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ACTION OF TRIFLUOROMETHANESULFONIC ACID ON HIGHLY GLYCOSYLATED REGIONS OF HUMAN BRONCHIAL MUCINS*

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ABSTRACT

Highly glycosylated glycopeptides were prepared from human bronchial mucus. They were heterogeneous and contained an average of 45 residues of glycosylated hydroxyamino acid per 100 amino acid residues. The kinetics of deglycosylation of these glycopeptides by trifluoromethanesulfonic acid–anisole mixtures at 25° was monitored by chemical analysis and by polyacrylamide gel electrophoresis. The peripheral sugars were almost completely cleaved in 45 min with 3:2 and 2:1 CF_3SO_3H–anisole. A maximum of 75% of the O-linked *N*-acetylgalactosamine residues were released and mixtures of glycopeptides and peptides were obtained. Increasing the reaction time caused peptide bond cleavage. Rather mild conditions (1.2:1 CF_3SO_3H–anisole at 25° for 90 min) gave limited deglycosylation of highly glycosylated bronchial glycopeptides, allowing the uncovering of GalNAc–peptide linkages and peptide regions able to induce the formation of specific antibodies in the rabbit.

INTRODUCTION

Respiratory mucus glycoproteins, like all mucins, are high molecular-weight, polydisperse, and highly glycosylated molecules. They contain hundreds of carbohydrate chains which are very heterogeneous; their structures are being slowly elucidated.

There are considerable differences in the estimated molecular weight of mucins and of their reduction products. These differences may be explained by proteolytic or mechanical modifications of the molecules during extraction and handling[1]. The polydispersity of several mucin preparations has recently been revealed by electron microscopy: bronchial and submaxillary mucin molecules appear mainly as flexible threads with a relatively broad distribution of length[2,3].

In respiratory mucins, as in most mucins, the proteolytic susceptibility of differ-

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ent parts of the molecules has led to the concept of "naked" regions, almost devoid of carbohydrate chains and susceptible to proteolysis, and of highly glycosylated regions resistant to proteolysis[4]. Mucin glycopeptides, which correspond to those highly glycosylated regions, are also heterogeneous when examined by electron microscopy[2,5].

The deglycosylation of purified bronchial mucin or of purified, highly glycosylated regions (the most characteristic part of mucins from the content of hydroxyamino acids), is an obligatory step in the chemical study of the mucin peptide. The peptide of the highly glycosylated region is most probably completely covered by the multiple carbohydrate chains and may be a good antigen only after deglycosylation. The preparation of an immune serum specific for this peptide would be extremely valuable in biosynthesis experiments, to isolate the nascent mucin peptide within the bronchial mucosa and during cell-free translation of mRNAs from human bronchial mucosa.

Trifluoromethanesulfonic acid has been introduced as a reagent for deglycosylation of glycoproteins[6]. As enzymic deglycosylation of human respiratory mucins is rather limited[7] and since a preliminary report by Woodward *et al.*[8] indicated a possible, although incomplete, deglycosylation of human bronchial mucins by CF_3SO_3H at 0°, the aim of the present work was (*i*) to study the effect of CF_3SO_3H at 25° on highly glycosylated glycopeptides from human bronchial mucins, (*ii*) to prepare rabbit antibodies against such deglycosylated bronchial mucus glycopeptides, and (*iii*) to determine the specificity of these antibodies.

EXPERIMENTAL

Materials. — ECTEOLA-cellulose (Cellex E) was from Bio-Rad Laboratories (Richmond, CA). Sepharose 4B and the molecular-weight electrophoresis kit were from Pharmacia Fine Chemicals (Uppsala, Sweden). 2-Mercaptoethanol was from Eastman Kodak, Coomassie Brilliant Blue from Gurr, Tween 20 and anisole were from Merck, and trifluoromethanesulfonic acid was obtained from Aldrich Chemical Inc. Nitrocellulose sheets (0.45 μm) were purchased from Schleicher and Schüll. Bovine serum albumin (fraction V), calf serum, and wheat germ and *Helix pomatia* agglutinins (WGA and HPA) were from IBF (France). Peroxidase (type VI), peroxidase-labelled agglutinin from *Ricinus communis* (RCA 120), and bovine submaxillary mucin were from Sigma.

