Phagocytosis stimulates alternative glycosylation of macrosialin (mouse CD68), a macrophage-specific endosomal protein

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Macrosialin (mouse CD68), a macrophage-specific member of the lysosomal-associated membrane protein family, displays N-linked glycosylation and a heavily sialylated, mucin-like domain. We show that phagocytosis of zymosan by inflammatory peritoneal macrophages potently alters glycan processing of macrosialin *in vitro*. The phagocytic glycoform is not induced by other forms of endocytosis and depends on particle internalization. Zymosan uptake does not influence macrosialin protein synthesis, but increases the specific incorporation of D-[2-^{3}H]mannose, D-[6-^{3}H]galactose, *N*-acetyl-D-[1-^{3}H]glucosamine and L-[5,6-^{3}H]-fucose by 2–15-fold. The phagocytic glycoform displays increased binding of agglutinins from peanut, *Amaranthus caudatus* and *Galanthus nivalis*, whereas binding of the sialic-acid-specific *Maakia amurensis* agglutinin is slightly reduced. Digestion by N-Glycanase abolishes the incorporation of [^{3}H]mannose label and *Galanthus nivalis* agglutinin binding activity, but preserves the incorporation of galactose and *N*-acetylglucosamine and specific lectin binding. We also show that phagocytosis increases the complexity and length of O-linked chains. The data presented highlight the importance of differential glycosylation in the biology of macrosialin, phagosomes and macrophages in general.

Key words: agglutinins, endocytosis, glycans, lysosomal-associated membrane glycoproteins.

INTRODUCTION

Macrosialin (mouse CD68) is a heavily glycosylated, macrophage (MØ)-specific protein that is localized intracellularly, mainly in late endosomes [1,2]. From a precursor protein of ~ 42 kDa, macrosialin is processed to a mature product that runs as a broad band in SDS/PAGE from 85 to 115 kDa, via an intermediate endoglycosidase H-sensitive product of 66 kDa. The processing steps responsible for this increase in molecular mass involve the addition of both N- and O-linked glycans, and glycosylation of the protein varies in different MØ peritoneal populations [1,3].

On cloning and sequencing of the encoding DNA, we found that macrosialin is a member of the lysosomal-associated membrane glycoprotein (LAMP) family [4]. Macrosialin is the murine CD68 homologue, and the protein is well conserved between humans and mice [4,5]. In common with the LAMPs, macrosialin, a type 1 membrane protein, has a short and highly conserved cytoplasmic tail, followed by a transmembrane domain that precedes the intralumenal portion of the peptide. Two distinct domains separated by a proline-rich hinge region are found in the intralumenal region. The more internal domain I shares 50 % similarity with the sequences found in other LAMPs, such as LEP100 and LAMP1. However, in the more distal, N-terminal, domain II, the macrosialin sequence is completely novel. The sequence of this domain is more comparable with that of mucins, and macrosialin displays abundant O-linked glycosylation [4]. Several reports have shown that macrosialin binds to oxidized low-density lipoproteins, and this raises the possibility of a receptor function for this protein [6,7].

Altered glycosylation of a particular backbone peptide has often been related to important biological processes. Leukosialin (CD43), a molecule that also contains mucin domains and is apparently involved in lymphocyte interactions, is a well studied example in which the presence of a specific glycosyltransferase determines the nature of its glycans during T-cell activation [8–12]. Specific glycosyltransferase regulation and consequent changes in oligosaccharide expression are associated with other adhesion molecules, such as the selectins and CD22 [13,14]. Tumour differentiation and metastasis have also been related to differentially expressed oligosaccharides carried by proteins such as LAMP1 and LAMP2 [15].

Changes in the glycosylation patterns of various MØ resident populations have been extensively documented [1,3,16,17]. Proteins such as CD44 and macrosialin itself have been shown previously to be differentially glycosylated in various MØ populations [18]. In comparison with the resident peritoneal cells, macrosialin in thioglycollate-elicited MØ (TMØ) displays altered O-linked sialylation and poly-*N*-acetyl-lactosamines, and these differences account for the binding of wheat-germ agglutinin (WGA) to inflammatory MØ [1,3]. However, very little is known about which aspects of MØ function are responsible for changes in glycosylation and how overall MØ biology might be affected.

