A mucus-secreting human colonic cancer cell line

Purification and partial characterization of the secreted mucins

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A stably differentiated clonal derivative (Cl.16E) of the human colonic adenocarcinoma cell line HT29 secretes in culture high- M_r glycoproteins that were purified from the serum-free conditioned medium by preparative SDS/polyacrylamide-gel electrophoresis. Analysis of the oligosaccharides released from the [3 H]glucosamine-labelled high- M_r glycoproteins by alkaline-borohydride treatment showed that this material consisted of *O*-linked oligosaccharides (without any detectable *N*-linked oligosaccharides) that were eluted as three fractions from Bio-Gel P-6 columns. The main oligosaccharide fraction obtained after such treatment and desialylation was eluted together with a six-unit glucose polymer from a Bio-Gel P-4 column. Polyclonal antibodies were raised against the high- M_r glycoproteins, and in immunoblot analysis they reacted specifically with the high- M_r glycoproteins present in the conditioned medium. Furthermore, immunohistochemical staining of sections in paraffin wax revealed that these antibodies labelled normal human gastrointestinal mucins. We conclude that (1) the high- M_r glycoproteins prepared by SDS/polyacrylamide-gel electrophoresis are pure mucus glycoproteins on the basis of sensitivity to alkaline-borohydride treatment, monosaccharide composition and immunochemical and immunohistological findings, and (2) these mucins have antigenic determinants in common with the normal human gastrointestinal mucins.

INTRODUCTION

Colonic mucins are high- M_r glycoproteins secreted by the epithelial goblet cells. The structure of the colonic mucins, their synthesis and their secretion mechanisms are still poorly understood. Among the main reasons for this slow progress are, first, the technical difficulties associated with the collection and purification of non-degraded mucins, which are highly contaminated by many other secretions and by bacteria present in the colonic lumen (Allen *et al.*, 1982; Carlstedt *et al.*, 1985; Neutra & Forstner, 1987), and, secondly, the need of model systems *in vitro* for studying the biosynthesis and the secretion of these substances. Although several studies have demonstrated the usefulness of organ-culture techniques for the study of the biosynthesis and secretion of mucins from goblet cells (Forstner *et al.*, 1973; MacDermott *et al.*, 1974; LaMont & Ventola, 1977; Neutra *et al.*, 1977; Smith & Podolsky, 1987), these systems suffer from some limitations.

The limitations of these organ cultures result essentially from their considerable cellular heterogeneity and from their limited viability. These difficulties might in principle be overcome by a monolayer culture of intestinal epithelial cells. Such a system would have several advantages: (1) the mucins would be produced in aseptic conditions; (2) they would be less contaminated by several types of glycoproteins than those produced *in vivo* or in organ culture; (3) a continuous monolayer culture would provide mucins with stable characteristics

allowing reproducible experiments. However, the long-term culture of differentiated epithelial cells from the normal human colonic mucosa is notoriously difficult (Whitehead *et al.*, 1987a; Keding *et al.*, 1987; Labois, 1989).

Recently, considerable attention has been focused on the differentiation characteristics of some human colonic cancer cell lines, which were found to be in close analogy to those of normal epithelial cells. In particular, several lines of investigation have shown that some of these cancer cell lines offer valid models for the study *in vitro* of specialized characteristics of the normal human intestinal mucosa, such as brush-border enzymes or ion transport (Pinto *et al.*, 1983; Grasset *et al.*, 1984; Hauri *et al.*, 1985; Augeron *et al.*, 1986). Following this approach, we were prompted to extend these investigations to the study of the production of mucins from human colonic cancer cells maintained in long-term culture. The model that we used in the present study is a mucus-secreting clonal derivative (Cl.16E) (Augeron & Labois, 1984) of the human colonic adenocarcinoma cell line HT29 (Fogh & Trempe, 1975). Under standard culture conditions these cells differentiate upon reaching confluence into typical goblet cells, which are arranged in a monolayer (Augeron & Labois, 1984; Augeron *et al.*, 1986). These cells are highly polarized, and they secrete their mucins into the culture medium as a visco-elastic gel. These characteristics of differentiation are very stable throughout long-term culture.

In the studies reported in the present paper, we

Abbreviations used: HMGs, high- M_r glycoproteins; PAGE, polyacrylamide-gel electrophoresis.

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developed a simple preparative method for purifying the mucins from the other glycoproteins secreted into the culture medium. This simple purification scheme yielded pure mucins, which were evaluated according to biochemical, immunochemical and immunohistological criteria.