Bovine submaxillary mucin was purified by gel chromatography: it was excluded from a column of Sepharose 4B. Desialylation of this mucin was carried out with 0.5M sulfuric acid at 80° for 30 min.

Isolation of bronchial-mucus glycoproteins. — Bronchial sputum was obtained from two blood group O patients, one suffering from cystic fibrosis and the other from chronic bronchitis. The sputum was collected daily and kept frozen until use. Acidic bronchial-mucus glycoproteins were isolated from the reduced mucus according to Lamblin *et al.*[5] with slight modifications: the reduction was performed

on the whole mucus and the acidic fractions F2 or F3 eluted from the ECTEOLA-cellulose column were collected, dialyzed, and freeze-dried.

Pronase hydrolysis. — Fractions F2 or F3 obtained from ECTEOLA chromatography (Fig. 1) were treated with pronase, in 0.15M Tris acetate buffer (pH 7.8), for 72 h, using an enzyme–substrate ratio of 1:40; additional enzyme was added at 24 and at 48 h.

After 72 h, the mixture was centrifuged and the supernatant solution was dialyzed, freeze-dried, and submitted to chromatography on a column of Sepharose 4B as already described[5].

Deglycosylation procedure. — Trifluoromethanesulfonic acid was used as a reagent for deglycosylation according to Edge *et al.*[6], with some modifications. Before treatment, the mucin glycopeptide samples were lyophilized for 48 h and dried over P_2O_5 under vacuum at 60° overnight. Anisole and CF_3SO_3H were mixed in a glass tube with a Teflon-lined screw-cap and cooled to 4°. CF_3SO_3H and anisole were used in ratios (v/v) of 1, 1.2, 1.5, and 2. Before opening the flask containing CF_3SO_3H, it was cooled with acetone–Dry Ice to keep it liquid.

In a typical experiment, 10 mg of dry mucin glycopeptides were added to 1 mL of the different CF_3SO_3H–anisole mixtures in a 5-mL Reacti-Vial (Pierce Chemical Co.). Nitrogen was bubbled through the solution for 30 s and the vial left at 25° with occasionnal stirring for 45, 90, and 150 min.

The reaction was terminated by placing the vial in acetone–Dry Ice. The deglycosylated peptides were freed of reagents and low molecular-weight sugars by successive additions of cold diethyl ether (2 mL) and cold 50% pyridine (2 mL); the suspension was mixed with a vortex mixer and two ether extractions were performed, followed by dialysis as described by Edge *et al.*[6].

The non-dialyzable fraction was then lyophilized and precipitated at 0° with 2 mL of acetone to remove the remaining salts. After centrifugation at 3000*g*, the pellet containing the deglycosylated mucin glycopeptides was dissolved in 1 mL of distilled water and freeze-dried.

Electrophoresis studies. — Polyacrylamide slab gels (5–18%) were prepared according to Kerckaert[9] and the electrophoresis was conducted according to Laemmli[10]. After electrophoresis, the gels were stained for protein with Coomassie Blue[10] and silver nitrate[11], and for carbohydrate by the procedure of Zacharius *et al.*[12]. The amount of product applied onto each well varied according to the staining procedure used subsequently: 100 μg was required for silver nitrate or periodate–Schiff staining and 3 μg for subsequent blot analysis with antibodies or with lectins.

Blot analysis. — Glycopeptides were electrophoretically transferred onto nitrocellulose as described by Towbin *et al.*[13] with a voltage of 12 V/cm for 3 h.

(*a*) *Immuno-overlays.* Nitrocellulose strips were soaked in 4% bovine serum albumin for 1 h at 37°. After a 15-min wash in 0.01M Tris–HCl buffer (pH 7.4) containing 0.15M NaCl and 0.05% Tween 20 (Tris-buffered saline containing Tween 20), the strips were incubated with rabbit immune serum directed against CF_3SO_3H-treated glycopeptides for 1 h at room temperature. This immune serum

was diluted 1/110 in Tris-buffered saline containing 1% bovine serum albumin and 10% calf serum, and 5.5 mL of diluted immune serum were used for each strip. Two 15-min washes with Tris-buffered saline containing Tween 20 were then performed before incubating the strips with a 1/2400 dilution of peroxidase-conjugated anti-rabbit IgG immune serum. Before staining the strip with 0.0001% *o*-dianisidine and 0.0015 v H_2O_2 in Tris-buffered saline for 10 min, two 15-min washes with Tris-buffered saline containing Tween 20 were performed.

