Macrosialin, a Macrophage-restricted Membrane Sialoprotein Differentially Glycosylated in Response to Inflammatory Stimuli

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Summary

Rat monoclonal antibody FA/11 has been used to identify macrosialin, a sialoglycoprotein confined to murine mononuclear phagocytes and related cells. Originally identified as a macrophage-associated glycoprotein predominantly localized in intracellular membranes (Smith, M.J., and G.L.E. Koch. 1987. *J. Cell Sci.* 87:113), the antigen is widely expressed on tissue macrophages, including those in lymphoid areas, and is expressed at low levels on isolated dendritic cells. Immuno-adsorption experiments reported here show that macrosialin is identical to the major 87–115-kD sialoglycoprotein previously identified by lectin blotting in exudate but not resident peritoneal macrophages (Rabinowitz, S., and S. Gordon. 1989. *J. Cell Sci.* 93:623). Resident peritoneal macrophages express low levels of macrosialin antigen in a glycoform that does not bind ^{125}I wheat germ agglutinin or ^{125}I peanut agglutinin; inflammatory stimuli upregulate expression of this antigen (up to 17-fold), in an alternative glycoform that is detected by these lectins. Pulse-chase experiments reveal a 44-kD core peptide that initially bears high-mannose chains (giving M_r 66 kD) and is subsequently processed to a mature protein of M_r 87–104 kD. Each glycoform contains N-linked glycan, as well as O-linked sugar structures that show alternative processing. Poly-*N*-acetyllactosamine structures are detected in the exudate cell glycoform only. This new marker for mononuclear phagocytes illustrates two strategies by which macrophages remodel their membranes in response to inflammatory stimuli. Its predominantly intracellular location and restricted cell distribution suggest a possible role in membrane fusion or antigen processing.

Functional activation of macrophages (MØ)[1] is accompanied by substantial remodelling of the cell surface. The mouse peritoneal cavity has been a useful system for studying phenotypic differences between the resident MØ population and exudate MØ, whether recruited in response to intraperitoneal pathogens (activated MØ) or sterile inflammatory stimuli (elicited MØ). Resident, elicited, and activated MØ have distinct surface profiles of enzymes, antigens, and receptors (1–3); different cell surface proteins are independently up- or downregulated by cytokines, which may have antagonistic effects (4).

Membrane remodeling also affects oligosaccharides and leads to enhanced binding to the surface of exudate MØ by wheat germ agglutinin (WGA) (5–7) and several other lectins (8–11). Inflammatory recruitment enhances the expression of defined sugar structures, such as lactosaminoglycans (12), and of relevant glycosyltransferases (13).

Changes in surface sugars may simply reflect altered expression of the proteins with which they are associated. However, there is emerging evidence for fine regulation of oligosaccharide structure (14), which may differ at a given *N*-glycosylation site on the same protein in different cell types, or between different *N*-glycosylation sites on the same protein (15). O-linked sugars on a single protein may likewise be modulated by cell type (16) and activation state (17).

We recently showed that mouse peritoneal exudate MØ express a group of membrane proteins with abundant sialic acid, probably on O-linked glycans (18). Our lectin-based system could not detect these glycoproteins in resident peritoneal MØ. In the present study, we show that the most abundant of these sialoglycoproteins, for which we propose the name macrosialin, is a MØ-restricted marker originally identified as a MØ-associated glycoprotein of intracellular membranes (19). Macrosialin is present in both resident and exudate MØ; differential glycosylation accounts for the differences in lectin binding. Its core peptide of 42 kD is abundantly glycosylated, giving the mature protein M_r 87–104 kD. Glycosidase studies reveal poly-*N*-acetyllactosamine structures in the exudate, but not the resident cell glycoform. Subtle differences also exist in O-glycan structure.

[1] *Abbreviations used in this paper:* Ag, antigen; MØ, macrophage; RαR, rabbit IgG anti-rat IgG; RPM, resident peritoneal macrophage; TPM, thioglycollate-elicited peritoneal macrophage; TX-100, Triton X-100; WGA, wheat germ agglutinin.

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Materials and Methods

Animals and Cells. Resident peritoneal cells were obtained from C57BL/6/Ola mice bred at the Sir William Dunn School of Pathology. Peritoneal exudate cells were obtained either 3–5 d after intraperitoneal injection of (*a*) 1.5 ml Brewer's complete thioglycollate broth, (*b*) 1 ml 5 mM sodium periodate in normal saline, or (*c*) 0.3 ml Biogel P-100 polyacrylamide beads, 100–200 mesh (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), prepared as described (20); or 8–12 days after intraperitoneal injection of (*a*) *Bacillus Calmette Guerin* (Pasteur strain 1011, 10^7 live organisms in 0.1 ml; a kind gift from Dr. R. North, Trudeau Institute, Saranac Lake, NY), or (*b*) heat-killed *Corynebacterium parvum* (Wellcome Biotechnology Ltd.; 0.7 mg in 0.1 ml normal saline).

Preparation of Lysates. MØ were purified by adherent culture in 35- or 100-mm diameter tissue culture dishes in DMEM (Gibco Laboratories, Paisley, Scotland) with 20 μg/ml gentamycin (or 50 μg/ml penicillin G and 50 μg/ml streptomycin), 2 mM glutamine, and either 5% heat-inactivated FCS (Sera Lab Ltd., Crawley Down, UK) or 0.1% BSA. Nonadherent cells were removed by washing in PBS after 4–6 h, and the monolayers cultured in fresh medium for 24–48 h. To prepare lysates, monolayers were washed and scraped into 1–2% Triton X-100 (TX-100) with 3 mM iodoacetamide and 1.5 mM PMSF as described (18). For precise estimation of cell numbers, aliquots of lysate were taken and the nuclei counted in a haemocytometer under phase-contrast optics.

Monoclonal Antibodies. Hybridomas secreting the following rat anti-mouse mAbs were maintained in IMDM (Flow Laboratories, Rickmansworth, UK) with 10% FCS, 0.1 mM hypoxanthine, 16 μM thymidine, glutamine, and antibiotics: FA/11 (IgG2a) (19); 5C6 (IgG2b) (21), and M1/70 (IgG2b) (22), both against CD11b; F4/80 (IgG2b) against a 160-kD MØ-restricted glycoprotein (23); SER-4 (IgG2a) against a surface lectin of stromal and splenic marginal zone MØ (24); M3/84 (IgG1) against the lysosomal membrane protein Mac-3 (25); and I42/5.1 (IgG2a) against a monomorphic determinant on Pgp-1 (26). Medium conditioned with the rat mAb MOMA-2 (IgG2b) (27) was kindly provided by Dr. G. Kraal (Dept. of Histology, Free University, Amsterdam). The M1/70 and M3/84 cell lines were a kind gift from Dr. T. Springer (Dept. of Pathology, Harvard Medical School, Boston, MA). The FA/11 line was generously made available by Drs. M. Smith and G. Koch (MRC Laboratory of Molecular Biology, Cambridge, UK).

Immunochemical Staining of Cells and Tissues. Cytospin preparations were fixed in methanol or acetone (10 min at 4°C). Coverslip preparations of MØ were fixed in 2% freshly dissolved paraformaldehyde/PBS (10 min at room temperature), quenched in 0.1 M glycine/PBS, and permeabilized in methanol. Organs immersed in Tissue-Tek OCT compound were rapidly frozen in isopentane/liquid nitrogen; 5-μm sections were cut onto glass slides, stored at −20°C, and shortly before use thawed and fixed in acetone (10 min, room temperature). Antigens were detected using a commercial avidin-biotin-peroxidase kit (Vectastain PK-4004; Vector Laboratories, Peterborough, UK). Negative controls included omission of first antibody or substitution of rat mAbs to unrelated antigens.