MATERIALS AND METHODS

Cell culture and preparation of conditioned medium

The Cl.16E cells were routinely cultured in 25 cm² flasks (Falcon, Oxnard, CA, U.S.A.) in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated foetal bovine serum. The culture medium was changed daily. The cells were routinely screened for mycoplasma contamination by using Chen's (1977) method and they were always found to be negative.

Preparation of conditioned medium. Post-confluent cultures of Cl.16E cells, secreting copious amounts of mucins, were used for the preparation of conditioned medium. The onset of mucus secretion was easily detected, since the mucus gel covering the monolayers was visible to the naked eye. Conditioned medium was prepared according to the following schedule. The cells were washed with serum-free medium composed of a 1:1 (v/v) mixture of Ham F12 medium and Dulbecco's modified Eagle's medium for 24 h to eliminate serum proteins. This wash solution was discarded, and subsequent 12 h collections were taken as conditioned medium. Before the collection of the conditioned medium, the mucus gel covering the cell layer was solubilized by repeatedly pipetting the conditioned medium over the cell layer. The conditioned medium was collected in plastic centrifuge tubes in the presence of 100 μ M-phenylmethanesulphonyl fluoride, centrifuged at low speed to remove cells and debris, dialysed for 48 h against several changes of deionized water, and concentrated by freeze-drying. Conditioned medium was prepared repeatedly from the same cultures for approx. 1 week.

Metabolic radiolabelling of the mucins. Post-confluent cultures of Cl.16E cells in 25 cm² flasks were washed for 24 h in serum-free medium as described above before the incubation of the cells with the radioactive agents. Then the monolayers were incubated for 24 h with one of the following radiolabelled compounds (Amersham), in 5 ml of culture medium: 20 μ Ci of D-[6-³H]glucosamine (22 Ci/mmol) or 70 μ Ci of D-[2-³H]mannose (16.4 Ci/mmol). After the incubation, the conditioned medium was extensively dialysed against several changes of deionized water at 4 °C. Incorporation of radioactivity into the glycoproteins of the conditioned medium was determined by counting the radioactivity of a sample of the dialysed medium in a β -radiation scintillation counter. Then the radioactive medium was freeze-dried.

Preparative and analytical SDS/PAGE of HMGs

SDS/PAGE. The freeze-dried material was resuspended in Laemmli sample buffer and boiled for 5 min. SDS/PAGE 3–16% gradient slab gels were prepared and run as described by Laemmli (1970). In all cases a 3% stacking gel was placed on top of the gel. For reduced samples, a sample buffer containing freshly diluted 5% (v/v) 2-mercaptoethanol was used.

Elution of glycoproteins from slab gels. In order to elute the HMGs from the stacking gel, the latter was cut out and then incubated in a solution of 5 mM-NaHCO₃ containing 0.1% SDS for 24 h at 37 °C with constant agitation (Takacs, 1979). Then the eluate was dialysed for 48 h against double-distilled water and freeze-dried. The elution yield, as determined by radioactivity counting before and after elution of a pure preparation of ³H-labelled HMGs of known radioactivity, was about 42%.

Electrotransfer of proteins on to nitrocellulose sheets. Components that were subjected to SDS/PAGE were transferred on to nitrocellulose sheets in the presence of 10% (v/v) methanol/25 mM-Tris/192 mM-glycine buffer, pH 8.3, with electrophoresis for 4–5 h at 150 V in a Bio-Rad Trans-Blot cell equipped with a cooling device and model 250/2.5 power supply. The nitrocellulose blots were reversibly stained with Ponceau S.

Biochemical and immunochemical analyses

Release of oligosaccharide chains from ³H-labelled glycoproteins by alkaline-borohydride treatment. Mild alkaline-borohydride treatment of [³H]glucosamine-labelled HMGs was performed with 0.05 M-NaOH/1 M-NaBH₄ for 72 h at 45 °C (Carlson, 1968). Afterwards the reaction mixture was cooled to 4 °C and adjusted to pH 5.0 by the addition of 2 M-acetic acid, and the sample was desalted through a column of AG 50-X8 (H⁺ form). The excess borate was removed with methanol as methyl borate by rotary evaporation. The resulting oligosaccharides were loaded on to a column (1.2 cm $\times$ 115 cm) of Bio-Gel P-6 and eluted at a constant flow rate of 8 ml/h with 0.1 M-pyridine/acetate buffer, pH 5.0. The column was calibrated with bovine serum albumin and mannose to determine the void volume and the included volume respectively. The bovine serum albumin was detected by measuring the absorbance at 280 nm, and mannose was detected by using the phenol/H₂SO₄ method (Dubois *et al.*, 1956). Fractions (1 ml) were collected and the radioactivity was determined on 100 μ l samples in a β -radiation scintillation counter.