(*b*) *Lectin overlays.* Nitrocellulose strips were soaked in 4% bovine serum albumin for 1 h at 37°. After a 15-min wash in phosphate-buffered saline (PBS), the strips were incubated for 16 h at room temperature with peroxidase-labelled lectins. *Helix pomatia* and wheat germ agglutinins were labelled with peroxidase according to Avrameas[14]. Labelled ricin was diluted 1 to 12.5 in PBS containing 0.05% Tween, whereas labelled *Helix pomatia* and wheat-germ agglutinins were diluted in PBS without Tween (1 to 5000 for labelled WGA and 1 to 10000 for labelled HPA). After 15 min in PBS, the strips were stained with *o*-dianisidine as already described.

Immune serum preparation. — Immune serum was prepared in a New Zealand rabbit by repeated injections of 200 μg of fraction F2a (from the patient suffering from chronic bronchitis) mixed with complete Freunds' adjuvant every 4 weeks over the course of 3 months. Saturation of the immune serum (50 μL) was carried out with 1 mg of desialylated bovine submaxillary mucin.

Chemical composition. — Amino acid analyses were performed as already described[15] and quantitative gas–liquid chromatography of carbohydrates was conducted as described by Lamblin *et al.*[16].

RESULTS AND DISCUSSION

Purification of highly glycosylated glycopeptides from bronchial mucins of a patient suffering from cystic fibrosis. — Lyophilized bronchial mucus (5 g) obtained from the patient suffering from cystic fibrosis was solubilized by reduction with 1% 2-mercaptoethanol. After centrifugation of the reduced mucus, the sediment was discarded and the supernatant solution was dialyzed and lyophilized (2 g); it was submitted to ion-exchange chromatography on a column of ECTEOLA-cellulose that was eluted with NaCl solutions of increasing molarity (Fig. 1A): three carbohydrate fractions were obtained.

The more-acidic fraction, F3 (200 mg), was subjected to digestion by pronase for 72 h. After proteolysis, the mixture was submitted to gel-filtration chromatography on a column of Sepharose 4B (Fig. 1B), which separated the acidic glycopeptides (F3a) from degraded peptides (F3b). The electrophoretic behavior of this glycopeptide fraction was checked: a fraction that hardly penetrated into polyacrylamide was strongly stained by periodate–Schiff (Fig. 2); this fraction was not stained by Coomassie Blue and was poorly revealed by silver nitrate. In addition, two very faint bands with apparent molecular weights of 90,000 and 60,000 were observed.