In the present paper we investigate the mechanisms responsible for triggering differential glycosylation in MØ. We have focused on macrosialin, an endosomal membrane protein whose function is potentially associated with changes in its glycan moiety. Macrosialin is a MØ-specific, heavily O- and N-glycosylated protein that is abundantly and universally expressed by all mouse MØ populations studied to date. Thus it affords unique insights into the cell-specific regulation of glycosylation during MØ activation. The data presented further clarify the biochemical

Abbreviations used: LAMP, lysosomal-associated membrane protein; MØ, macrophage(s); BMØ, Biogel-elicited MØ; TMØ, thioglycollate-elicited MØ; CG, control glycoform; PG, phagocytic glycoform; MMR, macrophage mannose receptor; WGA, wheat-germ agglutinin; GNA, *Galanthus nivalis* agglutinin; PNA, peanut agglutinin; ACA, *Amaranthus caudatus* agglutinin; DSA, *Datura stramonium* agglutinin; MAA, *Maackia amurensis* agglutinin; SNA, *Sambucus nigra* agglutinin.

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processes involved in remodelling MØ membranes to match functional requirements during cellular activation.

MATERIALS AND METHODS

Cell culture

Peritoneal MØ that had been elicited with Biogel polyacrylamide beads (BMØ) were used. Briefly, Balb/c mice were injected intraperitoneally with 1.0 ml of a 1 % (w/v) suspension of Biogel P-100 (Bio-Rad, Richmond, CA, U.S.A.), 4 or 5 days before harvesting. After collection by peritoneal lavage, the cells were washed once in PBS, resuspended in OPTIMEM 1 (GIBCO, Paisley, Scotland, U.K.), plated on plastic tissue-culture dishes and analysed immediately. When assaying the effects of endocytic uptake, particulate or soluble tracers were added for 2 h as follows: 10 zymosan particles (Sigma, Poole, Dorset, U.K.) or polystyrene beads (Polyscience, Northampton, U.K.) per cell, 5 mg/ml mannan (Fluka, Gillingham, U.K.), 30 μg/ml mannosylated BSA (E-Y Laboratories, San Mateo, CA, U.S.A.), 20 μg/ml WGA (Vector Laboratories, Burlingame, CA, U.S.A.), or 100 ng/ml PMA (Sigma). For blockade of uptake, cytochalasin B (Boehringer Mannheim, Mannheim, Germany) was added at 0.2 μM and the cells were pre-incubated for 1 h before addition of zymosan. Cell viability and inhibition of particle internalization were verified by phase-contrast microscopy.

Metabolic labelling

Cells were plated in 6-well tissue-culture clusters (Costar, Cambridge, MA, U.S.A.) at 3×10^6 cells per well. After 1 h at 37 °C, adherent BMØ were washed three times with PBS and the cells were placed in labelling medium.

For protein labelling, we used methionine-free minimal essential medium (GIBCO) supplemented with 5 % (v/v) dialysed fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. After a 1 h incubation to deplete the cells of free methionine, 50 μCi/ml Tran^{35}S protein labelling mix (ICN, Thame, Oxon., U.K.) was added to 1.0 ml of fresh labelling medium and the cells were incubated at 37 °C for the periods of time shown. Cells were then washed three times with ice-cold PBS plus 2 % (v/v) standard fetal calf serum and lysed in 1 ml/well lysis buffer [2 % *N*-octyl β-D-glucopyranoside, 200 mM PMSF, 1 mM iodoacetamide, 2 mM EDTA, 1.4 mg/ml pepstatin and 1 mg/ml leupeptin in 10 mM Tris buffer, pH 8.0 (all from Sigma)]. Cells were scraped, and the lysates were incubated on ice for 1 h and then centrifuged in a Microfuge at 10000 ***g*** for 15 min to clear nuclei and debris.