In order to release both N- and O-linked oligosaccharides, a harsh alkaline-borohydride treatment was performed by incubating the [³H]glucosamine-labelled HMGs in 1 M-NaOH/4 M-NaBH₄ for 24 h at 60 °C (Zinn *et al.*, 1978).

Acid hydrolysis of ³H-labelled oligosaccharides. Oligosaccharides released by alkaline-borohydride treatment were hydrolysed in acetic acid/5 M-H₂SO₄ (19:1, v/v) for 4 h at 80 °C (Ogier-Denis *et al.*, 1988). The resulting sample was chromatographed on an AG 3-X4 column equilibrated with methanol and eluted with methanol. The eluates were dried by using an Evapomix apparatus. The hydrolysates were dissolved in saturated NaHCO₃ and acetylated by adding acetic anhydride at room temperature for 10 min. The hydrolysates were desalted through coupled columns of AG 50-X8 and AG 3-X4. The eluates were dried and then separated by chromatography for 40 h on Schleicher and Schüll 2043b paper in butan-1-ol/pyridine/water solvent (6:4:3, by vol.) (Youakim & Herscovics, 1985). Before application of the sample the paper was impregnated with borate by dipping it in a solution of 0.57% Na₂B₄O₇·10H₂O in 10 mM-NaCl followed by drying. Guide strips contained standards of N-acetylgalactosamine, N-acetylglucos-

amine, *N*-acetylglucosaminitol and *N*-acetylgalactosaminitol. Both *N*-acetylhexosaminotols were obtained after NaBH_4 reduction of *N*-acetylhexosamines. Standards were stained by AgNO_3 reagents (Trevelyan *et al.*, 1950). Radioactivity was determined in 1 cm strips cut out of the chromatogram.

To assess the proportion of labelled sialic acids produced from $[^3\text{H}]$ glucosamine, the oligosaccharides were hydrolysed with 0.01 M-HCl at 80°C for 1 h and chromatographed on a Bio-Gel P-6 column as described above. The proportion of shifted radioactivity was considered to be associated with released sialic acid. Authentic *N*-acetylneuraminic acid was co-injected as a standard and its elution pattern was determined by using the thio-barbiturate assay (Warren, 1959).

Determination of the M_r values of the oligosaccharides. Neutral oligosaccharides eluted in fraction I after chromatography on a Bio-Gel P-6 column (see above) were chromatographed at 56°C in water on a Bio-Gel P-4 column (2.6 cm $\times$ 100 cm), eluted at a constant flow rate of 1 ml/min. The sample was co-injected with a dextran hydrolysate used as an M_r standard (Yamashita *et al.*, 1982). The elution pattern of the dextran hydrolysate was determined by using a refractometer. Fractions (3 ml) were collected and samples were taken for counting of radioactivity.

Preparation of polyclonal antibodies. Following the separation of the glycoproteins of the conditioned medium on 3–16% slab gels, the stacking gels were cut out and crushed. Before immunization, the rabbits (giant Flemish rabbits) were bled and the pre-immune serum was kept frozen. The crushed gels were injected subcutaneously and intramuscularly into the rabbits. Then the animals were boosted at 3 weeks and 11 weeks after the priming dose. Blood samples were taken 2 weeks after the last booster. The antiserum was absorbed as follows: it was heated at 56°C for 30 min, allowed to cool and then mixed with packed erythrocytes (1:1, v/v) of A, B and O blood groups. Three successive absorptions were performed for each blood group.

Immunolabelling of nitrocellulose filters. The proteins transferred on to the nitrocellulose filters (immunoblot) were immunolabelled by sequential incubation of the filter with blocking buffer, anti-mucin polyclonal antibodies (1:500) and horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibodies (Bio-Rad Laboratories) (1:2000). Peroxidase activity was revealed by using diaminobenzidine as the substrate.

Immunohistological techniques

Samples of normal human gastric and colonic mucosae were fixed in 95% (v/v) ethanol according to the method of Sainte-Marie (1962), and then embedded in paraffin wax. The sections were treated with the anti-mucin antibodies and then with horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibodies. Peroxidase activity was revealed as described above.