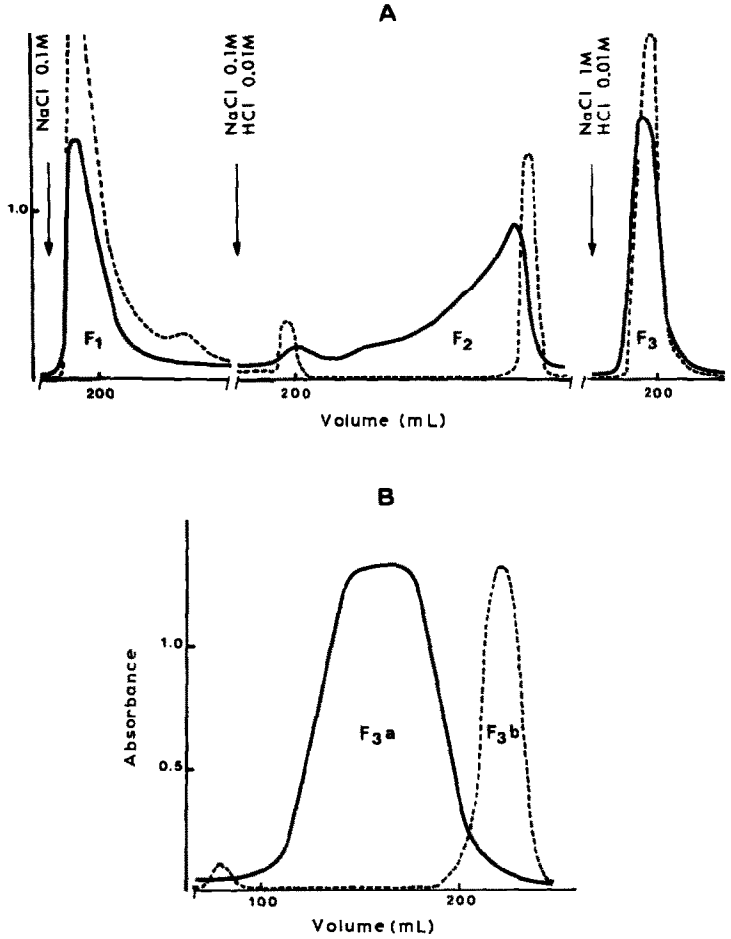

Fig. 1. A. Ion-exchange chromatography of reduced mucus (500 mg) on a column of ECTEOLA-cellulose (2.5 × 48 cm). The ECTEOLA column was eluted with 0.1M NaCl, 0.1M NaCl–0.01M HCl, and M NaCl–0.01M HCl solutions. Fractions (10 mL) were analyzed for hexose (solid line) and for absorbance at 278 nm (dashed line). Three fractions were pooled, dialyzed and lyophilized (F1 = 140 mg; F2 = 76 mg; F3 = 94 mg). B. Gel filtration chromatography of acidic bronchial glycopeptides (100 mg) on a column of Sepharose 4B (2.5 × 48 cm). The column was eluted with 0.1M Tris-HCl buffer pH 8.0 containing 0.2M NaCl and 0.02% NaN_3. The collected fractions (5 mL) were analyzed as indicated in Figure 1A. Fractions F3a and F3b were dialyzed and lyophilized (F3a = 72 mg; F3b = 20 mg).

Fraction F3a contained 77.5% carbohydrate and 9.3% amino acid. Its detailed composition is indicated in Table I, expressed as number of residues per 100 amino acid residues. The number of hydroxylated amino acids and of *N*-acetylgalactosamine residues are about the same. As the glycopeptides were prepared from the bronchial secretion of a patient with blood group O, assuming that all the *N*-acetylgalactosamine residues are involved in O-glycosylic linkages with the peptide, these results indicate that all of the serine and threonine residues are

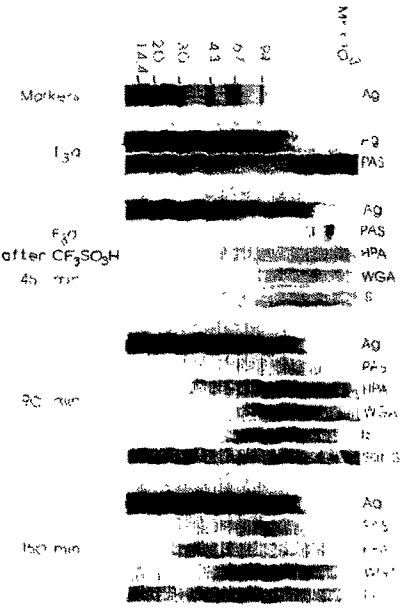

Fig. 2. Polyacrylamide gel electrophoresis of glycopeptides fraction F3a before and after treatment with 3:2 CF_3SO_3H–anisole for 45, 90, and 150 min. Gels were stained by silver nitrate (Ag) or periodate–Schiff (PAS). Blots were revealed by peroxidase-labelled *Helix pomatia* and wheat-germ agglutinins (HPA and WGA), or by immune serum (IS) or immune serum saturated with desialylated bovine submaxillary mucin (Sat IS).

glycosylated and that the glycopeptides contain an average of one carbohydrate chain almost every other amino acid.

Kinetic study of the deglycosylation of bronchial glycopeptides F3a. — A kinetic study of deglycosylation was carried out with increasing amounts of CF_3SO_3H. The study was monitored by determination of chemical composition and by polyacrylamide gel electrophoresis of the products.