Purification of FA/11 IgG. FA/11, a rat-mouse hybrid, could not be established as a productive ascites in rats or nude mice. IgG-depleted FCS was prepared by sodium sulphate precipitation, extensively dialyzed against PBS, and filter sterilized. FA/11 cells, grown in conventional medium, were seeded into T200 flasks at 3×10^7 cells in 100 ml DMEM with 5% IgG-depleted FCS, glutamine, and antibiotics. Spent medium was centrifuged to remove cell debris and concentrated 10-fold by pressure filtration in an Amicon cell with a Spectra/Por type C membrane (100-kD cutoff). Sodium sulphate precipitation yielded IgG >95% pure as assessed in Coomassie blue–stained gels.

SDS-PAGE and Western Blots. Whole cell lysates were subjected to SDS-PAGE (10% gels) and Western blotting to nitrocellulose membranes as described (18). Blots were probed with radioiodinated WGA (18) (Vector Laboratories) or ^{125}I-FA/11 IgG. Autoradiography was performed with preflashed X-ray film at −70°C with an intensifying screen; the signal was quantified with a laser densitometer (Ultroscan XL; LKB, Piscataway, NJ).

Metabolic Labeling and Immunoprecipitation. Washed cultures (10^7 MØ plated per 100-mm dish) were re-fed with labeling medium (methionine-free Eagle's MEM [Gibco Laboratories], 8% FCS dialyzed against PBS, 2% undialyzed FCS, glutamine, streptomycin, penicillin). Labeling was initiated by adding 30 μCi/ml ^{35}S-methionine (Amersham International, Amersham, UK). After 18 h, the monolayers were washed in PBS and extracted for 45 min on ice in TX-100 with iodoacetamide and PMSF. Postnuclear supernatant was taken after microfugation; radioactivity was typically 99% TCA precipitable, as assessed on GF/C filters (Whatman) (28). Pulse-chase studies were performed in suspension on freshly harvested thioglycollate-elicited peritoneal exudate cells. Cells were preincubated in labeling medium (4×10^7 MØ/ml, 1 h at 37°C); 1-ml aliquots were individually pulse labeled for 7.5 min with 660 μCi/ml ^{35}S-methionine. Where indicated, an excess of chase medium (complete MEM, 10% undialysed FCS, glutamine, antibiotics) was added. Processing was stopped by adding a threefold excess of ice-cold PBS; labeled cells were washed three times in cold PBS, pelleted, and lysed in TX-100 with protease inhibitors. Lysate was clarified by ultracentrifugation at 100,000 *g* in a TLA 100.3 rotor (Beckman Instruments, Palo Alto, CA) (4°C for 30 min); immunoprecipitation was carried out as described (21). The samples were analyzed by SDS-PAGE with ^{14}C-methylated protein markers (Amersham International, Amersham, UK; code CFA 626).

Immunoadsorption of Unlabeled FA/11 Antigen. FA-11-coated sepharose beads were prepared by incubating protein A–Sepharose beads (Pharmacia Fine Chemicals, Hounslow, UK) first with rabbit IgG anti-rat IgG (RαR) and subsequently with FA/11-conditioned medium. These beads were allowed to react with concentrated MØ lysate overnight at 4°C. Microfugation was used to separate the antigen-depleted supernate from the pelleted beads, which were washed as for immunoprecipitation. Pellet and supernate were analyzed by SDS-PAGE; Western blots were probed with ^{125}I WGA, or with FA/11-conditioned medium followed by ^{125}I RαR. Control immunoadsorptions were performed with culture medium or mAb 5C6.

Glycosidase Digestion. Glycosidase preparations (from Boehringer-Mannheim Biochemicals, Lewes, UK, except where otherwise noted) were used as recommended (29). Washed beads bearing immunoprecipitated, metabolically labelled antigen were boiled for 5 min in 0.5% SDS/0.1 M 2-ME/H_2O. Aliquots (5 μl) were mixed with buffers and reagents at the following final concentrations: (*a*) Glycopeptidase, F (*N*-glycanase) (E.C. 3.2.2.18) (20 U/ml), 10 mM orthophenanthroline, 1.5% (wt/vol) NP-40, 0.25 M sodium phosphate buffer, pH 8; (*b*) Endo-β-*N*-acetylglucosaminidase F (Endo-F) (E.C. 3.2.1.96) (10 U/ml): 10 mM orthophrenanthroline, 1.5% NP-40, 0.25 M sodium acetate buffer, pH 6.0; (*c*) Endo-β-*N*-acetylglucosaminidase H (Endo-H) (E.C. 3.2.1.96) (0.1 U/ml): 1.5% NP-40, 1 mg/ml BSA, 50 mM sodium acetate, pH 5.8; (*d*) Endo-β-galactosidase (E.C. 3.2.1.103) (0.25 U/ml): 10 mM orthophrenanthroline, 0.2 mg/ml BSA, 50 mM sodium acetate, pH 5.8; (*e*) *Vibrio cholerae* Neuraminidase (E.C. 3.2.1.18) (Calbiochem Corp.) (0.1 U/ml): 1% NP-40, 25 mM sodium phosphate, pH 6.0. Digestions were performed at 37°C for 16 h, and terminated by

adding 7.5 μg BSA/tube and precipitating with 20% cold TCA; washed TCA precipitates were boiled in single-strength reducing buffer for electrophoresis. As control, a mock digestion was performed as for *b*, omitting the enzyme; in some experiments, duplicate neuraminidase digestions were performed with and without NP-40. For Endo-α-*N*-acetylgalactosaminidase (*O*-glycanase) (E.C. 3.2.1.97) digestion, labeled glycoproteins were preincubated with neuraminidase (0.1 U/ml, in 20 mM sodium phosphate, pH 6.0, 10 mM calcium chloride at 37°C for 5.5 h) to remove potentially blocking sialic acid residues; 1.5% NP-40 and 25 mU/ml *O*-glycanase were then added and digestion continued for 10.5 h before TCA precipitation. Control digestions were performed with buffer instead of *O*-glycanase.

Results

Antibody FA/11 Recognizes a Macrophage-restricted Antigen. mAb FA/11 was originally reported by Smith and Koch (19) as identifying a predominantly intracellular membrane protein of isolated murine MØ. Immunocytochemistry on normal mouse spleen (Fig. 1) and other organs (manuscript in preparation) suggests that the antigen is confined to cells of the monocyte/macrophage lineage. The monoclonal markers F4/80 (Fig. 1 *a*) and SER-4 (Fig. 1 *b*) identify nonoverlapping macrophage subsets in the mouse spleen; FA/11 labels both these groups (Fig. 1 *c*) as well as macrophages in the white pulp, many of which contain ingested nuclei (Fig. 1, *d*) and probably represent tingible body MØ. The antigen (Ag) is found on circulating monocytes, but not neutrophils, lymphocytes, or red cells (Fig. 2 *a*); on resident peritoneal MØ, but not lymphocytes (Fig. 2 *b*); and on dendritic cells isolated from mouse spleen as described (30) in which low-level staining outlines a single perinuclear dot (not shown). The Ag is particularly abundant in thioglycollate-elicited MØ (Fig. 2 *c*), which contain the peripheral FA/11$^+$ granules seen in other MØ, as well as strongly staining phagolysosomal vacuoles. This pattern resembles the distribution of WGA binding sites in these cells (Fig. 2 *d*), though the lectin more prominently labels the surface membrane.