For glycan labelling, cells were incubated in OPTIMEM 1 containing 100 μCi/ml of various ^{3}H-labelled sugars for different periods of time. Sugars used were D-[2-^{3}H]mannose (370–740 GBq/mmol), D-[6-^{3}H]galactose (0.74–1.50 TBq/mmol), *N*-acetyl-D-[1-^{3}H]glucosamine (74–370 GBq/mmol), L-[5,6-^{3}H] fucose (1.66–2.60 TBq/mmol) and D-[6-^{3}H]glucose (0.74–1.50 TBq/mmol) (all from Amersham). After metabolic labelling, cells were washed, lysed, scraped and processed for immunoprecipitation.

Immunoprecipitation

Labelled lysates were pre-cleared by adding 10 μg/ml of an isotype-matched antibody and incubated for 1 h on ice, and then 50 μl of a 20 % (w/v) suspension of Protein G-coupled Sepharose (Gamma-Bind Plus; Pharmacia, Uppsala, Sweden) was added for precipitation. Tubes were rotated for 2 h at 4 °C, after which beads were removed by centrifugation (10000 ***g*** for 3 min) and the supernatant was transferred to another tube. For macrosialin precipitation, affinity-purified FA-11 monoclonal antibody [19] was added at 10 μg/ml, lysates were incubated for 1 h on ice and Protein G beads were added as above. Tubes were rotated overnight at 4 °C. Beads were then washed sequentially, twice, in buffers containing 500 and 150 mM NaCl prepared with 0.5 % Triton X-100, 0.5 % sodium deoxycholate and 0.05 % SDS in 10 mM Tris buffer, pH 8.0. Finally, beads were washed once in Tris buffer containing 0.05 % SDS and boiled in SDS/PAGE sample buffer for 2 min. Samples were analysed by PAGE and processed for fluorography. For quantification, radioactivity in a 10 μl aliquot from each SDS/PAGE sample was counted by liquid scintillation spectrometry, or bands developed on X-ray films were measured by densitometry in an Ultroscan XL Laser Densitometer (LKB).

Lectin-probed Western blots

BMØ (5×10^7) were plated in OPTIMEM 1 on 15 cm-diam. tissue culture dishes. After a 1 h incubation, cells were washed three times with PBS, 10 ml of fresh OPTIMEM 1 was added and, for dishes to be assayed for phagocytic uptake, zymosan was added at 10 particles/cell for 3 h. After phagocytosis, all dishes, including controls, were washed three times with PBS and OPTIMEM 1 was replaced before overnight incubation at 37 °C. Cells were again washed three times with PBS and the dishes were drained carefully. Lysis buffer (1.0 ml) was added to the centre and the dish was rotated gently to ensure that buffer covered all cells. Cells were then scraped, incubated for 1 h on ice and processed by immunoprecipitation and SDS/PAGE as described above. Gels were Western-blotted as described elsewhere [3] and the blots were blocked, probed and developed using a Digoxigenin Glycan Differentiation Kit (Boehringer-Mannheim) according to the manufacturer's instructions. FA-11 monoclonal antibody was labelled using a Digoxigenin Antibody Labelling Kit (Boehringer-Mannheim) and blots were processed as for digoxigenin-labelled lectins.

Glycosidase digestion of immunoprecipitates

Beads from immunoprecipitation were processed as described above, with the exception that, after the last wash, they were resuspended and boiled in 50 μl of 10 mM Tris buffer, pH 8.0, containing 0.05 % SDS. The SDS was quenched by adding 10-times excess (0.5 %) octyl glucoside (Sigma) before adding enzymes. Glycosidases were obtained from Oxford GlycoSystems (Abingdon, Oxon., U.K.) and used according to the manufacturer's instructions.