Aliquots of bronchial glycopeptides F3a (10 mg) in 1 mL of CF_3SO_3H–anisole mixtures of different proportions (v/v ratio: 1, 1.5, or 2) were kept at 25° for 45, 90, and 150 min. The yields, calculated by measuring the amino acid content of the deglycosylated glycopeptides, varied from 23 to 60%.

The chemical composition of the different deglycosylated fractions is indicated in Table I. These data demonstrate that treatment of such glycopeptides with 1:1 CF_3SO_3H–anisole progressively removes most of the peripheral fucose, galactose, and *N*-acetylglucosamine residues, and a small proportion of the peptide-linked *N*-acetylgalactosamine residues. By increasing the reaction time, more elimination of *N*-acetylgalactosamine residues occurs but, even after 150 min, the galactose content is still ~14% of the starting glycopeptides and the *N*-acetylgalactosamine content is about two-thirds of the original glycopeptides (Table I

TABLE I

COMPOSITION OF GLYCOPEPTIDE FRACTION F3a BEFORE AND AFTER TREATMENT BY CF_3SO_3H UNDER DIFFERENT CONDITIONS[a]

Component	*Untreated*	*1:1 CF_3SO_3H–anisole*			*3:2 CF_3SO_3H–anisole*			*2:1 CF_3SO_3H–anisole*		
		45 min	*90 min*	*150 min*	*45 min*	*90 min*	*150 min*	*45 min*	*90 min*	*150 min*
Asp	2.8	2.0	2.6	1.9	1.3	1.8	1.2	1.6	1.1	1.2
Thr	33.7	31.4	31.0	35.0	34.6	34.5	35.7	34	36.1	35.8
Ser	12.2	14.9	15.0	14.0	16.5	15.9	15.7	15.1	15.7	15.4
Glu	3.2	3.3	3.2	3.0	2.6	2.8	2.6	3.0	2.7	2.7
Pro	12.9	12.5	11.4	12.3	12.2	12.3	12.5	12.1	12.5	12.4
Gly	5.7	8.1	8.1	6.4	6.4	6.1	6.0	6.5	5.8	6.1
Ala	9.5	9.8	9.8	9.8	9.7	9.7	9.8	9.9	9.8	9.9
Val	5.6	4.0	4.0	3.9	3.8	3.7	3.7	4.2	3.7	3.8
Cyst A + Cys					0.3					
Met	0.3	0.2	0.4	0.3	0.3	0.2	0.6	0.1	0.4	0.6
Ile	2.3	2.3	2.3	2.2	2.1	2.1	2.2	2.3	2.2	2.2
Leu	4.3	4.3	4.3	4.3	4	4.1	4.1	4.5	4.2	4.2
Tyr		0.5	0.5	0.5	0.2	0.2	0.2	0.3	0.2	0.3
Phe	1.6	1.2	1.2	0.9	0.8	0.8	0.7	0.9	0.7	0.8
Lys	1.4	1.5	1.5	1.2	1.1	1.1	1.1	1.3	1.1	1.1
His	2.3	2.5	2.5	2.2	2.1	2.0	2.0	2.2	2	1.8
Arg	2.2	1.9	1.9	1.9	1.8	1.9	1.8	2.0	1.9	1.8
GalNAc	47.8	45.4	39.0	36.1	28	24.6	18.7	25.7	19.4	12.3
GlcNAc	93.5	51.8	31.5	15.7	9.1	4.4	3.9	8.2	4.6	2.1
Gal	139.5	64.4	30.7	21.1	3.7	3.1	1.8	4.7	2.9	1.8
Fuc	100.2	32.9	14.0	8.3	0	0	0	0	0	0
NeuAc	22.3	18.4								
GalNAc/Ser + Thr	1.00	0.98	0.84	0.73	0.54	0.49	0.36	0.52	0.37	0.24

[a]Results are expressed as residues per 100 amino acid residues.

and Fig. 3). With 3:2 or 2:1 CF_3SO_3H–anisole for 90 min, all of the fucose, >97% of the galactose, and 95% of the *N*-acetylglucosamine residues are removed. The resistant glycopeptides have an average of one *N*-acetylgalactosamine residue for every four or five amino acids. In all instances the hydroxy amino acid composition remains almost invariable. These results are slightly different from those of Woodward and Davidson[8] who indicated that all of the *N*-acetylgalactosamine residues were resistant to CF_3SO_3H at 0°.