FA/11 Antigen Is the Major WGA-binding Protein of Exudate MØ. The M_r reported for immunoprecipitated FA/11 antigen (19) was compatible with the electrophoretic behavior of the major WGA-binding protein band of mouse exudate MØ (18); moreover, their staining patterns in thioglycollate-elicited peritoneal macrophages (TPM) were similar. We therefore tested for identity by using protein A–sepharose beads coated with FA/11 IgG to immuno-deplete FA/11 antigen from TPM lysate. After centrifugation, the antigen-depleted lysate and antigen-rich pellet were analyzed by Western blotting to compare their WGA-binding profiles (Fig. 3). ^{125}I-WGA identifies a major 86–108-kD band in untreated lysate and in the FA/11-rich pellet, but not in the FA/11-depleted supernate, in which only minor WGA-binding bands remain. A control antibody (against the C3bi receptor) did not remove WGA-binding proteins from the supernate. The major WGA-binding band, despite its broad appearance, thus consists of a single antigen, which is fortuitously recognized by mAb FA/11.

In Western blots of TPM (see Fig. 4 *a*), ^{125}I-FA/11 IgG detects an antigen migrating as a broad band with a constant leading edge (M_r = 84 ± 2.0 kD, n = 5) and a trailing edge that lags progressively as the MØ are cultured from 4 to 50 h before lysis (not shown). Treatment of the lysate with neuraminidase before electrophoresis causes paradoxically slowed migration, as previously described for the major WGA-binding protein (18). In matched blots of the same lysate, the fastest-migrating component of the antigen, as detected by FA/11, does not bind WGA, giving a difference of 3–5 kD in the position of the leading edge. The lectin, by contrast, often detects slow-moving components beyond the trailing edge identified by the antibody.

FA/11 Ag Is Differentially Glycosylated in TPM and Resident Peritoneal Macrophages (RPM). Although RPM lack the glycoproteins detected in exudate MØ by ^{125}I-WGA blotting, immunocytochemistry shows that they express FA/11 antigen (Fig. 2), suggesting that this protein is differentially glycosylated in resident and exudate MØ. Our immunocytochemical findings also suggest upregulation of FA/11 antigen in TPM. To test these observations directly, we used ^{125}I-FA/11 IgG and ^{125}I-WGA to probe Western blots of TPM and RPM (Fig. 4 *a*); both glycoforms have identical electrophoretic mobility. Densitometry of the autoradiograms reveals that the antibody and lectin signals are linearly proportional to the number of cells loaded until the photographic plate is maximally darkened (Fig. 4 *b*). Equivalent quantities of FA/11 Ag are contained in 2.6 × 10^5 RPM and 1.5 × 10^4 TPM (Fig. 4 *c*), a difference of 17-fold per cell. With matched quantities of antigen, ^{125}I-WGA gives a 29-fold greater signal with the TPM glycoform (Fig. 4 *c*), confirming the occurrence of differential glycosylation. Exudate macrophage populations elicited with periodate, Biogel beads, live BCG, or heat-killed *C. parvum* contain intermediate levels of FA/11 antigen that binds WGA in Western blots (not shown). Antigen affinity purified from normal mouse spleen likewise binds WGA (not shown). TPM express the exudate glycoform within 24 h of an intraperitoneal eliciting stimulus, and the resident or exudate cell glycoforms are stably expressed for at least 72 h in culture (not shown).

FA/11 Ag Is an Endogenous MØ Product Distinct from Known MØ Antigens. To establish definitively whether FA/11 is MØ-derived, we performed immunoprecipitations on TPM metabolically labeled with an 18-h pulse of ^{35}S-methionine (Fig. 5), [^{3}H]mannose, or [^{3}H]galactose. FA/11 immunoprecipitated a single molecular species that migrated as a broad band, M_r 87–104 kD, with identical mobility under reducing and nonreducing conditions (not shown). The antigen differed in electrophoretic mobility from other MØ proteins of similar size (Fig. 5), including Mac-3, MOMA-2, the integrin β_2 chain immunoprecipitated by Mac-1, and Pgp-1 (not shown). In Western blots, FA/11 does not cross-react with lgp 110 or lgp 120 (R. da Silva, unpublished observations).

Post-translational Processing. Our previous study showed that WGA recognition of TPM-derived FA/11 Ag depends on sialic acid residues. The known specificity of WGA implies that these are located on multiple, clustered sugar side chains.

