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α-D-Galactose-bearing glycoproteins on the surface of stimulated murine peritoneal macrophages

Biochemical and immunochemical characterization of purified glycoproteins

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Two glycoproteins were isolated from lysates of thioglycollate-stimulated, murine peritoneal macrophages by affinity chromatography on immobilized *Griffonia simplicifolia* I lectin and by preparative SDS/PAGE. The glycoproteins were readily labeled on the surface of intact macrophages with ^{3}H and ^{125}I. The labeled glycoproteins migrated as broad bands of molecular mass 92–109 kDa and 115–125 kDa. The mobility of the glycoproteins decreased only slightly after reduction with dithiothreitol, indicating the absence of intersubunit disulfide bridges. The 92-kDa and 115-kDa glycoproteins had p*I* 5.2–5.4 and p*I* ≤ 4, respectively. Digestion of both glycoproteins with α-galactosidase released 23% of their ^{3}H content and abolished their ability to bind to the *G. simplicifolia* I lectin, showing that they contain terminal α-D-galactosyl groups. After reduction with 2-mercaptoethanol, each glycoprotein fraction was sensitive to *N*-glycanase; the 115-kDa glycoproteins produced a smear with the front at approximately 67 kDa, whereas the 92-kDa glycoprotein gave two bands of 61 kDa and 75 kDa. Unreduced glycoproteins were insensitive to *N*-glycanase, suggesting the presence of intramolecular disulfide bonds. Although each glycoprotein fraction was sensitive to endoglycosidase H, this enzyme produced only slight changes in molecular mass when compared with *N*-glycanase. From these results as well as from the specificity of the enzymes involved, it is concluded that each glycoprotein fraction contains complex-type oligosaccharides and a small amount of high-mannose and/or hybrid-type oligosaccharides. While each glycoprotein fraction was bound to *Datura stramonium* lectin, they failed to react with anti-[i-(Den)] serum and their digestion with endo-β-galactosidase did not cause a band shift in SDS/PAGE. Taken together, these results suggest the presence of *N*-acetyllactosamine units which are not arrayed in linear form but occur as single units, bound either to C2 and C6, or to C2 and C4, or both, of outer mannosyl residues on complex-type oligosaccharides. The glycoprotein(s) fraction precipitated with anti-[I (Step)] serum, suggesting the presence of branched lactosaminoglycans. Digestion of both glycoprotein fractions with a mixture of sialidase and *O*-glycanase did not alter their mobility in SDS/PAGE, suggesting a lack or low content of *O*-linked trisaccharides and tetrasaccharides. Each glycoprotein fraction was bound specifically to *Sambucus nigra* and *Maackia amurensis* immobilized lectins, indicating the presence of sialic acid linked α2,6 to subterminal D-galactose or *N*-acetylgalactosamine residues, and α2,3 to *N*-acetyllactosamine residues, respectively.

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Abbreviations. EDA, ethylenediamine; GSI, *Griffonia simplicifolia* I lectin; GSI-B_4, *G. simplicifolia* I-B_4 isolectin; LacNAc, *N*-acetyllactosamine; MeGal, methyl-α-D-galactoside; WGA, wheat-germ agglutinin.

Enzymes. Endoglycosidase H, endo-β-*N*-acetylglucosaminidase H (EC 3.2.1.96); endo-β-galactosidase (EC 3.2.1.103); α-D-galactosidase, α-D-galactoside galactohydrolase (EC 3.2.1.22); galactose oxidase, D-galactose:oxygen oxidoreductase (EC 1.1.3.9); neuraminidase, acylneuraminyl hydrolase (EC 3.2.1.18); *N*-glycanase, peptide-N_4-(*N*-aetyl-β-glucosaminyl)-asparagine amidase (EC 3.5.1.52); *O*-glycanase, endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97); trypsin (EC 3.4.21.4).

Cell-surface carbohydrates participate in recognition phenomena which include cell-milieu and cell-cell interactions [1, 2]. α-D-Galactose groups occur at the non-reducing termini of oligosaccharides linked to cell-surface lipids [3–11], proteins [12–22] and unidentified glycoconjugates [23–33]. Some α-D-galactose groups have been implicated in the function of various biological systems. For example, Bleil and Wassarman [20] have shown that α-D-galactose groups present on glycoproteins from zona pellucida of mouse ova are involved in the binding of sperm to egg. Varani et al. [27], have shown a strong positive correlation between expression of α-D-galactose groups and malignant behavior of murine fibrosarcoma cells. Moreover, the results of Grimstad et al.

[17] indicate that α-D-galactose groups play a role in the attachment of murine fibrosarcoma cells to various collagen species. Additionally, Castronovo et al. [29] observed that anti-(α-D-galactose) antibodies reduced lung colonization by the malignant murine tumor cell line MO4 and inhibited binding of some tumor cell lines to human umbilical chord and laminin [31].

Stimulated and activated, but not resident macrophages, express α-D-galactose groups on their surface glycoproteins [13, 34]. Moreover, surface glycoproteins containing α-D-galactose groups, in the presence of *Griffonia simplicifolia* I-B_4 isolectin (GSI-B_4) participate in binding of macrophages to tumor cells which result in lysis of the tumor cells [35]. Takacs and Staehli [36] postulated that α-D-galactose residues participate in a recognition system that permits activated macrophages to discriminate between normal and tumor cells. Preliminary studies by others showed that peptides obtained by pronase digestion of stimulated and activated macrophages contained lactosaminoglycans bearing α-D-galactose end groups [37]. Stimulated macrophages bind to *G. simplicifolia* I-B_4 [13], *Evonymus europea* [38, 39] and *Datura stramonium* lectins [39], and the physiochemical parameters of lectin binding have been determined [39].

In the present study, we report the isolation of α-D-galactose-bearing glycoproteins from the surface of stimulated murine peritoneal macrophages and their partial biochemical and immunochemical characterization. We suggest that identification of α-D-galactose-bearing molecules on the macrophage surface, as well as isolation and characterization of oligosaccharides from these molecules, will further our understanding of these biological processes at the molecular level.

MATERIALS AND METHODS

Chemicals

Lactose, methyl-α-D-galactopyranoside (MeGal) and *N*-acetyl-D-glucosamine were products of Pfanstiehl Laboratories. *N*-Acetyllactosamine (LacNAc) was available in this laboratory from previous studies [40]. The following compounds were obtained from the sources indicated: Iodogen from Pierce; $Na^{125}I$ (carrier free) and scintillator fluid (En^3Hance) from New England Nuclear/Du Pont; sodium [^3H] borohydride of specific activity 1.4 Ci/mmol from ICN Radiochemicals; crystallized bovine serum albumin from ICN ImmunoBiologicals; thioglycollate brewers medium from Difco; toluene-based scintillation fluid from Research Products Int. Corp.; Triton X-100 from Sigma.

Enzymes

Galactose oxidase (*Dactylium dendroides*) was obtained from Sigma; coffee bean α-D-galactosidase from Boehringer Mannheim Biochemicals; neuraminidase from *Vibrio cholerae*, Serva; tosylphenylalaninechloromethane-treated trypsin, Worthington Biochemical Corporation. *N*-Glycanase and *O*-glycanase were purchased from Genzyme Corporation. Endo-β-*N*-acetylglucosaminidase H and endo-β-glactosidase were generous gifts from Dr. F. Maley [41] and Dr. Y. T. Li, respectively.