The identification of the deglycosylated products on polyacrylamide gel electrophoresis is difficult as they are not stained by Coomassie Blue. They may be revealed with silver nitrate provided that the gels are loaded with relatively large quantities of material, suggesting that only limited regions of the glycopeptides react with silver nitrate.

Polyacrylamide gel electrophoresis of glycopeptides treated with 1:1 CF_3SO_3H–anisole still shows some fractions, stained by periodate–Schiff and which resist deglycosylation, and a broad zone corresponding to products of lower molecular mass revealed only by silver nitrate. These products may be mixtures of polypeptides and/or incompletely deglycosylated glycopeptides (data not shown). With increasing concentration of CF_3SO_3H and increasing reaction time, the electrophoretic mobility and the broadness of the zone stained by silver nitrate increase. Polyacrylamide gel electrophoresis of the fractions obtained after treat-

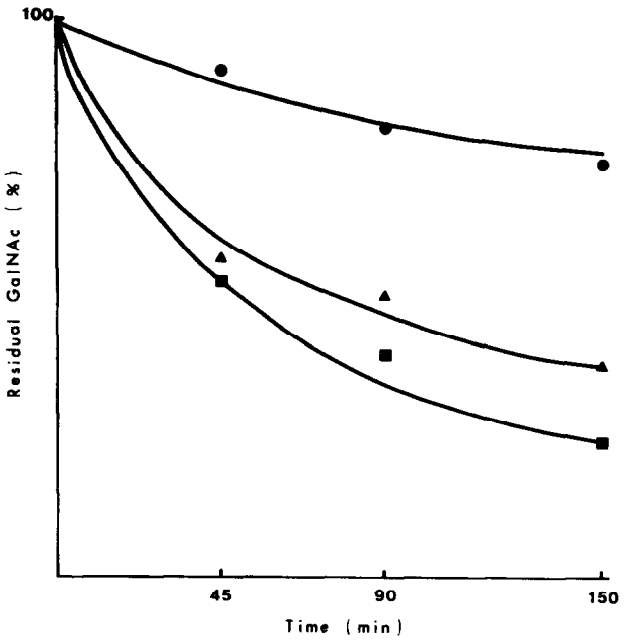

Fig. 3. Kinetics of removal of *N*-acetylgalactosamine in bronchial mucin glycopeptides (F3a) treated by increasing amounts of CF_3SO_3H. The proportions of CF_3SO_3H–anisole mixtures were 1 to 1 (●), 1.5 to 1 (▲) and 2 to 1 (■).

ment with 1.5:1 CF_3SO_3H–anisole showed broad zones of increasing mobility, scarcely stained by periodate–Schiff (Fig. 2). Silver nitrate revealed broadening zones of increasing mobility mainly in fractions treated for 90 and 150 min (from mol. wt. 200,000 to <20,000, Fig. 2). After treatment with 2:1 CF_3SO_3H–anisole, the zone stained by periodate–Schiff was even fainter and the zone revealed by silver nitrate increased even more in extent and mobility (data not shown).

Blot analysis of these fractions was performed with peroxidase-labelled lectins from *Ricinus communis* (RCA 120), wheat germ, and *Helix pomatia*. For the fractions treated with 3:2 CF_3SO_3H–anisole, the zones detected with *Ricinus communis* agglutinin corresponded to the zones stained by periodate–Schiff (data not shown). The zones stained by peroxidase-labelled HPA became broader with increasing reaction time: they were a little more diffuse than the zones revealed by peroxidase-labelled WGA but less than the zones stained by silver nitrate (Fig. 2).

All of these data probably indicate that, with increasing CF_3SO_3H concentration and reaction time, an increasing number of peptide bonds are cleaved, although most of the resistant sugars (GalNAc) remain attached to the peptide. Therefore a mixture of progressively smaller glycopeptides containing primarily *N*-acetylgalactosamine is obtained. Furthermore, ~40% of the total amount of amino acids is recovered after CF_3SO_3H treatment, thus suggesting some peptide loss either during dialysis or during acetone precipitation.