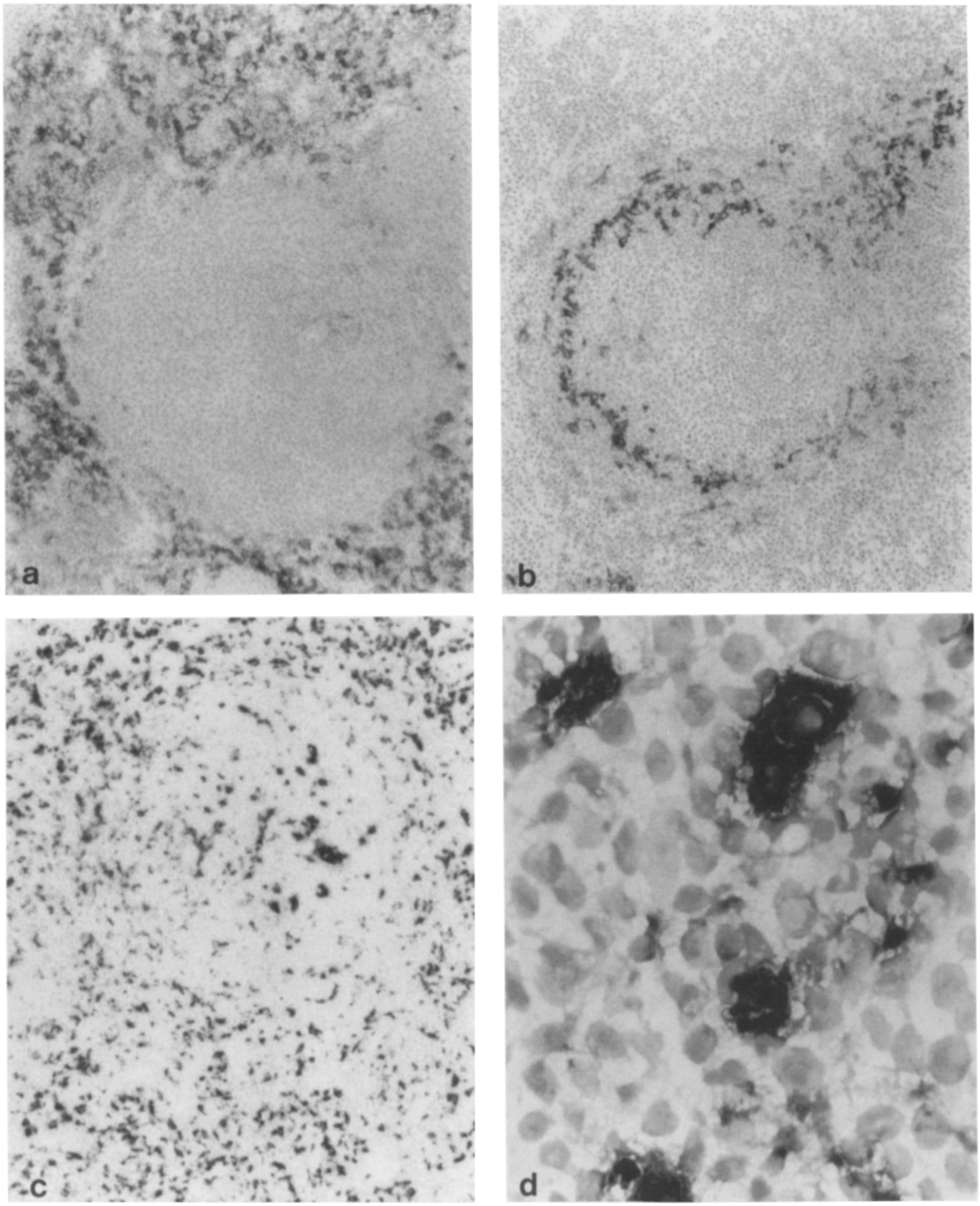

Figure 1. Expression of FA/11 Ag and other MØ markers in the mouse spleen. 5-μm cryostat sections were fixed in acetone and stained by the avidin-biotin-peroxidase method. (*a*) F4/80 outlines the surface and processes of red pulp MØ; (*b*) SER-4 gives surface staining of marginal zone MØ; (*c*) FA/11 labels intracellular structures in both these populations, as well as cells in the white pulp, which at higher power (*d*) are seen to contain ingested nuclei (*a–c*, ×20; *d*; ×100).

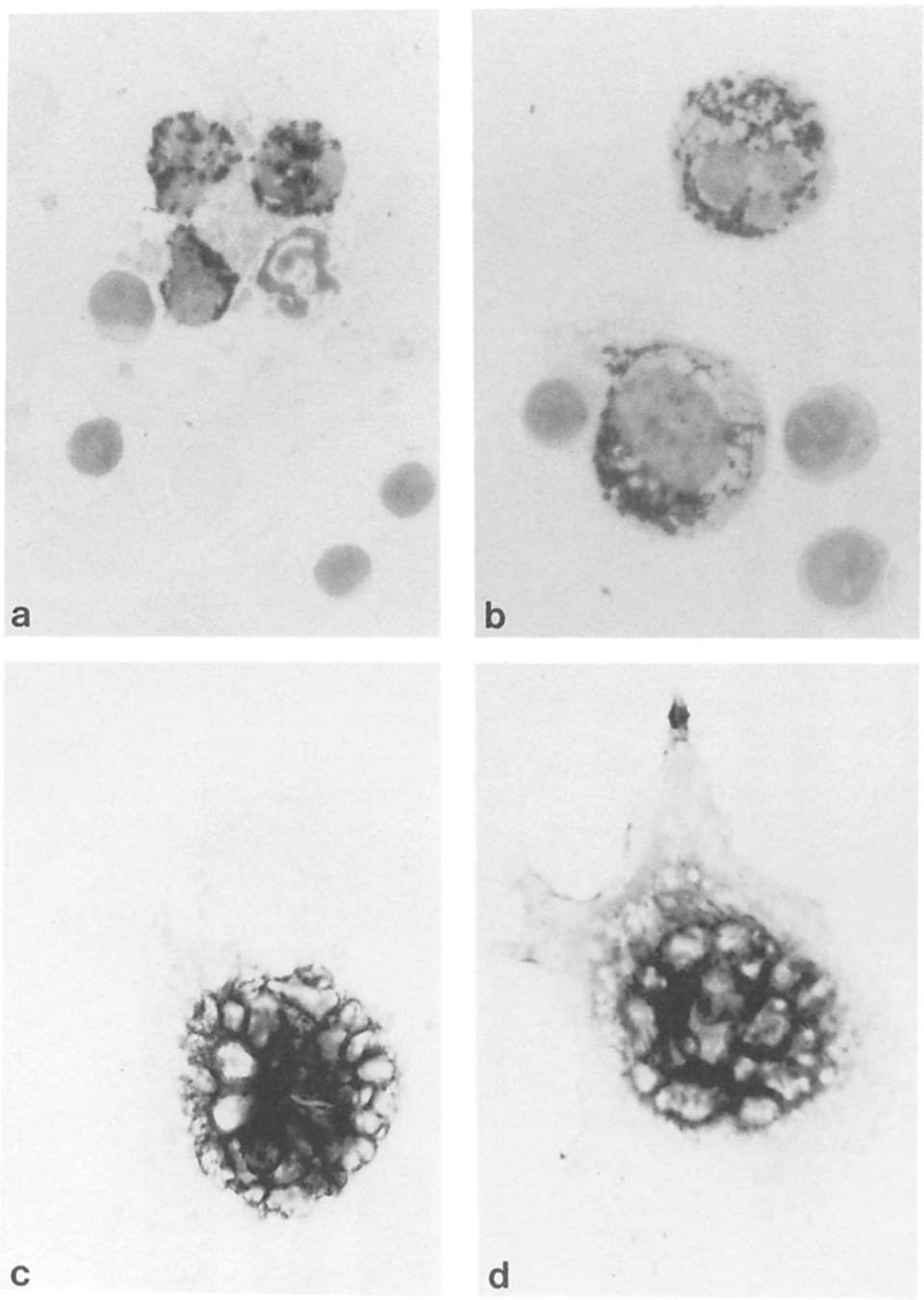

Figure 2. Expression of FA/11 Ag in (*a*) circulating leukocytes, (*b*) resident peritoneal cells, and (*c*) TPM. Cytospin preparations were fixed in acetone and stained by the avidin-biotin-peroxidase method. (*a*) Monocytes, but not neutrophils, lymphocytes, or red cells are labeled; (*b*) RPM, but not lymphocytes, express the Ag; (*c*) the phagolysosomal membranes of TPM show heavy staining, almost obscuring labeled peripheral vacuoles; (*d*) TPM processed for detection of WGA binding sites show similar labeling of phagolysosomes, and also of the plasma membrane (×100).

The polydisperse migration of the immunoprecipitated antigen (Fig. 5) supports the case for abundant sugar. To study the glycosylation and processing of newly synthesized FA/11 Ag in TPM, we performed pulse-chase studies with ^{35}S-methionine-labeled cells and analyzed the susceptibility of the immunoprecipitated antigen to digestion with Endo-H (Fig. 6). FA/11 recognizes an early precursor (7.5-min pulse, no chase), M_r 66 kD; Endo-H digestion generates a tight band, M_r 42 kD, which presumably represents the core protein. The early precursor is converted ($t_{½}$ = 14 min) without detectable intermediates to a single higher molecular mass species that migrates as a progressively broader band (after 45 min, M_r = 87–95 kD, though after 18 h it is broader still; compare Figs. 5 and 6). Endo-H sensitivity is partly retained (M_r decreased to 80–95 kD).

Carbohydrate Structures of RPM and TPM Glycoforms. These results confirm that FA/11 Ag is highly glycosylated, with carbohydrate accounting for over half the apparent molecular mass. To explore the structure of these sugar side-chains and to delineate differences between the alternative glycoforms, we compared the susceptibility of TPM and RPM glycoforms to digestion with various endo- and exoglycosidases (Table

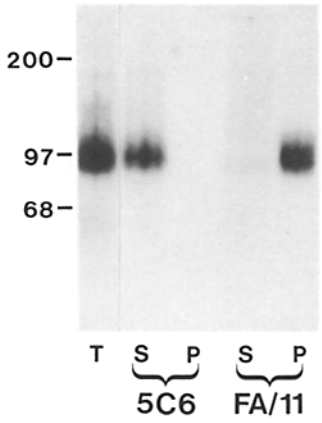

Figure 3. Immunoadsorption of the major WGA-binding protein by FA/11 mAb. ^{125}I-WGA was used to probe Western blots of total TPM lysate (T), and of the supernate (S) and pellet (P) fractions of lysate after immunoadsorption by protein A–sepharose beads bearing FA/11 IgG. The major WGA-binding protein is specifically adsorbed from the supernate, leaving only minor WGA-binding bands. Beads coated with control mAb 5C6 do not immunoadsorb WGA-binding proteins.