Lectins and sera

G. simplicifolia I lectin (GSI) and GSI-B_4 were purified from seeds provided by Calbiochem, as previously described [42]. GSI was coupled to Sepharose 4B by the CNBr technique [43]; the conjugate contained 1.1 – 2.4 mg lectin/ml settled beads. *D. stramonium, Maackia amurensis* and wheat-germ agglutinin (WGA) lectins coupled to agarose were obtained from E-Y Laboratories and contained 3 – 5 mg/ml, 9 mg/ml and 4 – 5 mg/ml purified lectin, respectively, on settled beads. *Sambucus nigra* lectin, purified from elderberry bark [44], was coupled to Sepharose 4B according to the procedure of March et al. [45]. The conjugate contained approximately 1 mg purified lectin/ml gel. Antisera to purified GSI were obtained by immunization of rabbits according to the procedure described earlier [46]. Anti-[i (Den)] and anti-[I (Step)] antisera were generously provided by B. Croucher and M. C. Crookstone from Toronto General Hospital. Rat monoclonal antibody against murine Mac-3 antigen was from Hybritech. Rabbit antibodies against rat IgG were from ICN ImmunoBiologicals and goat serum against human IgM was provided by the International Immunology Corporation.

Protease inhibitors

Aprotinin (10 μg), antipain (50 μg), chymostatin (100 μg), leupeptin (10 μg), α_2-macroglobulin (100 μg), pepstatin (100 μg) and 4-amidinophenylmethylsulfonyl fluoride (50 μg) were obtained from Boehringer Mannheim. Benzaminide hydrochloride (100 μg), phenylmethylsulfonyl fluoride (2 mM), 1,10-phenanthroline (5 mM) and *N*-ethylmaleimide (0.4 mM) were from Eastman Kodak. Iodoacetamide (1 mM) was from Aldrich Chemical Co. Numbers in parentheses indicate final concentrations of inhibitors in 1 ml solution present during the purification procedure.

Macrophages

Thioglycollate-stimulated macrophages were obtained from the peritoneal cavity of C57BL/6J female mice, 6 – 10 weeks of age, as described previously [39]. The mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Macrophage surface labeling

Approximately $9 - 12 \times 10^7$ thioglycollate-stimulated macrophages in 2 – 2.5 ml 10 mM phosphate and 0.15 M NaCl, pH 7.1 (NaCl/P_i) containing 0.02% azide (buffer A) was digested wtih 6 μg trypsin for 30 s. The reaction was terminated by addition of a solution containing 1 mg α_2-macroglobulin, then benzamidine/HCl and phenylmethylsulfonyl fluoride at a final concentration of 100 μg/ml and 2 mM, respectively. The macrophages were washed three times at 4 °C with 15 ml buffer A, centrifuged at $400 \times g$ for 5 min, and labeled with ^3H or ^{125}I.

Iodination was carried out by the iodogen method [47]. The pelleted cells were suspended in 2 ml buffer A, in a glass vial coated with 800 μg iodogen. 1.5 mCi $Na^{125}I$ was added and the suspension was stirred magnetically for 12 – 15 min at room temperature. The cell suspension was washed as described above.

Labeling with ^3H was carried out as described elsewhere [48, 49]. Briefly, a suspension of trypsinized macrophages contained 14×10^7 cells, 0.15 U of neuraminidase, 450 U of galactose oxidase and protease inhibitors (except iodoacetamide, α_2-macroglobulin and 4-aminidinophenylmethylsulfonyl fluoride) in a final volume of 2 ml. In control experiments, galactose oxidase was omitted. The suspension was incubated at 37 °C for 2 h, followed by removal of enzymes

by washing the cells three times with 3 ml cold $NaCl/P_i$, pH 7.4, and centrifuging at $400 \times g$ for 5 min. Washed cells were suspended in 2 ml $NaCl/P_i$, pH 7.4, and proteases inhibitors were added as before. Reduction was carried out by adding to this suspension 1.5 mCi NaB^3H_4 in 15 µl 0.1 M NaOH and mixing the suspension for 30 min at room temperature. An additional 1.5 mCi NaB^3H_4 was added and incubation was continued for 30 min. The cells were washed three times with 3 ml buffer A and were used immediately for lysis and affinity purification of glycoproteins.

Procedure for purification of macrophage glycoproteins

All purification steps were carried out at 4 °C unless stated otherwise. ^3H-labeled or ^{125}I-labeled macrophages (approximately $9-12 \times 10^7$ cells) were lysed on an ice/water bath for 30 min at pH 8.2 in 0.01 M Tris/HCl containing 0.15 M NaCl, 1% bovine serum albumin and 1% Triton X-100. To this lysis buffer, a freshly prepared full set of protease inhibitors except for α_2-macroglobulin and 4-amidinophenylmethylsulfonyl fluoride was added just prior to lysis. The lysate was spun either on a preparative ultracentrifuge at $100000 \times g$ for 1 h or in a microfuge (Eppendorf) at $15100 \times g$ for 15 min (the results being the same), and the ^3H-labeled supernatant was applied immediately to a GSI-Sepharose column (1.7 cm × 14 cm) equilibrated with buffer A containing 0.1 mM $CaCl_2$ (buffer B). The column was washed consecutively with buffer B containing 1% Triton X-100 and buffer B with 0.01% Triton X-100 (buffer C) until the radioactivity dropped below 1000 cpm/fraction. Buffers contained 1 mM phenylmethylsulfonyl fluoride and 0.4 mM *N*-ethylmaleimide. The glycoprotein fractions bound to the column were eluted with 3 column vol. 100 mM MeGal in buffer C followed by the same volume of 20 mM EDA. Appropriate aliquots were sampled from each fraction were assayed for liquid scintillation counting. The fractions eluted with EDA were neutralized to pH 8 using 0.5% acetic acid (other details are given in the figure legend). The labeled fractions eluted with MeGal and EDA were poooled separately, concentrated in a Centricon 10 (Amicon) and suitable aliquots removed for analysis. The ^{125}I-labeled material was applied to a GSI-Sepharose column (0.7 cm × 13 cm) equilibrated with 0.01 M Tris/HCl, pH 8.2, containing 0.14 M NaCl, 0.1 mM $CaCl_2$ and 0.02% sodium azide (buffer D). The column was washed with buffer D containing 1.5% Triton X-100, then with 0.2 M Tris/HCl, pH 8.2, containing 0.1% Triton X-100 (buffer E) until the radioactivity present in the effluent was less than 1000 cpm/fraction. Buffers contained 1 mM phenylmethylsulfonyl fluoride and 0.4 mM *N*-ethylmaleimide. Absorbed material was eluted with 100 mM MeGal in buffer E. Following addition of 0.1 M MeGal (approximately equal to the V_o of the column), the column was incubated for 1 h, then eluted. Proteases inhibitors, including 100 µg α_2-macroglobulin were added to each fraction as eluted from the column (other details in the legend to the figure). The fraction eluted with MeGal was further purified by preparative SDS/PAGE.

