proteins in rat stomach, has identified several putative mucin precursors of less than 80 kDa molecular mass [24]. A 60 kDa bovine submaxillary mucin precursor has been identified by translation *in vitro* [6], but the threonine-rich composition of LS174T-colon-cancer mucin makes it unlikely that it could have the same polypeptide core as submaxillary mucins. In preparations of gastric and small-intestinal mucin, a 70–118 kDa disulphide-bonded protein has been described [25,26]. Although the 70 kDa and 90 kDa bands that are detected with anti-HFB and anti-HFA antibodies are not reduction-sensitive, it is possible that they could represent non-covalently bound 'link' peptide.

Immunohistochemical expression of antigens recognized by anti-HFA and anti-HFB antibodies: LS174T-cell mucin, HFA and HFB

In the normal colon, anti-(native LS174T-cell mucin) antibody strongly stained the luminal secretions (ten out of 13 specimens), which contain mature mucin (Fig. 7a). Goblet-cell vacuoles were weakly stained in nine cases, but there was no detectable cytoplasmic staining. Anti-HFA-antibody staining, like that for native mucin, was found in secretions and some goblet cells (eight cases), but, in addition, occurred in the cytoplasm of six cases (Fig. 7b). Anti-HFB-antibody staining was strictly localized to the supranuclear cytoplasm of colonocytes (ten out of 13 cases) and was not found in goblet-cell mucin or secretions (Fig. 7c). This cellular location is consistent with the concept that the antigen recognized by anti-HFB antibody is apo-mucin.

The numbers of cancer specimens that were reactive with the three antisera were as follows: anti-(native mucin) antibody, 14/15; anti-HFA antibody, 15/15; anti-HFB antibody, 13/15. With all three antisera, the predominant staining occurred in the cell cytoplasm (Fig. 7d). However, in addition, anti-HFA and anti-(native mucin) antibodies also stained cell membranes

and luminal secretions in the well-differentiated and mucinous carcinomas. Cytoplasmic staining with anti-HFB and anti-HFA antibodies was increased in colon cancers relative to normals, and extracellular staining with anti-HFA and anti-(native mucin) antibodies was decreased in cancers relative to normal. This suggests that both glycosylation and secretion of colonic mucin are less efficient in colon cancer than in normal colon.

A common immunohistochemical finding in colon cancers is that carbohydrate antigens that correspond to incompletely glycosylated glycoproteins, for example T antigen, Gal β 3GalNAc, are found [27]. Monoclonal antibodies against Tn antigen, GalNAc α Ser/Thr, have also been found to bind to gastrointestinal cancers more than to normal gastrointestinal tissues [28]. Although the polyclonal anti-HFA antibody used here seems less specific for cancerous tissues than is monoclonal anti-Tn antibody, the increased cytoplasmic staining with anti-HFA antibody is in agreement with the suggestion that Tn antigen (i.e. GalNAc-mucin) accumulates in colon cancers. The increased staining with anti-HFB antibody suggests that there may also be an accumulation of apomucin. Preparation of monoclonal antibodies to HFA and HFB and immunohistochemical studies of other tissues should help to elucidate the mechanisms of mucin biosynthesis and the alterations in disease states.

This work was supported by the Veterans Administration Medical Research Service (J. C. B., B. S. and Y. S. K.), by Grant nos. AM17938 and CA47551 (R. E. and Y. S. K.) and Grant no. CA42981 (S. H. I.) from the National Institutes of Health, and by Department of Energy Contract no. DE-AC 02-76 ER 01 338 (D. T. A. L.). We acknowledge the excellent technical assistance of Mr. Pat Muldoon and Mr. Robert Lagac, and the assistance of Ms. Rita Burns in manuscript preparation.

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Received 3 August 1988/3 January 1989; accepted 12 January 1989