1). The undigested RPM form comigrated with its TPM equivalent, though as a slightly tighter band. Both glycoforms lost M_r 6 kD on treatment with Endo-H or Endo-F. Digestion with *N*-glycanase removed more sugar, decreasing M_r by 21-kD. Endo-β-galactosidase substantially cleaved the TPM form, suggesting the presence of poly-*N*-acetyllactosamine structures, but had no effect on the RPM form.

On digestion with neuraminidase, both glycoforms showed the paradoxical behavior typical of mucin-like proteins rich in O-linked sialyloligosaccharides. In the presence of the denaturing detergent SDS, there was no change in migration. If excess NP-40 was added before digestion (to segregate SDS into mixed micelles), the M_r was paradoxically increased; similar results were obtained if SDS was omitted. Differences in O-linked sugar structure were revealed by digestion with O-glycanase (Fig. 7). Preliminary digestion with neuraminidase/SDS was performed to remove blocking sialic acid. NP-40 was then added, together with O-glycanase (or buffer as control). Neuraminidase treatment by this protocol produced a slight increase in M_r of the TPM form only, revealing subtle differences in O-linked sugar structure. This increase was partially reversed by endo-*N*-acetylgalactosaminidase, which had no evident effect on the RPM form. An aliquot of these RPM was tested to control for unintentional macrophage activation: negative WGA blotting confirmed that the resident phenotype was expressed.

Discussion

FA/11 Antigen Is a Novel Macrophage-restricted Sialoglycoprotein. FA/11 Ag is found on circulating monocytes, but not neutrophils, lymphocytes, or red cells; its staining pattern in tissue sections is compatible with restriction to the monocyte/MØ lineage. Two previously described markers for murine macrophages, F4/80 and SER-4, define distinct nonlymphoid macrophage subpopulations. FA/11 labels both, as well as MØ of T and B cell areas in lymph nodes, thymus, and spleen, which lack these antigens. It resembles MOMA-2 in its expression on mononuclear phagocytes and dendritic cells, but is also found on microglia (not shown), which do not express MOMA-2 (27).

The antigen is metabolically labeled in culture. Its M_r is

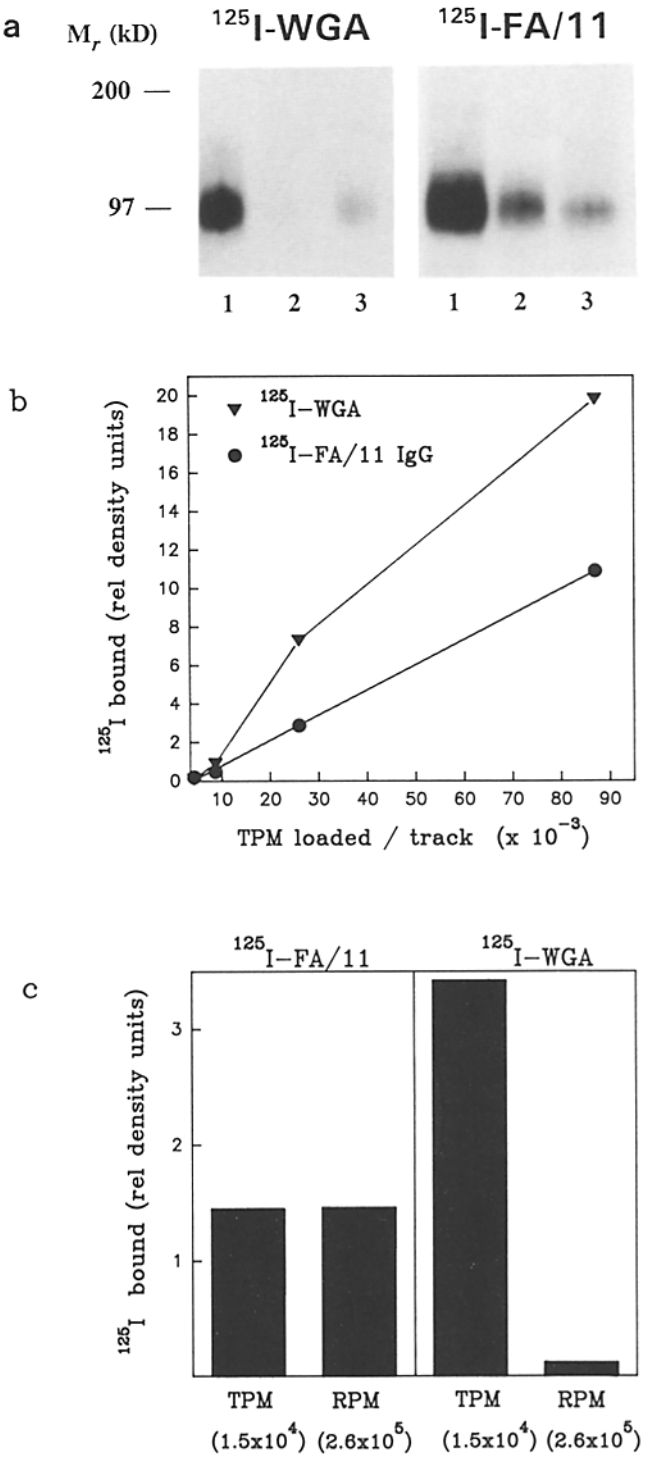

Figure 4. (*a*) Differential glycosylation of FA/11 Ag in exudate and resident peritoneal MØ. Lysate equivalent to (*1*) 2.6 × 10^6 TPM, (*2*) 2.6 × 10^7 RPM, and (*3*) 8.7 × 10^5 TPM was analyzed in Western blots probed with ^{125}I-WGA or ^{125}I-FA/11 IgG. FA/11 Ag was readily detectable in the RPM lysate, but did not bind WGA. (*b*) Quantification of ^{125}I-WGA and ^{125}I-FA/11 IgG signal. Densitometry of autoradiograms shows a signal linearly proportional to cell number loaded until the detection system saturates. (*c*) Binding of ^{125}I-WGA to matched quantities of FA/11 Ag from TPM and RPM. Interpolation from the binding curve in (*b*) shows that 2.6 × 10^5 RPM and 1.5 × 10^4 TPM contain equal quantities of FA/11 antigen, but differ 29-fold in their capacity to bind ^{125}I-WGA.

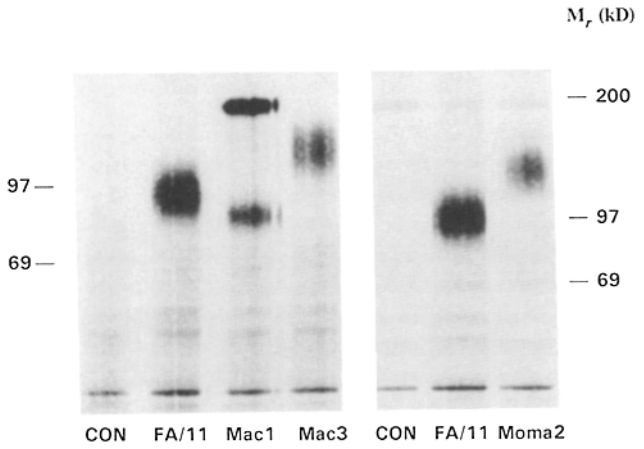

Figure 5. Immunoprecipitation of metabolically labeled FA/11 Ag. TPM were labeled with ^{35}S-methionine for 18 h; FA/11, or other antigens as indicated, were immunoprecipitated and analyzed by SDS-PAGE in 10% uniform gels under reducing conditions. FA/11 Ag is electrophoretically distinct from CD18 (precipitated with Mac-1), Mac-3, and MOMA-2.