Preparative PAGE

The ^{125}I-labeled fractions were precipitated at −20 °C with 3 vol. absolute ethanol for 4 − 16 h. The precipitate was centrifuged at $15100 \times g$ for 15 min, washed twice with 1 ml 75% ethanol (−20 °C) and centrifuged again. The pellet was dissolved in sample buffer, incubated at room temperature for 10 min, centrifuged at $15100 \times g$ for 5 min and the supernatant solution was either frozen or used immediately for electrophoresis. The sample buffer was prepared according to Laemmli [50] except that it contained 4% SDS, 100 mM MeGal and a full complement of proteases inhibitors. The material was subjected to preparative PAGE in 1.5-mm 5% slab gels at constant current of 25 mA. ^{125}I-labeled bands were detected by overnight exposure of the gel to Kodak X-Omat XAR 5 film at 4 °C. The glycoprotein bands were excised from the gel and eluted in an Amicon eletroeluter at room temperature for 2 h at 200 V. The eluted glycoprotein fractions were concentrated on a Centricon 30 apparatus (Amicon) and stored at 4 °C in 0.1% SDS.

Analytical PAGE

Analytical PAGE was carried out in 5% or 7.5% acrylamide gel according to Laemmli [50]. Other conditions were the same as for preparative PAGE. Gradient gel electrophoresis was performed in linear 3 − 20% gradient gels at constant voltage of 50 V. The sample buffer contained 4% SDS. Unless otherwise stated, all samples were incubated at 100 °C for 5 min. Reduction was carried out in the presence of either 0.1 M 2-mercaptoethanol or 0.13 M 1,4-dithiothreitol. The isotopically labeled glycoprotein bands were detected by autoradiography or fluorography. For autoradiography, gels were dried and exposed to Kodak X-Omat XAR 5 films for 3 − 14 days at −70 °C in cassettes with intensifying screens. For fluorography, gels were fixed in a solution of 5% (by vol.) methanol and 7.5% (by vol.) glacial acetic acid with constant shaking for 4 h. Subsequently, gels were impregnated with scintillator fluid and dried according to the directions of the manufacturer (Du Pont). Dried gels were exposed to Kodak films (see above) for 2 − 3 weeks.

Digestion of glycoproteins with glycosidases

α-D-Galactosidase

^3H-labeled glycoproteins were used for quantitation of the release of α-linked D-[6-^3H]galactose, whereas ^{125}I-labeled glycoproteins were assayed for binding to GSI-Sepharose by a batch technique. The reaction mixture in 0.22 ml 50 mM citric acid/100 mM Na_2HPO_4, pH 5.5, contained either 40000 cpm ^3H or 6000 cpm ^{125}I, 2 U α-galactosidase and 0.01% Triton X-100. The mixture was incubated at 37 °C for 3.5 h, filtered on a Centricon 10 and the D-[6-^3H]galactose released was quantified in a liquid scintillation counter (Beckman LS 5801). For binding by the batch technique, the digest was mixed with 100 µl settled beads of GSI-Sepharose suspended in 0.25 ml buffer B containing 0.1% Triton X-100. The suspension was incubated at 4 °C for 30 min with occasional mixing. The beads were pelleted by centrifugation at $400 \times g$ for 1 min and washed three times with 1 ml buffer B containing 0.1% Triton X-100, then the radioactivity bound to the beads was determined. For digestion with other enzymes, only ^{125}I-labeled glycoprotein fractions were used. A typical reaction mixture contained 3000 cpm ^{125}I-labeled glycoprotein and, unless otherwise stated, was incubated at 37 °C for 24 h.

Neuraminidase

The reaction mixture, in a final volume of 0.1 ml 50 mM acetate buffer, pH 5.5, contained 1.3 U enzyme, 1 mM $CaCl_2$, 10 µg bovine serum albumin and 0.01% SDS. After incu-

bation for 6 h, the mixture was applied to a *Maackia* or *Sambucus* lectin-Sepharose column.

N-*Glycanase*

The reaction mixture contained in 50 µl 0.2 M phosphate buffer, pH 8.6, 1.25 U enzyme, 1.25% Triton X-100, 0.17% SDS and 10 mM phenanthroline. The mixture was incubated in the presence or absence of 0.1 M 2-mercaptoethanol.

Endo-β-galactosidase

The reaction mixture contained, in 30 µl 50 mM phosphate buffer, pH 6.0, 130 mU enzyme, 0.01% SDS and was incubated in the presence or absence of 0.1 M 2-mercaptoethanol.

Endoglycosidase H

The reaction mixture contained, in 50 µl 50 mM acetic acid/sodium acetate buffer, pH 5.5, 0.84 U or 1.26 U enzyme, 0.2% SDS and 10 mM 1,10-phenanthroline.

O-*Glycanase*

Prior to digestion with *O*-glycanase, glycoproteins were digested wtih neuraminidase as described above, except that 50 mM Tris/maleate buffer, pH 6, was used, and the incubation was extended to 11 h. The desialized glycoproteins were incubated in the reaction mixture which contained in a final volume of 50 µl, 10 mU *O*-glycanase, 1.25% Triton X-100, 0.17% SDS, 0.1 M 2-mercaptoethanol and 25 mM β-galactonolactone.

Immunoprecipitation

The reaction mixtures contained, in a final volume of 0.25 ml 10 mM Tris/HCl, pH 8.2, 0.14 M NaCl, 0.5% Triton X-100, 0.1 mM $CaCl_2$ and approximately 3000 cpm ^{125}I-labelled glycoprotein. To this mixture, the following amounts of lectin, serum or monoclonal antibodies were added: 5 µg GSI-B_4 and 50 µl rabbit serum raised against GSI; 7 µl anti-i serum and 2 mg goat serum raised against human IgM; 7 µl anti-I serum and 2 mg goat serum raised against human IgM; 9 µg anti-(Mac-3) rat monoclonal antibodies and 30 µg rabbit IgG against rat serum. Before second antibodies were added, the mixtures were incubated at 4 °C for 1 h. After addition of second antibodies, incubation was continued at 4 °C for 48 h. The precipitate was centrifuged and washed as described previously [51] and quantified in a γ-counter.