β-Elimination

O-linked glycans were hydrolysed by β-elimination, and released glycans were run on a 1 cm × 50 cm P4 Biogel column as detailed elsewhere [20]. Briefly, 5×10^7cells were plated on 175 cm^2-area tissue-culture flasks, cultured and washed as above. MØ were subsequently labelled with [^{3}H]galactose at 100 μCi/ml overnight. For phagocytosis assays, zymosan was added at 10 particles/cell. MØ were lysed and macrosialin was immunoprecipitated as described above. Proteins were re-precipitated with 20 % (w/v) trichloroacetic acid on ice for 1 h, spun down in a Microfuge, resuspended in 250 μl of 0.2 M NaOH, vortexed for 2 min for complete solubilization, and then 250 μl of 2 M $NaBH_3$ was added. Samples were incubated overnight at 37 °C, after which 50 % (v/v) acetic acid was used for pH neutralization. Samples were then added to the column and eluted with 0.1 M

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ammonium acetate at 4 ml/min. The void column volume was discarded, and 1.0 ml fractions were collected and processed for liquid scintillation counting.

RESULTS

Phagocytosis alters macrosialin glycosylation without affecting specific protein synthesis

We tested the effect of zymosan phagocytosis on the biosynthesis and processing of macrosialin, and found dramatic changes in processing of the glycan moiety following phagocytic activation of the cells. Protein synthesis, however, remained unaltered. Figure 1 shows a comparative assay in which the same cell preparation was labelled either with [^{3}H]mannose (Figures 1A and 1B) as glycan label or with Tran^{35}S* (Figures 1C and 1D) as protein label. As early as 3 h after zymosan phagocytosis, a large increase in radioactivity specifically associated with the macrosialin phagocytic glycoform (PG) was detected after [^{3}H]mannose metabolic labelling. Adhesion to tissue-culture plastic caused less stimulation, as shown by the increase in radioactivity associated with the control glycoform (CG) in culture. The band representing the mature macrosialin protein (85–110 kDa) had a broader electrophoretic mobility, as shown in the SDS/PAGE analysis, and the endoglycosidase H-sensitive macrosialin precursor protein (66 kDa) was also affected (Figure 1A). Small increases in the amount of radioactivity immunoprecipitated after Tran^{35}S labelling were sometimes observed, but these did not prove to be reproducible (Figure 1C). In contrast, processing of the glycan moiety consistently showed substantial differences and increased with time up to 16 h of labelling. After phagocytosis, a 3–5.5-fold increase in macrosialin-associated immunoprecipitated radiolabel was observed for the mature, fully processed protein (Figure 1B). Increases for the endoglycosidase H-sensitive precursor protein were between 6- and 9-fold (Figure 1B). There was no consistent difference in the levels of macrosialin polypeptide synthesis between the CG and the PG of the protein (Figure 1D). The protein synthesis data were further confirmed by Northern blot analysis of transcriptional levels, which remained unchanged after phagocytosis or stimulation with PMA ([4]; and results not shown).

Differential glycosylation is stimulated by receptor-mediated phagocytosis, and not by receptor-mediated endocytosis or fluid-phase uptake

We investigated the relationship between various endocytic processes and altered glycosylation by measuring the incorporation of [^{3}H]mannose into immunoprecipitated macrosialin after the uptake of different tracers targeted to different compartments of the MØ endocytic pathway. We included ligands that bind to the macrophage mannose receptor (MMR), one of the receptors implicated in zymosan uptake. Zymosan can also bind, with or without complement opsonization, to the β-glucan receptor and type 3 complement receptor [21–24]. Mannosylated BSA is taken up by clathrin-coated-receptor-mediated endocytosis, and was used as a soluble ligand for the MMR at 30 μg/ml. Mannan (used at 5 mg/ml), another soluble ligand for