RESULTS

Biochemical characterization of the Cl.16E mucins

When the glycoproteins of the Cl.16E conditioned medium were analysed by Coomassie Blue staining of the

Fig. 1. Gel-filtration chromatography on a Bio-Gel P-6 column of oligosaccharides released after mild alkaline-borohydride treatment of $[^3\text{H}]$ glucosamine-labelled HMGs

The sample was applied to a column (1.2 cm $\times$ 115 cm) equilibrated with 0.1 M-pyridine/acetate buffer, pH 5.0. Fractions (1 ml) were collected and 20 μl samples were taken for counting of radioactivity. Fractions 83–91, 93–99 and 100–108 were pooled and designated fraction I, II and III respectively. The broken line represents the elution pattern of the HMGs before the alkaline-borohydride treatment. The values above the arrows indicate the K_{av} values for the corresponding peaks. V_0 and V_t are the excluded volume and total volume respectively.

protein bands after electrophoretic separation in 3–16% slab gels, it was found that a fraction of these glycoproteins was blocked in the 3% stacking gel. When the cells were metabolically labelled with $[^3\text{H}]$ glucosamine, this fraction, representing the putative mucins, accounted for 27% of the radioactivity incorporated into the glycoproteins of the conditioned medium. When the $[^3\text{H}]$ -mannose-labelled glycoproteins of the conditioned medium were analysed by gel electrophoresis, it was found that the radioactivity of the HMGs accounted for only 1.4% of the radioactivity of the glycoproteins of the conditioned medium.

To examine the nature of these HMGs further, they were eluted from the polyacrylamide gels and analysed by biochemical and immunological techniques.

The carbohydrate chains of the mucins are known to be linked *O*-glycosidically to the protein core through *N*-acetylgalactosamine. This bond is alkali-labile and it is selectively cleaved by mild alkaline-borohydride treatment.

All the carbohydrates present in the HMGs were released by mild alkaline-borohydride treatment (Fig. 1). Oligosaccharide fractions I (K_{av} 0.53), II (K_{av} 0.66) and III (K_{av} 0.76) were isolated by chromatography on Bio-Gel P-6 columns. These fractions represented 56, 16 and 28% of the radioactivity respectively.

The nature of the hexosamines linked to the polypeptide chain was determined after acid hydrolysis and paper chromatography (Fig. 2) of the oligosaccharide chains. From the chromatogram presented in Fig. 2, it was deduced that *N*-acetylgalactosaminitol represented 60% of the radioactivity, *N*-acetylgalactosamine 29% and *N*-acetylglucosamine 11%. No *N*-acetylglucosaminitol was found even when the HMG monosaccharides were analysed after a harsh alkaline hydrolysis, a treat-

Fig. 2. Paper chromatography of strong-acid hydrolysates obtained from the [^3H]glucosamine-labelled HMGs after mild alkaline-borohydride treatment

The hydrolysates were subjected to paper chromatography as described in the Materials and methods section. Arrows indicate the migration of standards: 1, *N*-acetylglucosaminitol; 2, *N*-acetylgalactosaminitol; 3, *N*-acetylgalactosamine; 4, *N*-acetylglucosamine.

ment that is thought to cleave both the *N*- and *O*-linked oligosaccharides (Zinn *et al.*, 1978). Thus we conclude that all the oligosaccharides were originally *O*-glycosidically linked via *N*-acetylglucosamine to threonine or serine residues in the polypeptide chain.

When sialic acids were removed by mild acid hydrolysis only fractions I and II were shifted to an elution pattern (Figs. 3*a* and 3*b*) similar to that observed for fraction III (Fig. 1) (this fraction is unaffected by hydrolysis). In each case an additional fraction with a K_{av} of 0.85 was produced. This material was considered to be sialic acid released after mild acid hydrolysis, since it was co-eluted with the standard *N*-acetylneuraminic acid. This material accounted in both cases for 38 % of the radioactivity in peaks I and II.

Finally, the metabolic conversion of the [^3H]glucosamine incorporated into the oligosaccharides

Fig. 4. Gel-filtration chromatography on a Bio-Gel P-4 column of neutral (desialylated) fraction I

The elution pattern of the dextran hydrolysate standard was determined by using a refractometer. The elution pattern was determined by counting the radioactivity of 100 μl samples. The numbers above the arrows indicate the number of glucose units, two to eight, per glucose oligomer eluted.

was inferred from the bulk of the results presented in Figs. 2, 3(*a*) and 3(*b*). The radioactivity was mainly found to be associated with sialic acid (27 %) and *N*-acetylgalactosaminitol (44 %), whereas *N*-acetylglucosamine and *N*-acetylglucosamine represented 21 % and 8 % respectively.