RESULTS

Purification of macrophage glycoproteins by affinity chromatography and SDS/PAGE analysis

Affinity chromatography of ^{3}H-labeled lysates from stimulated macrophages, on GSI-Sephrose yielded two peaks: one peak was eluted with 100 mM MeGal and a second with

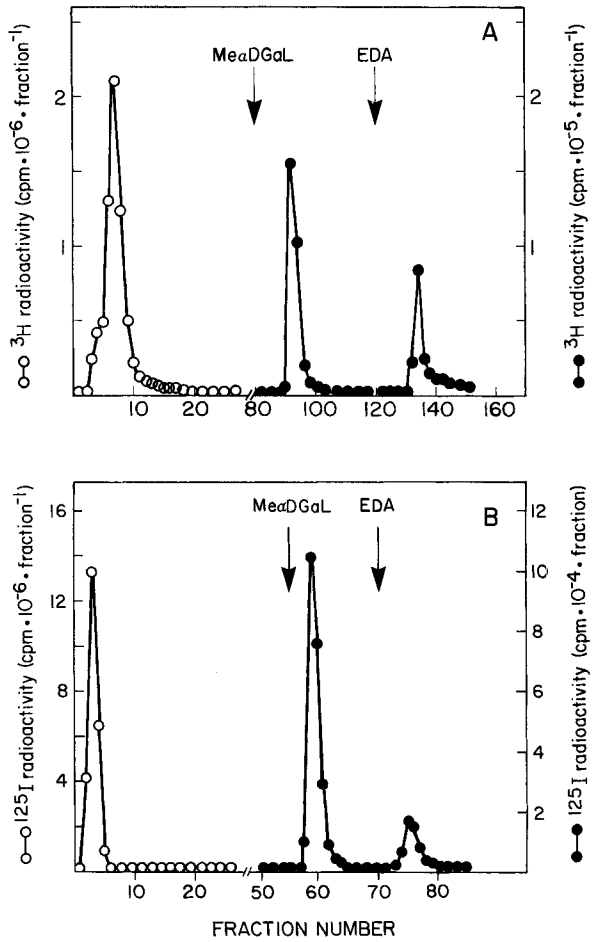

Fig. 1. Affinity chromatography on GSI-Sepharose of lysates from stimulated macrophages. (A) ^{3}H-labeled lysate from 14×10^7 thioglycollate-stimulated macrophages was applied to a GSI-Sepharose column (1.7 cm × 14 cm) equilibrated with buffer B. The column was washed successively with 300 ml buffer B containing 1% Triton X-100 and 120 ml buffer C. Each buffer contained 1 mM phenylmethylsulfonyl fluoride and 0.4 mM *N*-ethylmaleimide. Adsorbed glycoproteins were eluted with 120 ml 100 mM MeGal in buffer C and with 120 ml 20 mM EDA containing 0.01% Triton X-100. Fractions of 3.5 ml were collected at a flow rate of 7 ml/h. (B) ^{125}I-labeled lysate from 5.3×10^6 thioglycollate-stimulated macrophages was applied to a column (0.7 cm × 13 cm) equilibrated with buffer D. The column was washed successively with 150 ml buffer E. Buffers contained 1 mM phenylmethylsulfonyl fluoride and 0.4 mM *N*-ethylmaleimide. Adsorbed glycoproteins were eluted first with 20 ml 100 mM MeGal in buffer E then with 20 ml 20 mM EDA containing 0.1% Triton X-100. Fractions of 1.3 ml were collected at a flow rate of 16 ml/h.

20 mM EDA (Fig. 1A). The same elution pattern was obtained with the ^{125}I-labeled lysate (Fig. 1B). These results indicate that each fraction contains cell-surface D-galactose and further suggest that the peak specifically eluted with MeGal contains terminal α-D-galactose residues.

Gradient SDS/PAGE analysis of the ^{3}H-labeled peaks, eluted with MeGal and EDA, yielded the same fluorographic profile (Fig. 2A, a and b). Each fraction exhibited one major band of 95–132 kDa. Additional bands of 144, 180 and greater than 200 kDa were noted and appeared to be aggregates of the major band (see next paragraphs). The ^{3}H-labeled major band of 95–132 kDa, when subjected to SDS/PAGE in a 7.5% gel, was resolved into two bands of 92–109 kDa

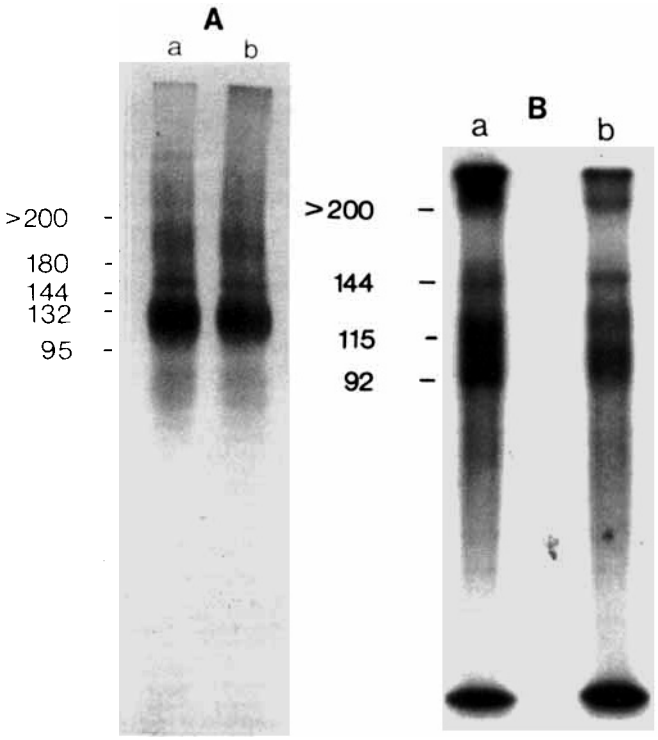

Fig. 2. SDS/PAGE of macrophage glycoproteins purified by affinity chromatography on GSI-Sepharose. (A) Fluorogram of ^{3}H-labeled glycoproteins: (a) MeGal-eluted fraction; (b) EDTA-eluted fraction. Electrophoresis was carried out in 3–20% gradient acrylamide gel at a constant voltage of 50 V. (B) Autoradiogram of ^{125}I-labeled glycoprotein fraction eluted with MeGal. (a) unreduced glycoproteins; (b) glycoproteins reduced with 2% dithiothreitol. Electrophoresis was carried out in a 7.5% gel at constant current of 25 mA.

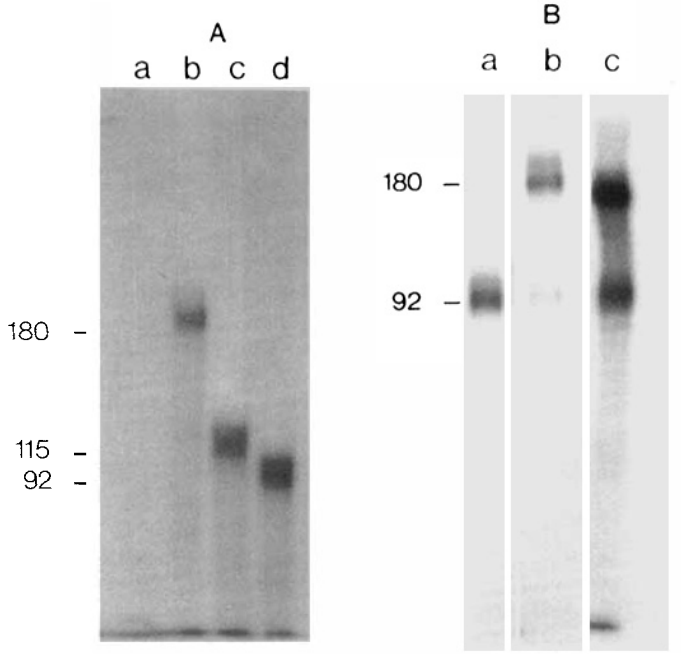

Fig. 3. SDS/PAGE of ^{125}I-labeled glycoprotein fractions purified by acrylamide gel electrophoresis. (A) SDS/PAGE of glycoprotein fractions. (a) > 200 kDa; (b) 180 kDa; (c) 115 kDa; (d) 92 kDa. Electrophoresis was carried out in a 5% gel under reducing conditions. (B) Spontaneous aggregation/dissociation of glycoprotein fractions; (a) aggregation of 92-kDa glycoprotein; (b) dissociation of 180-kDa glycoprotein; (c) dissociation of 180-kDa glycoprotein after prolonged storage. Electrophoresis was carried out in a 7.5% gel under reducing conditions. Molecular masses (kDa) are shown.

and 115–125 kDa and produced the same pattern as the ^{125}I-labeled glycoproteins (not shown).