The presence of broad zones in polyacrylamide gel electrophoresis is probably due to the additive effects of several factors responsible for heterogeneity: (*i*) the bronchial glycopeptides, whose lengths have been measured by electron microscopy, are already heterogeneous[5]; (*ii*) the deglycosylation procedure may not affect all of the carbohydrate chains equally, and (*iii*) some peptide cleavage may occur. Altogether, these factors probably account for the increasing polydispersity of the deglycosylation products.

Edge *et al.*[6] have used CF_3SO_3H treatment at 0° to deglycosylate fetuin and human M,N-active erythrocyte sialoglycoproteins that contain serine- or threonine-linked *N*-acetylgalactosamine and *N*-glycosylically linked *N*-acetylglucosamine. They observed a retention of *N*-glycosylically linked *N*-acetylglucosamine and a slow disappearance of serine- and threonine-linked *N*-acetylgalactosamine, which seemed to be complete after 5 h. For fetuin treated at 0°, they reported that no peptide fragmentation occurred, although the recovery of protein was 76% of the control.

Preparation of an immune serum directed against bronchial glycopeptides treated by CF_3SO_3H. — Another bronchial-glycopeptides fraction was prepared from the bronchial mucus of a patient suffering from chronic bronchitis. The fractionation procedure was similar to that used in the kinetic study, except that Fraction 2, which was eluted from the ECTEOLA-cellulose column by a solution of 0.1M NaCl, 0.01M HCl, was the major fraction. This glycopeptide fraction (F2) was then digested with pronase and subjected to chromatography on a column of Sepharose 4B, in order to obtain glycopeptide fraction F2a. The chemical com-

position of fraction F2a is indicated in Table II and its contamination by peptides was checked by polyacrylamide gel electrophoresis (Fig. 4). Fraction F2a was stained only by periodate–Schiff.

In order to obtain mucin peptide having only a limited peptide degradation, fraction F2a (230 mg) was deglycosylated with 1.2:1 CF_3SO_3H–anisole mixture for 90 min at 25°. The amino acid recovery of the deglycosylated fraction was 52%. The chemical compositions before and after deglycosylation are indicated in Table II. Most of the fucose, galactose, and *N*-acetylglucosamine residues were removed, but ~76% of the *N*-acetylgalactosamine residues remained attached to the peptide. The electrophoresis pattern on polyacrylamide gel of this fraction, and the blot analyses, are shown in Fig. 4. The deglycosylated fraction F2a is revealed by silver nitrate in two rather broad zones with average molecular mass of 120,000 and 60,000, but less diffuse than the zone observed after treatment of fraction F3a with 3:2 CF_3SO_3H–anisole (Fig. 2). The periodate–Schiff staining is weak and limited to a zone corresponding to 100,000–120,000. The staining of the blots with peroxidase-labelled *Helix pomatia* agglutinin is broader than the staining observed with labelled WGA (Fig. 4).

TABLE II

CHEMICAL COMPOSITION OF GLYCOPEPTIDE FRACTION F2a BEFORE AND AFTER TREATMENT BY CF_3SO_3H (CF_3SO_3H/ANISOLE = 1.2)[a]

Component	*Untreated*	*CF_3SO_3H-treated*
Asp	3.5	3.6
Thr	32.4	31.2
Ser	15.4	14.1
Glu	3.8	4.5
Pro	12.1	11.3
Gly	6.8	7.3
Ala	9.0	9.6
Val	3.6	3.8
Met	0.2	0.2
Ile	1.9	2.1
Leu	3.8	4.1
Tyr	0.4	0.5
Phe	1.5	1.2
Lys	1.4	2.0
His	2.1	1.9
Arg	2.0	2.3
GalNAc	45.5	34.6
GlcNAc	94.2	6.7
Gal	126.4	4.0
Fuc	57.4	1.3
NeuAc	11	
GalNAc/Ser + Thr	0.95	0.76

[a]Results are expressed as residue per 100 amino acid residues.