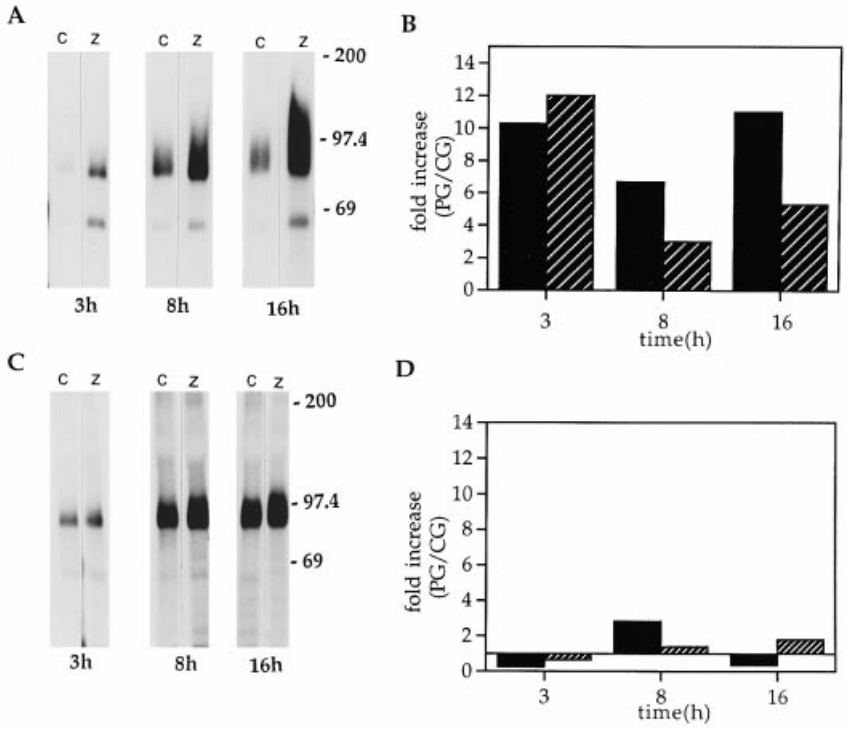

Figure 1 Phagocytic uptake induces increased incorporation of [^{3}H]mannose, without altering specific protein labelling

SDS/PAGE analysis and fluorography of immunoprecipitated macrosialin 3, 8 and 16 h after phagocytosis of zymosan (z). Note that (**A**) and (**B**) refer to glycan labelling and (**C**) and (**D**) refer to polypeptide labelling. Left panels: immunoprecipitation from equal numbers of phagocytosing (z) and control (c) BMØ labelled with [^{3}H]mannose (**A**) and Tran^{35}S protein labelling mix (**C**). Numbers on the right-hand side of gels are molecular mass markers (kDa). Panels (**B**) and (**D**) show fold increases in densitometric readings of the PG (z) compared with the CG (c) bands shown in (**A**) and (**C**) respectively. Solid bars represent results for the precursor protein (66 kDa), and hatched bars represent results for the mature protein (85–110 kDa). The data shown are representative of six experiments.

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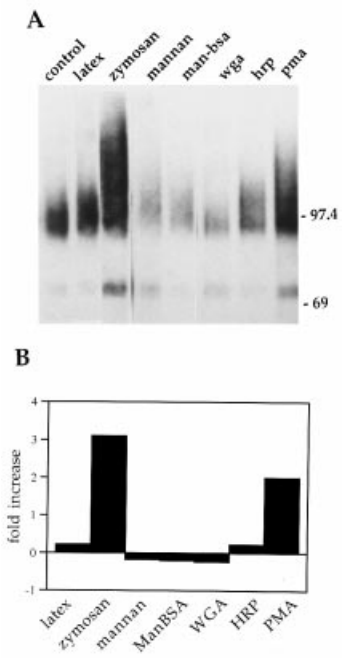

Figure 2 Increased [^{3}H]mannose labelling is associated with zymosan phagocytosis, but not with other forms of endocytic uptake

(**A**) SDS/PAGE analysis and fluorography of immunoprecipitated macrosialin labelled with [^{3}H]mannose, 16 h after phagocytosis of particles or treatment with soluble stimulants as indicated. Positions of molecular mass markers are shown on the right-hand side of the gel (kDa). (**B**) Fold increases in densitometric readings of bands shown in (**A**) relative to the control. 'ManBSA' indicates mannosylated BSA. The data shown are representative of five independent experiments. HRP, horseradish peroxidase.