The ^{125}I-labeled glycoprotein fraction eluted with MeGal, after separation in a 7.5% acrylamide gel, revealed one diffuse zone in which a doublet of 92–109 kDa and 115–125 kDa was observed (Fig. 2B, a). The same pattern of bands was obtained with the ^{125}I-labeled EDA fraction (not shown). These results suggest that the same moieties are labeled with ^{3}H and ^{125}I and that the MeGal and EDA fractions contain the same glycoproteins. Reduction of these glycoproteins with dithiothreitol only slightly decreased their mobility in SDS/PAGE, thus indicating the possible presence of intrachain and lack of intersubunit disulfide bonds (Fig. 2B, b). The ^{125}I-labeled glycoprotein fraction eluted from the column with MeGal was further resolved into individual components by preparative SDS/PAGE.

Analysis of purified glycoproteins

The SDS/PAGE profiles of the ^{125}I-labeled glycoprotein fractions separated by preparative electrophoresis are shown in Fig. 3. Glycoprotein fractions of 92, 115 and 180 kDa appeared in the acrylamide gel as single bands (Fig. 3A, b, c, d). The band greater than 200 kDa was unstable and appears to have degraded into low-molecular-mass products moving with the front (Fig. 3A, a). The glycoprotein fraction of 180 kDa yielded a 92-kDa band (Fig. 3B, b) and vice versa; the 92-kDa glycoprotein generated a 180-kDa band (Fig. 3B, a). These results suggest that the 180-kDa glycoprotein is a dimer of the 92-kDa component. Dissociation occurs spontaneously when the 180-kDa glycoprotein is stored at 4°C in 0.1% SDS (Fig. 3Bc).

The results of analytical isoelectrofocusing are presented in Fig. 4. Each glycoprotein fraction exhibited a distinct p*I*; the 115-kDa glycoprotein was strongly acidic and had p$I \leqslant 4$. The glycoprotein of 92 kDa gave a diffuse band with p*I* 5.2–5.4. When the ^{3}H-labeled glycoprotein fraction, eluted from the affinity column with MeGal (Fig. 1A), was digested with α-galactosidase, 23% of dialysable ^{3}H was released (not shown), and the component lost its capacity to bind to GSI-Sepharose (Fig. 5). This analysis of purified glycoproteins from the surface of stimulated macrophages indicates that α-D-galactosyl groups are located on two major, different glycoprotein moieties.

Digestion with endoglycosidases

Each glycoprotein fraction was digested with *N*-glycanase and the products of digestion were subjected to SDS/PAGE (Fig. 6). Each fraction was susceptible to the action of *N*-glycanase. The 115-kDa glycoprotein disappeared and produced a diffuse zone in which a few faint bands (with the fastest moving at approximately 67 kDa) were discernable (Fig. 6b). The 92-kDa glycoprotein gave two diffuse bands of 75 kDa and 61 kDa (Fig. 5d). These results indicate that each glycoprotein fraction contains Asn-linked oligosaccharides. Both glycoprotein fractions were sensitive to *N*-glycanase, but only after reduction with 0.1 M 2-mercaptoethanol, suggesting the presence of intrachain disulfide bonds. For *N*-glycanase digestion, purified glycoprotein fractions excised

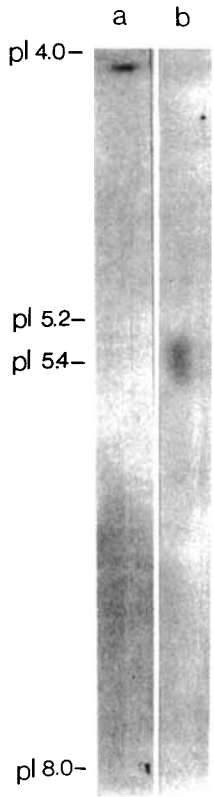

Fig. 4. Isoelectric focusing of ^{125}I-labeled macrophage glycoproteins. *Isoelectric* focusing was carried out in 5% acrylamide gel containing 2% carrier ampholyte with pH gradient of 4–8. (a) 115-kDa glycoprotein; (b) 92-kDa glycoprotein.

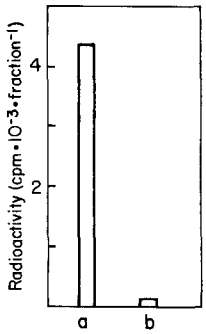

Fig. 5. Binding of macrophage glycoproteins to GSI-Sepharose following digestion with α-galactosidase. A mixture of 92-kDa and 115-kDa glycoprotein fractions was digested with coffee bean α-galactosidase and assayed for binding to GSI-Sepharose by a batch technique. (a) undigested; (b) digested glycoproteins.

from gels were employed. Therefore, appearance of additional bands of approximately 170 kDa and 160 kDa in the reaction mixture containing the 115-kDa and 92-kDa glycoproteins, respectively (Fig. 6a, c) indicates that each glycoprotein fraction underwent aggregation. An aggregate greater than 200 kDa was also formed from each glycoprotein. These aggregates were also sensitive to *N*-glycanase (Fig. 6b, d).

Each glycoprotein fraction was also sensitive to endoglycosidase H, and digestion resulted in an apparent decrease in the molecular masses of the 92-kDa and 115-kDa proteins (Fig. 7b, d) by 9 kDa and 6 kDa, respectively (Fig. 7b, d).

The large decrease in the apparent molecular masses of the two glycoproteins caused by *N*-glycanase, compared to

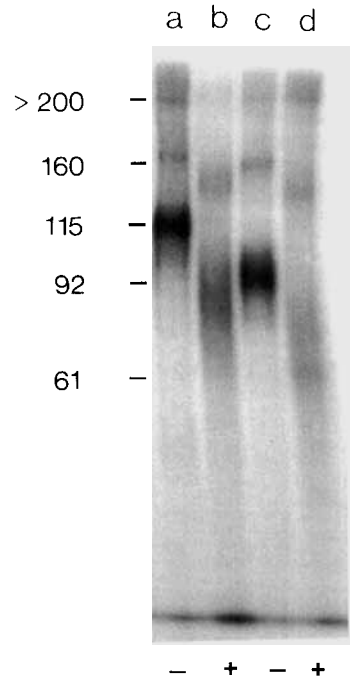

Fig. 6. SDS/PAGE of macrophage glycoproteins digested with *N*-glycanase. (A) ^{125}I-labeled glycoprotein fractions of 115 kDa and 92 kDa were incubated in the absence (lanes a and c, respectively) and presence (lanes b and d, respectively) of *N*-glycanase and subjected to SDS/PAGE in 7.5% acrylamide gel under reducing conditions.