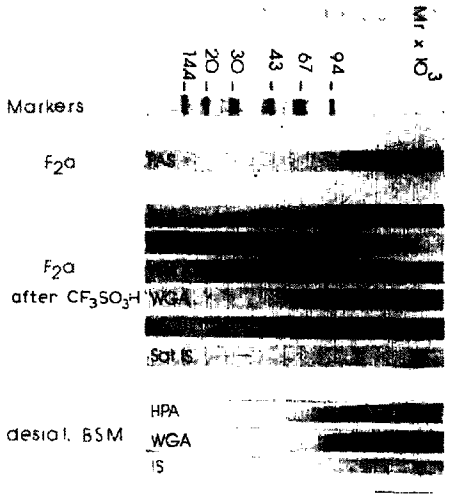

Fig. 4. Polyacrylamide gel electrophoresis of glycopeptide fraction F2a before and after treatment with CF_3SO_3H–anisole in a ratio of 1.2 to 1 for 90 min. Gels were stained by silver nitrate (Ag) or periodate–Schiff (PAS). Blots were revealed by peroxidase-labelled HPA and WGA, or by immune serum (IS) or immune serum saturated by desialylated bovine submaxillary mucin (Sat IS). Blots of desialylated bovine submaxillary mucin (desial. BSM) were also revealed by labelled lectins and immune serum.

The deglycosylated fraction F2a was used for immunization, and the production of antibodies was checked by immunoblotting. Within 3 months, an immune serum able to reveal the deglycosylated fraction F2a was obtained (Fig. 4); under our experimental conditions, it did not react with the untreated fraction F2a. This immune serum also reacted with the products obtained after deglycosylation of fraction F3 (Fig. 2).

Characterization of the antigenic determinants revealed by the immune serum. — As antibodies may have been produced against 2-acetamido-2-deoxy-α-D-galactosyl groups, the hemagglutinating properties of the immune serum were tested with A and B human red blood cells. A weak agglutination was observed with both types of cell, but without a higher affinity for A-blood cells.

The chemical composition of deglycosylated F2a suggested that it contained many GalNAc–peptide linkages in addition to longer carbohydrate chains containing *N*-acetylglucosamine. Such a composition resemble that of desialylated bovine submaxillary mucin, which is well revealed not only by *Helix pomatia* and wheat germ labelled agglutinins (Fig. 4), but also by the immune serum (Fig. 4).

Finally, using a blot of deglycosylated bronchial fraction F2a, a band having a molecular mass of ~120,000 was revealed by the same immune serum previously adsorbed with desialylated bovine submaxillary mucin (Fig. 4). Among all the fractions obtained after the different CF_3SO_3H treatments of fraction F3a (Table I), only one fraction could be revealed by this saturated immune serum: the fraction that was treated by 3:2 CF_3SO_3H–anisole for 90 min and which gave two small bands with an estimated molecular mass of 65,000–70,000 (Fig. 2).

All of the fractions obtained by deglycosylation of the glycopeptides from both patients, one suffering from chronic bronchitis and the other from cystic fibrosis, are revealed by *Helix pomatia* agglutinin and by the immune serum (Fig. 2 and Fig. 4). Most of these antigenic sites probably correspond to GalNAc–peptide linkages, as their broad staining on immunoblots disappears after prior adsorption of the immune serum with desialylated bovine submaxillary mucin.

However, this saturated immune serum still contains antibodies reacting with the deglycosylated fraction F2a from the patient with chronic bronchitis and with a deglycosylated fraction F3a from the patient with cystic fibrosis (Fig. 2).

The probability that these epitopes, present in the mucin peptides of two patients suffering from different bronchial disorders, are related to a specific bronchial disorder is weak. Moreover, these epitopes are very sensitive to CF_3SO_3H, as they are only observed after a treatment with CF_3SO_3H–anisole mixtures of 1.2 to 1 or 1.5 to 1 for 90 min, and not for a longer time or with a higher CF_3SO_3H–anisole ratio. The difference in molecular mass of the bands revealed in the two different experiments may correspond to a difference in the CF_3SO_3H sensitivity of the two glycopeptides (F2a and F3a) or to the slight difference in CF_3SO_3H–anisole ratio.

In conclusion, the present data strongly suggest that a treatment of highly glycosylated regions of human bronchial mucins with a CF_3SO_3H–anisole mixture of 1.5 or 1.2 to 1 for 90 min at 25° allows the uncovering of antigenic sites corresponding to GalNAc–peptide linkages and to small parts of the bronchial mucin peptides.

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