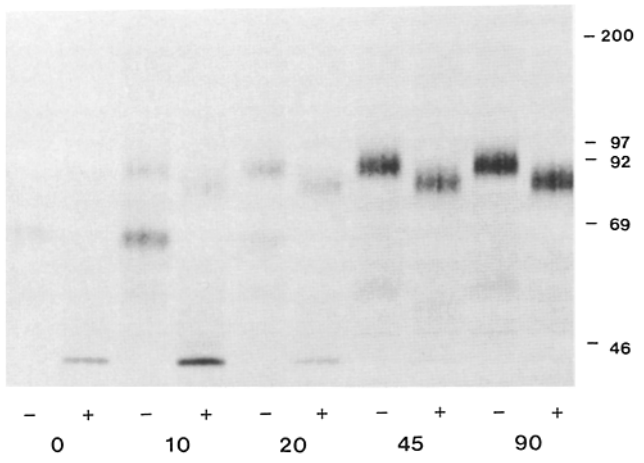

Figure 6. Post-translational processing of FA/11 Ag. TPM were metabolically labeled with ^{35}S-methionine for 7.5 min and chased with unlabeled medium for 0–90 min as indicated. Immunoprecipitated FA/11 Ag was analyzed by SDS-PAGE (10% gel, reducing conditions) after digestion with Endo-H (+) or without glycosidase treatment (–). The mAb recognizes an early 66-kD precursor digested by Endo-H to a core-peptide of 44 kD. The precursor is processed to a polydisperse protein (M_r 87–94 kD), which remains partially sensitive to Endo-H.

distinct from those of CD18, Mac-3, MOMA-2, and Pgp-1. In Western blots, it does not crossreact with lgp 110 or lgp 120 (R. da Silva, unpublished observations), and its cellular distribution distinguishes it from leukosialin. Since the epitope is expressed by an early 66-kD intermediate before processing of high-mannose oligosaccharide chains, it is unlikely to be sugar dependent. Taken together, these data show that mAb FA/11 defines a novel macrophage-restricted glycoprotein; any structural relationships with other leukocyte proteins will emerge once cDNA sequence is available.

Since FA/11 Ag corresponds with the major membrane sialoglycoprotein previously demonstrated in peritoneal exudate macrophages, we propose to name this protein macrosialin.

Table 1. *Effect of Glycosidases on the Resident and Exudate Glycoforms of Macrosialin*

Treatment	M_r TPM	M_r RPM	Change in M_r TPM	Change in M_r RPM
	kD		*kD*	
None	86–104	90–100		
Endoglycosidase H	80–97	84–96	–6	–6
Endoglycosidase F	80–97	84–96	–6	–6
N-glycanase	65–77	69–79	–21	–21
Endo-β-galactosidase	73–83	90–98	–13	0
Neuraminidase/NP-40	100–125	97–115	+14	+7
Neuraminidase/SDS	85–104	91–97	0	0

M_r was determined from the leading edge.

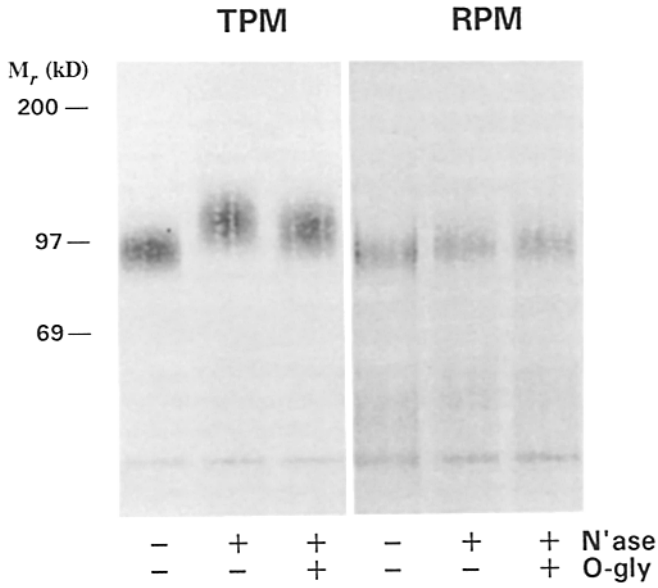

Figure 7. O-linked oligosaccharides of exudate and resident glycoforms of FA/11 Ag. TPM and RPM were metabolically labeled with ^{35}S-methionine for 18 and 24 h, respectively; FA/11 Ag was immunoprecipitated and analyzed in 10% gels (under reducing conditions) after digestion with neuraminidase, O-glycanase, both, or neither. With neuraminidase digestion by this protocol, FA/11 from TPM shows substantially reduced electrophoretic mobility, while FA/11 from RPM is only slightly affected. O-glycanase treatment reduces the M_r of the TPM glycoform, but has no detectable effect on the RPM glycoform.

Regulation by Inflammatory Stimuli. Macrosialin illustrates two levels of regulation in response to inflammatory stimuli. As with many other membrane proteins, antigen expression is enhanced during macrophage recruitment. Thioglycollate-elicited macrophages have the highest content of macrosialin Ag, perhaps reflecting expansion of the phagolysosomal com-

partment in which this protein is principally localized in these cells (Rabinowitz et al., manuscript in preparation); other sterile inflammatory (periodate, polyacrylamide) or fully activating (*C. parvum*, BCG) stimuli provoke a lesser increase.

In addition, macrosialin is expressed in two distinct glycoforms. In Western blots, the lectins WGA and peanut agglutinin recognize only the exudate form; further differences, revealed by glycosidase studies, are discussed below.

Changes in macrophage surface sugars during inflammatory recruitment are well described. Recruited murine peritoneal MØ differ from their resident counterparts in surface binding of several lectins (5, 7–9, 18, 31), and in expression of defined sugar structures on the surface membrane (11). Our data show that oligosaccharide changes are not merely incidental to altered expression of the associated proteins, but also arise from differential glycosylation of constitutively expressed proteins. This may be a general mechanism for modulating the composition of the leukocyte surface, since O-linked sugar structures on leukosialin are altered in activated human T cells (16), and the macrophage membrane protein Mac-3 is expressed by TPM and RPM with differing M_r (our unpublished observations), probably reflecting differences in glycosylation.

Macrosialin exudate glycoform appeared within 24 h of an intraperitoneal eliciting stimulus, and glycoform expression was maintained in culture for at least 72 h (not shown). Our data do not suggest whether glycosylation phenotype is determined during cell maturation, in circulating monocytes, or after extravasation, though circulating human monocytes resemble exudate murine MØ when analyzed by WGA blotting (data not shown).

Nature of the Variant Sugar Structures. Oligosaccharide accounts for over half the M_r of macrosialin. Studies with *N*-glycanase suggest that N-linked sugar contributes at least 21 kD to the M_r of the mature protein; high-mannose chains constitute a small proportion of this and confer partial residual sensitivity to Endo-H. No differences definitely attributable to N-linked sugar structures were demonstrated between the resident and exudate glycoforms.