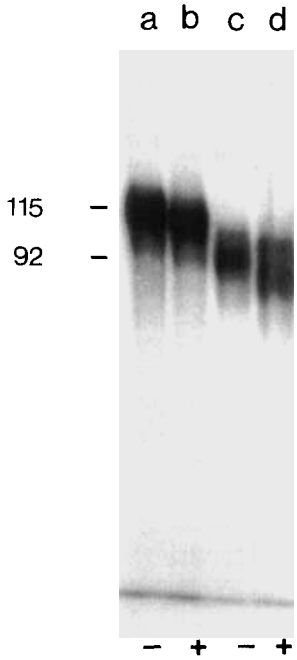

Fig. 7. SDS/PAGE of macrophage glycoproteins digested with endoglycosidase H. ^{125}I-labeled glycoprotein fractions of 115 kDa and 92 kDa were incubated in the absence (lanes a and c) and presence (lanes b and d) of enzyme, and the digest was subjected to SDS/PAGE in 7.5% gel under reducing conditions. Molecular masses (kDa) are shown.

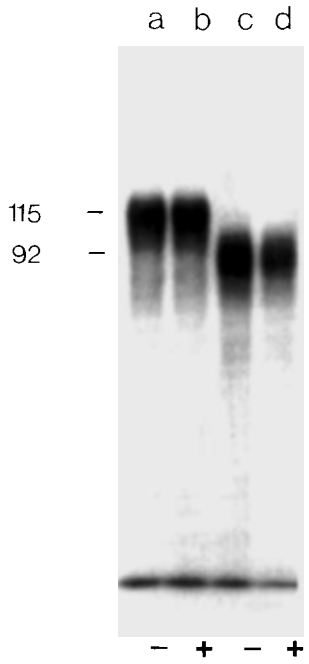

Fig. 8. SDS/PAGE of macrophage glycoproteins digested with endo-β-galactosidase. ^{125}I-labeled glycoprotein fractions of 115 kDa and 92 kDa were incubated in the absence (lanes a and c) and in the presence (lanes b and d) of enzyme. Digested glycoproteins were subjected to SDS/PAGE in a 7.5% gel under reducing conditions.

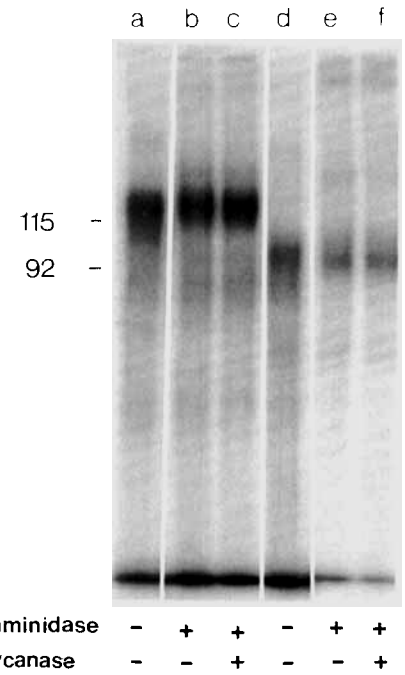

Fig. 9. SDS/PAGE of macrophage glycoproteins digested with neuraminidase and *O*-glycanase. ^{125}I-labeled glycoprotein fractions of 115 kDa and 92 kDa were incubated in the absence (lanes a and d) and presence (lanes b and c) of neuraminidase and neuraminidase with *O*-glycanase (lanes c and f). Digested glycoproteins were subjected to SDS/PAGE in 7.5% acrylamide gel under reducing conditions.

that obtained with endoglycosidase H, is ascribed to the presence of complex oligosaccharides inasmuch as endoglycosidase H is not able to digest complex oligosaccharides. Of the three types of Asn-linked oligosaccharides, high-mannose, hybrid and complex types, endoglycosidase H can split only the high-mannose and hybrid types [41, 52]. We conclude, therefore, that each glycoprotein fraction is composed largely of complex-type oligosaccharides, accompanied by small amounts of high-mannose-type and/or hybrid-type oligosaccharides. Digestion of each glycoprotein fraction with endo-β-galactosidase from *Escherichia freundi* did not alter their mobility in SDS/PAGE (Fig. 8), suggesting the absence of linear arrays of poly-(*N*-acetyllactosamine) units [53].

Digestion of each glycoprotein fraction with neuraminidase, as well as with a mixture of *O*-glycanase and neuraminidase, did not alter their mobility in SDS/PAGE (Fig. 9). The only effect of this treatment was a sharpening of the bands. These results suggest the lack of or low content of *O*-linked oligosaccharides in each glycoprotein fraction.

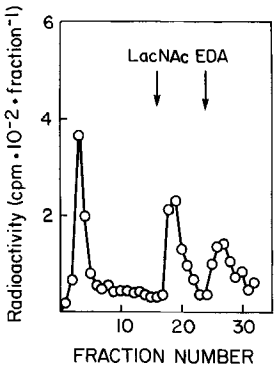

Fig. 10. Binding of ^{125}I-labeled macrophage glycoproteins to *D. stramonium* lectin coupled to Sepharose. The macrophage glycoprotein fractions were applied to a 0.7 cm × 2.1 cm column of immobilized *Datura* lectin, equilibrated and washed with 8 ml buffer E. The glycoproteins bound were eluted consecutively, first with 6 ml 10 mM *N*-acetyllactosamine (LacNAc) solution in the same buffer, then with 6 ml of 20 mM EDA containing 0.1% Triton X-100. The flow rate was 1 ml/3 min and fractions of 0.5 ml were collected.

Immunochemical studies

Macrophage glycoprotein fractions were applied to a *D. stramonium* lectin column (Fig. 10). Of the material applied, 76% was retained, of which approximately 23% was released by elution with *N*-acetyllactosamine and 18% with EDA. Elution with the sugar hapten showed that binding of glycoproteins to the lectin is specific. Binding of a compound to *Datura* lectin requires that the compound have at least two *N*-acetyllactosamine units in a linear array or be present as single *N*-acetyllactosamine units situated as adjacent antennae linked to outer mannose residues [40] of complex-type oligosaccharides. The finding that neither glycoprotein fraction reacted with anti-i serum (Fig. 13) suggests that *N*-acetyllactosamine residues are arrayed as single, multiple units on oligosaccharide chains. Multiple *N*-acetyllactosamine units are integral structural components of complex-type oligosaccharides; therefore, these results suggest the presence of complex oligosaccharides on macrophage glycoproteins.