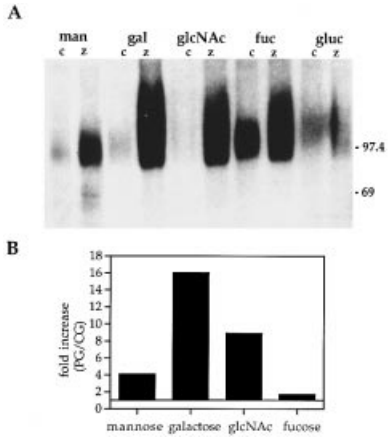

Figure 3 Zymosan uptake also induces increased incorporation of [^{3}H]galactose, [^{3}H]GlcNAc and [^{3}H]fucose

(**A**) SDS/PAGE analysis and fluorography of macrosialin immunoprecipitated from BMØ labelled with [^{3}H]mannose (man), [^{3}H]galactose (gal), [^{3}H]GlcNAc (glcNAc), [^{3}H]fucose (fuc) or [^{3}H]glucose (gluc), 16 h after phagocytosis of zymosan (z). c indicates macrosialin from control BMØ (no phagocytosis). Positions of molecular mass markers are indicated on the right-hand side of the gel (kDa). (**B**) Fold increases in densitometric readings of the bands representing PG (z) relative to CG (c). Specific incorporation of glucose did not prove to be consistent, and was therefore excluded from the analysis. The data shown are representative of four independent experiments.

Table 1 Specificity of lectins used

Lectin	Specificity
GNA	Manα(1-3)Man α1-3 α1-6 α1-2
PNA	Galβ(1-3)GalNAc
ACA	Galβ(1-3)GalNAc-α-Ser/Thr NeuNAc-Galβ(1-3)GalNAc-α-Ser/Thr
MAA	NeuNAcα(2-3)Gal
SNA	NeuNAcα(2-6)Gal/GalNAc
DSA	Galβ(1-4)GlcNAc GlcNAc-Ser/Thr
Aleuria aurantia agglutinin	L-Fucα(1-6)GlcNAc

the MMR, forms larger aggregates and served to cross-link this receptor. Horseradish peroxidase (used at 1 mg/ml) is frequently used as a fluid-phase marker of pinocytic uptake, but in MØ approx. 50% of this ligand can be taken up via the MMR [24]. Little is known about mechanisms involved in WGA endocytosis, but uptake of this lectin (used here at 20 μg/ml), which binds macrosialin and other MØ glycoproteins, generates large intracellular vacuoles due to cross-linking of MØ endosomal glycoproteins [25]. We used latex beads as an additional phagocytic particle.

A representative experiment is shown in Figure 2. Macrosialin immunoprecipitated from MØ after zymosan uptake contained approx. 3 times as much label as did that from control cells. Mannan, mannosylated BSA, WGA and horseradish peroxidase failed to induce significant stimulation. Latex beads were also a poor stimulant. We also tested the effects of PMA, an activator of protein kinase C-mediated intracellular signalling which reproduces many features of phagocytic activation. As expected, PMA was efficient in triggering the glycan changes (Figure 2). These data suggest a role for receptor-mediated phagocytic uptake in the induction of alternative glycosylation. After treatment with cytochalasin B (which allows receptor binding and clustering, but prevents internalization by interfering with the formation of the actin-based phagocytic cup [26]), incorporation of [^{3}H]mannose in the PG was comparable with that in the CG, showing that glycoform alteration depended on internalization of the phagocytic particle after binding to the receptor (results not shown).