Three lines of evidence point to the presence of sialylated O-linked sugar structures. (*a*) Neuraminidase digestion of both resident and exudate macrosialin produces the complex electrophoretic changes typical of mucin-like proteins (32). Differences between the glycoforms are revealed by brief neuraminidase/SDS treatment (not shown), or by neuraminidase digestion with nonionic detergent (Fig. 7), since resident cell macrosialin returns more rapidly to its native M_r. (*b*) O-Glycanase digestion directly demonstrates the presence of O-linked glycan in the exudate glycoform. (*c*) Lectin blotting with WGA and peanut agglutinin confirms that exudate cell macrosialin contains O-linked chains with terminal sialic acid residues (18). Failure of these lectins to recognize the resident cell glycoform suggests that in this system these lectins act as highly selective ligands, exposing subtle differences in O-linked sugar structure. Such findings are in keeping with previous reports that WGA distinguishes between singly and multiply sialylated sugar chains (33), and between different classes of cell surface sialic acid (34).

Digestion with endo-β-galactosidase reveals a further difference. The linear poly-*N*-acetyllactosamine structures cleaved by this enzyme were found only in the exudate cell glycoform. These (Galβ1-4GlcNAcβ1) polymers (also known as polylactosaminoglycans) may occur in N-linked (35) or O-linked (36) oligosaccharide chains. Their differential expression on macrosialin is in keeping with previous reports that surface protein-bound lactosaminoglycans of mouse macrophages alter with activation (11), and that several related enzymes are coordinately modulated (12). Immobilized WGA binds and retains poly-*N*-acetyllactosamines derived from membrane proteins (37), but the interaction is complex, appears to require fucose residues not directly involved in lectin-binding, and may be inhibited by adjacent sialic acid residues (38). These structures do not account for the binding of WGA by macrosialin, which is fully neuraminidase sensitive.

Functional Significance of the Sugar Structures. The nature and regulated expression of these sugar structures on a predominantly intracellular, macrophage-restricted membrane protein suggest several possible functional roles.

O-linked sugars and sialic acid contribute to phagocyte defence by allowing proteins to resist proteolysis (39) and to shield cell membranes from degradative enzymes (40) and complement (41). These properties would be useful in lysosomes and at the surface of cells acting in environments rich in acid, proteolytic enzymes, and complement. The occurrence of poly-*N*-acetyllactosamine structures on the myelomonocytic forms of leukosialin (42), on lysosomal integral membrane proteins (43), and on macrosialin raises similar possibilities.

Viral (44) and bacterial (45) lectins bind terminal sialic acid residues; this may contribute to ingestion of pathogens, or to their ability to interfere with intracellular fusion events. WGA binding sites have been implicated in macrophage binding of yeast particles (46), phagocytosis of bacteria (30), and killing of tumour cells (47–49).

WGA treatment causes mouse peritoneal exudate MØ to fuse into multinucleate giant cells (S. Rabinowitz, unpublished observations); a role in membrane fusion would be appropriate for a protein expressed on membranes of the cell surface, lysosomes, and endosomes. Macrosialin is also endocytically active, mediating internalization of surface-bound FITC-FA/11 IgG (S. Rabinowitz, unpublished observations). While many lysosome proteins are also found in membranes of other compartments (50–52), the restricted cellular distribution of macrosialin suggests a possible role in antigen processing.

Macrosialin shows promise as a widely expressed MØ marker, particularly for MØ of lymphoid areas. Its dual regulation by inflammatory stimuli, involving both increased levels of protein and changes in O-linked glycan and poly-*N*-acetyllactosamine, illustrates the plasticity of the MØ membrane in achieving phenotypic variation within a single lineage.

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References

1. Karnovsky, M.L., and J.K. Lazdins. 1978. Biochemical criteria for activated macrophages. *J. Immunol.* 121:809.
2. Hamilton, T.A., J.E. Weiel, and D.O. Adams. 1984. Expression of the transferrin receptor in murine peritoneal macrophages is modulated in the different stages of activation. *J. Immunol.* 132:2285.
3. Ezekowitz, R.A.B., M. Hill, and S. Gordon. 1985. Modulation of macrophage plasma membrane antigens and receptors by activation. *In* Mononuclear Phagocytes. Characteristics, Physiology, and Function. R. van Furth, editor. Martinus Nijhoff Publishers, Dordrecht. 513–520.
4. Ezekowitz, R.A.B., M. Hill, and S. Gordon. 1986. Interferon α/β selectively antagonises down-regulation of mannosyl-fucosyl receptors on activated macrophages by interferon-γ. *Biochem. Biophys. Res. Commun.* 136:737.
5. De Water, R., J.M. van 't Noordende, L.A. Ginsel, and W.T. Daems. 1981. Heterogeneity in wheat germ agglutinin binding by mouse peritoneal macrophages. *Histochemistry.* 72:333.
6. De Water, R., J.M. van 't Noordende, W.T. Daems, and L.A. Ginsel. 1984. Wheat germ agglutinin binding in four types of mouse peritoneal macrophages. A quantitative EM-cytochemical study. *Histochemistry.* 80:449.
7. Gallily, R., B. Vray, and N. Sharon. 1984. Wheat germ agglutinin potentiates uptake of bacteria by murine peritoneal macrophages. *Immunology.* 52:679.
8. Maddox, D.E., S. Shibata, and I.L. Goldstein. 1982. Stimulated macrophages express a new glycoprotein receptor reactive with *Griffonia simplicifolia* I-B_4 isolectin. *Proc. Natl. Acad. Sci. USA.* 79:166.
9. Petryniak, J., D. Dus, and J. Podwinska. 1983. Agglutinin of murine and guinea pig peritoneal cells by α-L-fucose-binding lectin: *Evonymus europaea*. *Eur. J. Immunol.* 13:458.
10. Petryniak, J., T.K. Huard, G.D. Noordblom, and I.J. Goldstein. 1986. Lectin binding studies on murine peritoneal cells: physicochemical characterization of the binding of lectins from *Datura stramonium*, *Evonymus europaea*, and *Griffonia simplicifolia* to murine peritoneal cells. *Arch. Biochem. Biophys.* 244:57.
11. Irimura, T., S.M. North, and G.L. Nicolson. 1987. Glycoprotein profiles of macrophages at different stages of activation as revealed by lectin binding after electrophoretic separation. *Eur. J. Immunol.* 17:73.
12. Mercurio, A.M., and P.W. Robbins. 1985. Activation of mouse peritoneal macrophages alters the structure and surface expression of protein-bound lactosaminoglycans. *J. Immunol.* 135:1305.
13. Sheares, B.T., and A.M. Mercurio. 1987. Modulation of two distinct galactosyltransferase activities in populations of mouse peritoneal macrophages. *J. Immunol.* 139:3748.
14. Rademacher, T.W., R.B. Parekh, and R.A. Dwek. 1988. Glycobiology. *Annu. Rev. Biochem.* 57:785.
15. Parekh, R.B., A.G.D. Tse, R.A. Dwek, A.F. Williams, and T.W. Rademacher. 1987. Tissue-specific N-glycosylation, site-specific oligo-saccharide patterns and lentil lectin recognition of rat Thy-1. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1233.
16. Carlsson, S.R., H. Sasaki, and M. Fukuda. 1986. Structural variations of O-linked oligosaccharides present in leukosialin isolated from erythroid, myeloid, and T lymphoid cell lines. *J. Biol. Chem.* 261:12787.
17. Piller, F., V. Piller, R.I. Fox, and M. Fukuda. 1988. Human T-lymphocyte activation is associated with changes in O-glycan biosynthesis. *J. Biol. Chem.* 263:15146.
18. Rabinowitz, S., and S. Gordon. 1989. Differential expression of membrane sialoglycoproteins in exudate and resident mouse peritoneal macrophages. *J. Cell Sci.* 93:623.
19. Smith, M.J., and G.L.E. Koch. 1987. Differential expression of murine macrophage surface glycoprotein antigens in intracellular membranes. *J. Cell Sci.* 87:113.
20. Fauve, R.M., H. Jusforgues, and B. Hevin. 1983. Maintenance of granuloma macrophages in serum-free medium. *J. Immunol. Methods.* 64:345.
21. Rosen, H., and S. Gordon. 1987. Monoclonal antibody to murine type 3 complement receptor inhibits adhesion of myelomonocytic cells in vitro and inflammatory recruitment in vivo. *J. Exp. Med.* 166:1685.
22. Springer, T., G. Galfre, D.S. Secher, and C. Milstein. 1979. Mac-1: a macrophage differentiation antigen identified by a monoclonal antibody. *Eur. J. Immunol.* 9:301.
23. Austyn, J.M., and S. Gordon. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* 11:805.
24. Crocker, P.R., and S. Gordon. 1989. Mouse macrophage hemagglutinin (sheep erythrocyte receptor) with specificity for sialylated glycoconjugates characterized by a monoclonal antibody. *J. Exp. Med.* 169:1333.
25. Springer, T. 1981. Monoclonal antibody analysis of complex biological systems. *J. Biol. Chem.* 256:3833.
26. Trowbridge, I.S., J. Lesley, R. Schulte, R. Hyman, and J. Trotter. 1982. Biochemical characterization and cellular distribution of a polymorphic murine cell-surface glycoprotein expressed on lymphoid tissues. *Immunogenetics.* 15:299.
27. Kraal, G., M. Rep, and M. Janse. 1987. Macrophages in T and B cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2. An immunohistochemical study. *Scand. J. Immunol.* 26:653.
28. Werb, Z., M.J. Banda, R. Takemura, and S. Gordon. 1986.