The presence of *N*-acetylneuraminic acid groups, as well as the nature of their linkage to subterminal galactose, was probed by utilizing *N*-acetylneuraminic-acid-specific lectins. The binding of macrophage glycoproteins to immobilized *S. nigra* lectin is shown in Fig. 11 A. 71% of macrophage glyco-

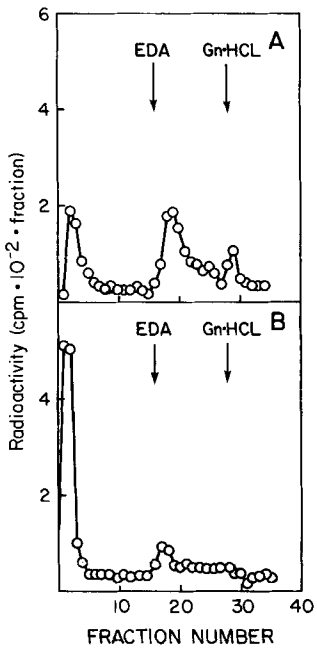

Fig. 11. Binding of ^{125}I-labeled macrophage glycoproteins to *S. nigra* lectin-Sepharose. (A) Undigested glycoproteins. (B) Digested with neuraminidase from *V. cholerae*. The column and flow rate were the same as in those in the legend to Fig. 10. The glycoproteins bound were eluted with 6 ml 20 mM EDA containing 0.1% Triton X-100 and subsequently with 6 ml 6 M guanidine/HCl (Gn · HCl) in 50 mM *glycine*/HCl, pH 3.4. Fractions of 0.5 ml (A) and 0.8 ml (B) were collected.

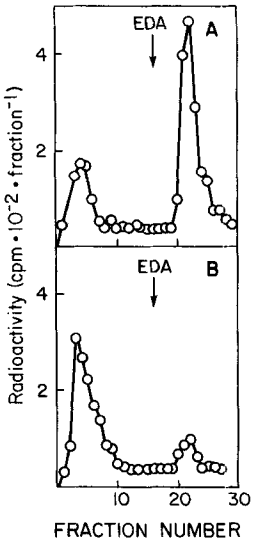

Fig. 12. Binding of ^{125}I-labeled macrophage glycoproteins to *M. amurensis* lectin-Sepharose. (A) Undigested glycoprotein. (B) Digested with neuraminidase from *V. cholerae*. The column and other conditions were the same as those in the legend to Fig. 10. The column was washed with 6 ml 20 mM EDA solution containing 0.1% Triton X-100.

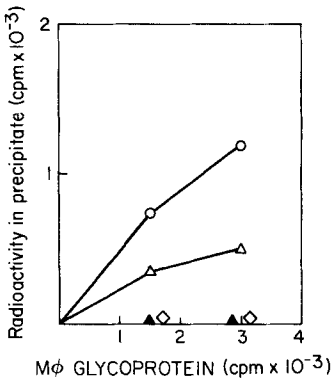

Fig. 13. Precipitation of ^{125}I-labeled macrophage (Mϕ) glycoproteins with antisera. (△) Anti-I serum (Step); (▲) anti-i (Den) serum; (◇) hybridoma antibody against Mac-3; (○) GSI-B_4.

proteins were strongly bound to the lectin and could not be released by the inhibitor hapten 0.2 M lactose (not shown). A portion of the bound glycoproteins (50%) was released by EDA and an additional 11% with 6 M guanidine/HCl. After neuraminidase treatment, only 23% of the glycoproteins were bound to the column (Fig. 11 B). Based on the known specificity of this lectin, the results obtained indicate that the macrophage glycoproteins contain *N*-acetylneuraminic-acid-linked α2,6 to subterminal D-glactose or *N*-acetylgalactosamine residues [54].

Binding of macrophage glycoproteins to *M. amurensis* lectin, which specifically recognizes *N*-acetylneuraminic acid residues linked α2,3 to subterminal *N*-acetyllactosamine residues [55, 56], is shown in Fig. 12. 73% of the untreated glycoproteins was bound to the lectin (Fig. 12A), whereas, after treatment with neuraminidase, only 16% remained associated with the immobilized lectin (Fig. 12B). These results suggest the presence of NeuNAcα2,3Galβ1,4GlcNAc units on macrophage glycoprotein fractions.

Further conclusions regarding the structure of macrophage glycoproteins can be drawn from their reactivity with anti-I sera (Fig. 13). From the specificity of this antiserum, it is concluded that macrophage glycoproteins contain, in addition to the already-mentioned oligosaccharides, branched *N*-acetyllactosamine units linked to the C3 and C6 hydroxyl groups of D-galactose in lactosaminoglycans [57, 58]. However, because only 17% of macrophage glycoproteins precipitated with this serum, one cannot, at this point, answer whether each glycoprotein fraction or only one of them contains branched lactosaminoglycans.

The macrophage glycoprotein fractions did not precipitate with anti-(Mac-3) monoclonal antibodies (Fig. 13) and were firmly bound (> 93%) to immobilized WGA (Fig. 14). This binding was not affected by neuraminidase digestion (not shown). These results indicate that the binding of each glycoprotein fraction to WGA is due to the *N,N'*-diacetylchitobiose core of Asn-linked oligosaccharides. They also indicate that the glycoproteins described have properties distinct from those of macrophage glycoproteins previously reported, including Mac-3 [59] and sialoglycoproteins [60].

DISCUSSION

Two glycoprotein fractions containing α-D-galactose end groups were purified from the surface of stimulated murine macrophages. These glycoproteins shared several structural features, i.e. lack of subunit structure, presence of intramolecular disulfide bonds and digestibility by the same panel of endoglycosidases. However, the glycoproteins differed in molecular mass, p*I* and susceptibility to *N*-glycanase. Despite

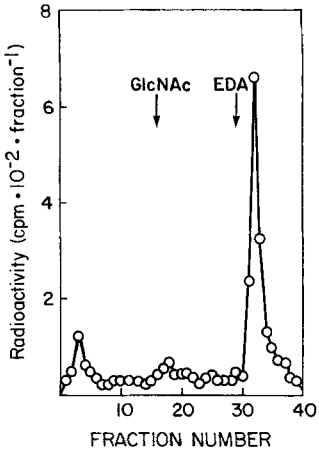

Fig. 14. Binding of ^{125}I-labeled macrophage glycoproteins to WGA-Sepharose. The column and other conditions were the same as those in the legend to Fig. 10. The glycoproteins bound were eluted with approximately 8 ml 0.1 M *N*-acetylglucosamine in buffer C and subsequently with 8 ml 20 mM EDTA (EDA). Each solution contained 0.1% Triton X-100.

the fact that both glycoprotein fractions were digested under identical conditions with *N*-glycanase, each glycoprotein exhibited a distinct profile in SDS/PAGE. This could indicate structural difference between the two fractions.

Each macrophage glycoprotein fraction purified by affinity chromatography on GSI exhibited several bands in SDS/PAGE gels. The profile of two major bands of 92 kDa and 115 kDa was reproducible and showed little variation in different preparations, provided a full set of protease inhibitors was used during purification. In preparations lacking some inhibitors, more diffuse zones with overlapping bands were obtained. Two additional bands of 144 kDa and 180 kDa showed variation both in their appearance as well as in molecular mass; in some preparations only one was apparent. Glycoproteins of 144 kDa and 180 kDa could be seen in the ranges 133–155 kDa and 160–184 kDa, respectively. Similar variability in molecular mass of macrophage glycoproteins was reported by Ho and Springer [59] and by Rabinowitz and Gordon [60]. The reason for this anomalous behavior remains unclear.