Changes in glycosylation after phagocytosis include elongation and increased complexity of O-linked glycans

We used metabolic labelling with a range of monosaccharides to probe for structural modifications occurring during phagocytic activation. Figure 3 shows an assay in which D-[2-^{3}H]mannose, D-[6-^{3}H]galactose, *N*-acetyl-D-[1-^{3}H]glucosamine ([^{3}H]GlcNAc), L-[5,6-^{3}H]fucose and D-[6-^{3}H]glucose were used. In this experiment, the PG of macrosialin incorporated 4 times as much mannose, 16 times as much galactose and 1.5 times as much fucose compared with the CG. GlcNAc was almost exclusively incorporated into the PG. Although some glucose incorporation was shown in this experiment, labelling of macrosialin by glucose was not reproducible, and no difference between the CG and the PG was ever found. Therefore phagocytic uptake seems to alter glycan biosynthesis in MØ broadly, involving increased transfer of different sugars associated with both N- and O-linked chains.

Labelled sugars may, however, be metabolized by the cells and incorporated in another form, so we set up experiments in which immunoprecipitated, unlabelled macrosialin was probed with a

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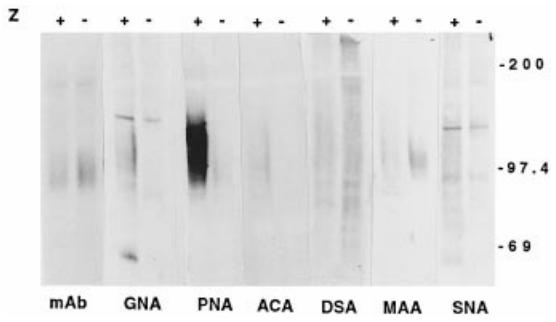

Figure 4 Zymosan uptake increases GNA and PNA binding, and decreases MAA binding, to macrosialin

Western blot analysis of macrosialin immunoprecipitated from BMØ, 16 h after phagocytosis of zymosan (Z). The blot was probed with digoxigenin-labelled FA-11 monoclonal antibody (mAb) or lectins as indicated. Positions of molecular mass markers are indicated (kDa). The blot shown is representative of five independent experiments.

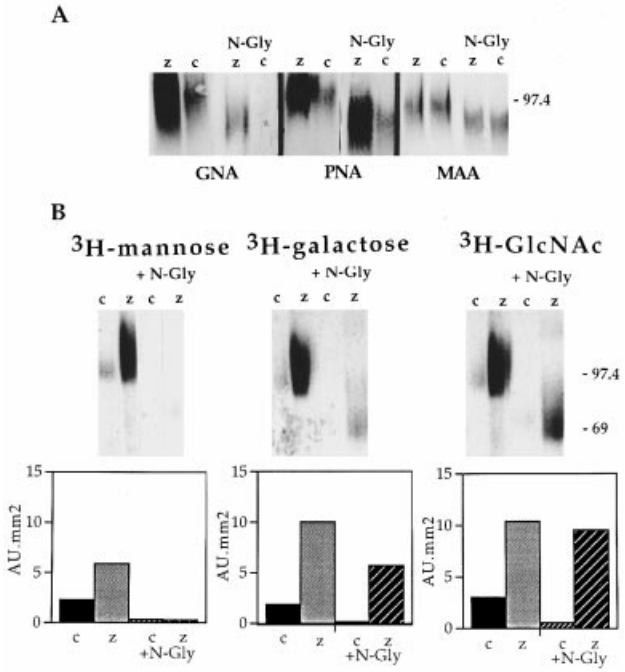

Figure 5 [^{3}H]Galactose- and [^{3}H]GlcNAc-specific labelling, and PNA and MAA reactivity, are resistant to N-Glycanase digestion

(**A**) Western blot analysis of the PG (z) and the CG (c) of macrosialin with and without N-Glycanase (N-gly) digestion. Blots were probed with lectins GNA, PNA and MAA as indicated. (**B**) SDS/PAGE analysis and fluorography of immunoprecipitated macrosialin from BMØ labelled with [^{3}H]mannose, [^{3}H]galactose or [^{3}H]GlcNAc as indicated. Graphs immediately below each gel represent densitometric readings of bands shown in each lane of the gels. AU (absorbance units) = (absorbance of band) × (area of peak in densitometric plot).