Secreted proteins of resting and activated macrophages. *In* Handbook of Experimental Immunology. D.M. Weir, editor. Blackwell Scientific Publications, Oxford. 47.1–47.29.
29. Keesey, J. 1987. Biochimica Information. Boehringer Mannheim Biochemicals, Indianapolis. 246 pp.
30. Austyn, J.M., R.M. Steinman, D.E. Weinstein, A. Granelli-Piperno, and M.A. Palladino. 1983. Dendritic cells initiate a two-stage mechanism for T-lymphocyte proliferation. *J. Exp. Med.* 157:1101.
31. Gallily, R., B. Vray, I. Stain, and N. Sharon. 1984. Wheat germ agglutinin potentiates uptake of bacteria by murine peritoneal macrophages. *Immunology.* 52:679.
32. Gahmberg, C., and L.C. Andersson. 1982. Role of sialic acid in the mobility of membrane proteins containing O-linked oligosaccharides on polyacrylamide gel electrophoresis in sodium dodecyl sulfate. *Eur. J. Biochem.* 122:581.
33. Bhavanandan, V.P., and A.W. Katlic. 1979. The interaction of wheat germ agglutinin with sialoglycoproteins. *J. Biol. Chem.* 254:4000.
34. Stanley, P., T. Sudo, and J. Carver. 1980. Differential involvement of cell surface sialic acid residues in wheat germ agglutinin binding to parental and wheat germ agglutinin-resistant Chinese Hamster Ovary cells. *J. Cell Biol.* 85:60.
35. Tsuji, T., T. Irimura, and T. Osawa. 1980. The carbohydrate moiety of band-3 glycoprotein of human erythrocyte membranes. *Biochem. J.* 187:677.
36. Zdebska, E., and J. Koscielak. 1978. Studies on the structure and I-blood group activity of poly(glycosyl)ceramides. *Eur. J. Biochem.* 91:517.
37. Gallagher, J.T., A. Morris, and T.M. Dexter. 1985. Identification of two binding sites for wheat germ agglutinin on polylactosamine-type oligosaccharides. *Biochem. J.* 231:115.
38. Ivatt, R.J., J.W. Reeder, and G.F. Clark. 1986. Structural and conformational features that affect interaction of polylactosaminoglycans with immobilized wheat germ agglutinin. *Biochim. Biophys. Acta.* 883:253.
39. Kozarsky, K., D. Kingsley, and M. Krieger. 1988. Use of a mutant cell line to study the kinetics and function of O-linked glycosylation of low density lipoprotein receptors. *Proc. Natl. Acad. Sci. USA.* 85:4335.
40. Jentoft, N. 1990. Why are proteins O-glycosylated? *TIBS (Trends Biol. Sci.).* 15:291.
41. Fearon, D.T. 1978. Regulation by membrane sialic acid of β1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. *Proc. Natl. Acad. Sci. USA.* 75:1971.
42. Fukuda, M., S. Carlsson, J.C. Klock, and A. Dell. 1986. Structures of O-linked oligosaccharides isolated from normal granulocytes, chronic myelogenous leukemia cells, and acute myelogenous leukemia cells. *J. Biol. Chem.* 261:12796.
43. Carlsson, S.R., J. Roth, F. Piller, and M. Fukuda. 1988. Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Major sialoglycoproteins carrying polylactosaminoglycan. *J. Biol. Chem.* 263:18911.
44. Paulson, J.C., J.E. Sadler, and R.L. Hill. 1979. Restoration of specific myxovirus receptors to asialoerythrocytes by incorporation of sialic acid with pure sialyltransferases. *J. Biol. Chem.* 254:2120.
45. Parkkinen, J., G.N. Rogers, T. Korhonen, W. Dahr, and J. Finne. 1986. Identification of the O-linked sialyloligosaccharides of glycophorin A as the erythrocyte receptors for S-fimbriated *Escherichia coli*. *Infect. Immun.* 54:37.
46. Kean, E.L., and N. Sharon. 1987. Inhibition of yeast binding to mouse peritoneal macrophages by wheat germ agglutinin: a novel effect of the lectin on phagocytic cells. *Biochem. Biophys. Res. Commun.* 148:1202.
47. Ogawara, M., T. Utsugi, M. Yamazaki, and S. Sone. 1985. Induction of human monocyte-mediated tumor cell killing by a plant lectin, wheat germ agglutinin. *Jpn. J. Cancer Res. (Gann).* 76:1107.
48. Ogawara, M., S. Sone, and T. Ogura. 1987. Human alveolar macrophages: wheat germ agglutinin-dependent tumor cell killing. *Jpn. J. Cancer Res. (Gann).* 78:288.
49. Kurisu, M., M. Yamazaki, and M. Den'ichi. 1980. Induction of macrophage-mediated tumor lysis by the lectin wheat germ agglutinin. *Cancer Res.* 40:3798.
50. Muller, W.A., R.M. Steinman, and Z.A. Cohn. 1980. The membrane proteins of the vacuolar system II. Bidirectional flow between secondary lysosomes and the plasma membrane. *J. Cell Biol.* 86:304.
51. Lippincott-Schwartz, J., and D.M. Fambrough. 1987. Cycling of the integral membrane glycoprotein, LEP100, between plasma membrane and lysosomes: a kinetic and morphological analysis. *Cell.* 49:669.
52. Croze, E., I.E. Ivanov, G. Kreiblich, M. Adesnik, and D.D. Sabatini. 1989. Endolyn 78, a membrane glycoprotein present in morphologically diverse compartments of the endosomal and lysosomal compartments: implication for lysosomal biogenesis. *J. Cell Biol.* 108:1597.