The M_r of the neutral oligosaccharide chains from fraction I was estimated by chromatography on Bio-Gel P-4. As shown in Fig. 4, these oligosaccharides behaved homogeneously and they represented six glucose units.

Taken together, these biochemical findings strongly suggest that the HMGs are mucins.

Immunological characterization of the Cl.16E mucins

Our simple procedure for rabbit immunization with purified HMGs in the presence of polyacrylamide gel resulted in the production of polyclonal anti-mucin antibodies. In the normal colonic mucosa, these anti-

Fig. 3. Gel-filtration chromatography on a Bio-Gel P-6 column of desialylated fractions I (*a*) and II (*b*) obtained after mild alkaline-borohydride treatment of [^3H]glucosamine-labelled HMGs

Each desialylated fraction was passed through a Bio-Gel P-6 column (1.2 cm $\times$ 115 cm). The elution pattern was determined by counting the radioactivity of 100 μl samples. The values above the arrows indicate the K_{av} values for the corresponding peaks. V_0 and V_t are the excluded volume and total volume respectively.

Fig. 5. Immuno-peroxidase staining of human colonic and gastric mucosae with rabbit antibodies to HMGs secreted by the CL16E cells

(a) In the colonic mucosa, specific reactivity was confined to the goblet cells. Magnification $\times 200$. (b) The staining was restricted to the mucus vacuoles of the goblet cells. Magnification $\times 550$. (c) In the gastric mucosa, the reactivity was found to be restricted to the superficial and to the neck mucous cells. Magnification $\times 70$.

bodies specifically stained the mucus content of the goblet cells of the crypt and of the surface epithelium (Figs. 5a and 5b). The mucin vacuoles of the goblet cells were not uniformly labelled. Interestingly, the reactivity of the immune serum was not restricted to the colonic mucus, since the superficial mucous cells of the gastric mucosa were also stained (Fig. 5c). The deep gland cells of the pyloric mucosa did not react with this immune serum.

These antibodies never reacted with cellular structures other than mucus vacuoles, nor did they react with the extracellular matrix. The reactivity was found to be restricted to the human mucins. Finally, when the pre-immune serum was used instead of the immune serum in the immunohistological assay, no labelling was observed.

To examine further the molecular species of the Cl.16E conditioned medium that reacted with these antibodies, the Cl.16E conditioned medium was concentrated by freeze-drying and then subjected to SDS/PAGE and then to immunoblot analysis. Before reduction, the HMGs were blocked in the wells of the stacking gel (Fig. 6, lane 2). The reactivity of the antibodies was restricted to the upper part of the gel near the stacking gel (Fig. 6, lane 3). The low- M_r species did not react with the antibodies. When the HMGs were purified by preparative electrophoresis and subjected to SDS/PAGE under reducing conditions, the mucins entered the gels and gave

Fig. 6. SDS/PAGE of glycoproteins secreted by the Cl.16E cells and of the HMGs purified from the conditioned medium

The glycoproteins secreted by the Cl.16E cells were electrophoresed in SDS/polyacrylamide gradient gel (3–16% acrylamide). Lanes 1 and 4, M_r standards in non-reduced and reduced conditions; lane 2, glycoproteins secreted by the Cl.16E cells (Coomassie Blue-stained); lane 5, after preparative SDS/PAGE, the HMGs were eluted from the stacking gels and run in reduced conditions (Coomassie Blue-stained); lanes 3 and 7, the same as lanes 2 and 5 respectively, but detection was by Western blot, the antibodies to HMGs being used; lane 6, the same as lane 5, but stained for glycoproteins with periodate/Schiff reagents.

several bands: a periodate/Schiff-stained band (Fig. 6, lane 6), which had barely entered the gel, corresponding to the glycosylated domains of the mucins, and several Coomassie Blue-stained bands, namely a band with an apparent M_r extending from 120 000 to 150 000 and several bands in the M_r range 40 000–66 000 (Fig. 6, lane 5). The broad periodate/Schiff-stained high- M_r band was barely observed when the gels were stained with Coomassie Blue. Upon immunoblot analysis after electrophoresis under reducing conditions, the reactivity of the immune serum was found to be restricted to the glycosylated domains of the mucins (Fig. 6, lane 7).