The 180-kDa and greater than 200-kDa glycoproteins represent aggregates of the 92-kDa glycoprotein. The glycoprotein of 115 kDa was also capable of forming aggregates in a similar molecular mass range. The tendency to form aggregates is a common phenomenon observed for membrane glycoproteins, as exemplified in the case of glycophorin [61].

It is clear from the present study that α-D-galactose groups appear to be expressed on the surface of stimulated macrophages, predominantly on two major glycoproteins. Many cell-surface glycoproteins exhibit a characteristic pattern of terminal glycosylation [2], and the nature of the sugar residues at their non-reducing termini influences many biological processes based on carbohydrate-recognition phenomena [1, 20]. Therefore, the distribution pattern of terminal non-reducing α-D-galactose groups on the cell-surface glycoproteins might be of importance for understanding these processes. In this regard, the only cells studied were Ehrlich ascites tumor cells which exhibited a family of glycoproteins bearing α-D-galactose end groups [14, 15]. α-D-Galactose groups on the surface of murine macrophage glycoproteins are predominantly expressed on two major glycoproteins. The question as to whether this is due to the specificity of α-D-galactosyltransferase for these glycoprotein molecules, or whether these results from the lack of acceptor species on other glycoprotein molecules, has yet to be determined. A similar rather-restrictive pattern of α-D-galactose-bearing glycoprotein molecules was also found on the human breast carcinoma cell line MCF-7 [62].

During storage, purified ^{125}I-labeled macrophage glycoprotein fractions underwent a constant process of degradation. This was envisaged in SDS/PAGE by broadening of the original bands, appearance of radioactive, lower-molecular-mass products and an increase in the amount of radioactive products moving with the front. There was also a time-dependent increase in the amount of dialysable radioactive products. These properties were intensified by incubation at 37°C. Whether this process of breakdown is caused by γ radiation emitted by ^{125}I and/or by products of ^{125}I decay, or whether this is an inherent proteolytic property of these isolated glycoprotein moieties cannot be answered at this point. However, because ^{3}H-labeled glycoproteins released far fewer dialysable products upon storage than the ^{125}I-labeled ones, it appears that the damage due to γ radiation might be the case.

The products of digestion of each glycoprotein fraction by *N*-glycanase treatment produced, in SDS/PAGE, a diffuse zone in which few bands could be discerned. This reflects a heterogeneous mixture of glycoproteins most likely resulting from the incomplete digestion of the original glycoproteins. Assuming that bands moving with the front of the digest represent the most deglycosylated glycoprotein(s), the carbohydrate content of the 92-kDa and 115-kDa species is at least 34% (by mass) and 42%, respectively. Complete deglycosylation of each glycoprotein fraction was a difficult task, as we found that the ^{125}I-labeled glycoproteins after treatment with endoglycosidases were more readily degraded to lower-molecular-mass radioactive products than their glycosylated counterparts.

Although each glycoprotein fraction was insensitive to *O*-glycanase treatment, one can not exclude the persence of a low content of O-linked oligosaccharides. The enzyme might require, for its activity, prior removal of Asn-linked oligosaccharides which constitute a substantial portion of each glycoprotein fraction. It has been reported by others that the removal of sialic acid from the glycoproteins containing a large number of O-linked oligosaccharides resulted in a decrease in their mobility in SDS/PAGE, signifying an increase of their apparent molecular mass [60, 63]. The mobility of glycoproteins described here was not altered after neuraminidase treatment thus providing additional support for the conclusion that they do not contain a large number of O-linked oligosaccharides.

Both glycoprotein fractions reacted with *D. stramonium* lectin. This lectin binds either to two or more consecutive *N*-acetyllactosamine units in a linear array, or to two single *N*-acetyllactosamine units located on neighboring antennae of the oligosaccharide [40]. However, macrophage glycoproteins did not precipitate with anti-i antiserum (Den) which recognizes poly(*N*-acetyllactosamine) units in a linear array, thus suggesting the presence of single, multiple Galβ1,4GlcNAc units. While *Datura* lectin shows preference toward *N*-acetyllactosamine units linked to the C2 and C6 hydroxyl groups of outer mannose residues [16, 40], it has been observed that complex type oligosaccharides with *N*-acetyllactosamine units linked to the C2 and C4 of outer mannose residues are retarded on immobilized *Datura* lectin [64]. Therefore, it is possible that the presence of multiple *N*-acetyllactosamine

units linked to C2 and C4 hydroxyl groups of mannose, linkages known to occur in glycoproteins, might provide sufficient energy for binding of these glycoproteins to *Datura* lectin. Thus binding of macrophage glycoproteins to *Datura* lectin could result from the presence on complex-type oligosaccharides of either or both of these structures.

Mercurio and Robbins [37] showed that thioglycollate-stimulated, but not resident macrophages, reacted with anti-I serum and that both kinds of cells were unreactive with anti-i serum. The reactivity of the macrophage glycoproteins which we isolated with anti-I serum, and lack of reactivity with anti-i sera, is in accordance with this observation.

Several macrophage glycoproteins which exhibit some properties similar to the glycoproteins containing α-D-galactose groups described here have been reported. These include Mac-3 antigen [59] and sialoglycoproteins [60]. The glycoproteins described here failed to react with anti-(Mac-3) antibodies; they also differed in their susceptibility to endo-β-galactosidase. Mac-3 showed increased mobility in SDS/PAGE after endo-β-galactosidase treatment [65], whereas our glycoproteins containing α-D-galactose groups did not.

The sialoglycoproteins described by Rabinowitz and Gordon [60] differed substantially from our glycoproteins despite the fact that they also produced a diffuse zone in SDS/PAGE in the same range as the glycoproteins described here. These sialoglycoproteins lost their capacity to bind to WGA when treated with neuraminidase, whereas the binding of the glycoproteins described here was not affected by neuraminidase digestion. Besides these differences, the sialoglycoproteins reported by Rabinowitz and Gordon [60] changed their mobility in SDS/PAGE after desialylation and *O*-glycanase treatment, whereas glycoproteins containing α-D-galactose groups did not. These results indicate that the macrophage glycoproteins described here have not been reported previously.

Takacs and Staehli [36] have hypothesized that α-D-galactose groups on the macrophage surface constitute part of their recognition system through which activated macrophages discriminate between normal and tumor cells. Kurisu et al. [66] reported that of the several lectins which can induce contact between macrophages and tumor cells, only WGA produced tumor cell lysis by macrophages. Maddox et al. [35] found that the GSI-B_4 also induced the same effect. Since both macrophage glycoproteins isolated here are on the cell surface and bind GSI-B_4 and WGA, it is possible that these glycoproteins are involved in the mechanism of recognition/cytotoxicity of tumor cells by activated macrophages. The glycoproteins described here may provide a tool to approach a solution of this phenomenon at the molecular level.

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