DISCUSSION

Several cell lines have been derived, either from colonic adenomas or from human colonic carcinomas (Tom *et al.*, 1976; Paraskeva *et al.*, 1984; Whitehead *et al.*, 1985, 1987b; Boland *et al.*, 1986; Willson *et al.*, 1987), that are potential candidates for studying mucins. The possibility of exploiting these cell lines depends primarily upon the availability of simple and reliable techniques for purifying and characterizing the secreted mucins. The experiments reported in the present paper were designed to purify the mucins secreted by a human colonic cancer cell line in culture and to characterize them according to biochemical and immunological criteria. On the basis of the very high resolution of SDS/PAGE, we reasoned that this method could be a very simple and powerful preparative technique for purifying the mucins secreted by the Cl.16E cells. Two lines of evidence strongly suggest that this method proved effective. First, the HMGs eluted from the gels were found to have the characteristically *O*-linked oligosaccharides of mucins; *N*-linked oligosaccharides were absent from the purified material. Secondly, the antibodies raised against these HMGs were specific for normal human gastrointestinal mucins, and did not react in immunohistological assays with non-mucin components.

Recently, these antibodies have been instrumental in developing an immunochemical assay for mucins secreted in culture. With the development of this assay method it becomes possible to quantify the mucins secreted either in basal conditions or under stimulation (Roumagnac & Labois, 1987). For example, the analysis of the incubation medium of post-confluent Cl.16E cells has shown that about 75 ng of mucins (expressed as protein) per 10^6 cells per 24 h was recovered from the conditioned medium (C. Augeron, unpublished work). Furthermore, recent investigations using this assay have shown that the cholinergic agent carbachol is able to stimulate the secretion of mucins from the Cl.16E cells (Roumagnac & Labois, 1987).

To date relatively few studies are available defining the oligosaccharide side chains of colonic mucins. In their work on the purification and characterization of rat colonic mucins, Slomiany *et al.* (1980) have found that the carbohydrate chains released by alkaline-borohydride reductive cleavage consist of oligosaccharides ranging in length from two to 12 sugar units. In his work on the structure of the carbohydrate units of human colonic mucus glycoproteins, Podolsky (1985a,b) has identified 21 discrete oligosaccharide structures in the major colonic mucin species, including ten acidic oligosaccharides and 11 neutral structures, which ranged in size from two to 12 sugar residues.

The present study indicates that the size heterogeneity of oligosaccharide chains is considerably less in the Cl.16E mucins than in normal colonic mucins. In agreement with our data are those of Kurosaka *et al.* (1983), who showed that four oligosaccharides, accounting for 50% of the total oligosaccharide side chains of mucins from a human adenocarcinoma, were two or three sugar residues in size. Since it has been shown that cancer mucins bear developmentally regulated antigenic determinants that are masked by further glycosylations in the adult, it is conceivable that the small size of the oligosaccharide side chains of cancer mucins represents the expression of a foetal program of maturation of mucus glycoproteins.

Several lines of investigation have clearly shown that the mucins isolated from the gastrointestinal tract are polymeric structures whose subunits are linked by disulphide bridges (reviewed in Allen *et al.*, 1982; Carlstedt *et al.*, 1985; Neutra & Forstner, 1987). In particular, it has been shown that with thiol reduction pig colonic (Marshall & Allen, 1978) and pig and rat small-intestinal (Mantle *et al.*, 1981; Fahim *et al.*, 1983) mucins release glycopeptides of high M_r , thereby providing evidence for a polymeric structure of the intestinal mucins. Our findings show that this concept of a polymeric structure for the normal intestinal mucins also applies to the Cl.16E mucins. Finally, several studies have shown that the reduction of small-intestinal mucins liberates a peptidic component having an M_r of 90000 for pig mucins (Allen *et al.*, 1982) and an M_r of 118000 for rat and human mucins (Fahim *et al.*, 1983, 1987; Mantle *et al.*, 1984), which may function as a discrete cross-linking agent for the glycopeptides. The fact that such a 'link' component is also present in the Cl.16E mucins can only be suggested on the basis of the present study, and further work is needed to clarify this important point.

This work supported in part by grants from the Association pour la Recherche sur le Cancer (A.R.C.), from the Ligue Nationale Française contre le Cancer and from the Faculté Xavier Bichat.

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