panel of lectins. Table 1 shows a list of the lectins used in our experiments and their specificities. The results obtained confirmed and extended our observations. Figure 4 shows an experiment in which the effects of *Galanthus nivalis* agglutinin (GNA), peanut agglutinin (PNA), *Amaranthus caudatus* agglutinin (ACA), *Datura stramonium* agglutinin (DSA), *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA) were tested. The digoxigenin-labelled FA-11 monoclonal antibody was used as a positive control to reveal total protein loaded. A consistent and distinctive pattern of exclusive, increased or diminished lectin binding emerged, whereas the reactivity developed for both the PG and the CG using digoxigenin-labelled FA-11 monoclonal antibody as probe remained unchanged (Figure 4). After phagocytosis, binding of GNA and PNA to the PG was increased. ACA bound exclusively to the PG, albeit weakly. MAA binding decreased after phagocytosis, and no specific binding of SNA was detected to either the PG or the CG. DSA, a Galβ(1-4)-GlcNAc- or GlcNAc-detecting lectin, bound very weakly but without specificity to macrosialin, and, if anything, bound less to phagocytosing MØ. This finding is at odds with our previous observations of polyacetyl-lactosaminoglycans on macrosialin [1] and the substantial increase in [^{3}H]GlcNAc incorporation into the PG shown in Figure 3. Presumably, GlcNAc newly transferred to the PG is in polyacetyl-lactosamine configurations that do not react with DSA. We did not detect specific binding of *Aleuria aurantia* agglutinin to either glycoform (results not shown), despite increased incorporation of [^{3}H]fucose in the PG. These data suggest that, after phagocytic uptake, macrosialin is expressed as a glycoform that contains altered O-linked chains, with increased expression of, or more exposed, Galβ(1-3)GalNAc structures, as shown by increased PNA and ACA reactivity. Sialylation of both glycoforms seems to be in an α(2-3) configuration with less capping of glycans in the PG, as demonstrated by the pattern of MAA binding and the absence of SNA reactivity. Additionally, there seems to be augmented retention of mannosylated oligosaccharides, as suggested by the pattern of GNA binding.

We extended these observations in experiments combining metabolic labelling, lectin probing and digestion of the macrosialin CG and PG by endoglycosidases. Figure 5(A) shows an experiment in which immunoprecipitated macrosialin was digested with peptide N-glycosidase F (N-Glycanase) and then probed with the lectins GNA, PNA and MAA. After N-Glycanase treatment, bands representing macrosialin migrated faster during SDS/PAGE, as expected. Not surprisingly, since no mannosylated O-linked oligosaccharide has ever been found in mammalian cells, N-Glycanase digestion of the glycoforms markedly reduced GNA reactivity. The faint reactivity remaining is probably due to incomplete digestion. However, this digestion made no difference to PNA or MAA binding (Figure 5A). Moreover, N-Glycanase removed the [^{3}H]mannose label from macrosialin, but preserved [^{3}H]galactose and [^{3}H]GlcNAc labelling (Figure 5B), showing that these sugars or their metabolites are transferred predominantly into O-linked glycans.

In the case of O-linked glycans, the resistance of PNA reactivity to N-Glycanase digestion is consistent with the fact that this lectin is highly specific in detecting O-linked Galβ(1-3)GalNAc (core I oligosaccharide). However, since PNA reactivity is blocked by sialic acid capping, the data presented so far do not distinguish between increased Galβ(1-3)GalNAc acquisition and diminished sialylation during processing of the PG. The latter would be a more attractive possibility, since we observed less MAA binding to macrosialin after phagocytic activation. However, in disagreement with this rationale, we also found increased binding of ACA, which can bind core I oligosaccharide even after sialic acid capping. To clarify this point, we carried out *Vibrio cholerae* sialidase digestion of immunoprecipitated macrosialin and probed both the CG and the PG with PNA and MAA. As shown in Figure 6, sialidase digestion did reveal cryptic epitopes for PNA binding in the CG, but after complete sialic acid digestion (as proved by the abrogation of MAA reactivity), the PG showed stronger reactivity with PNA, albeit to a small extent.

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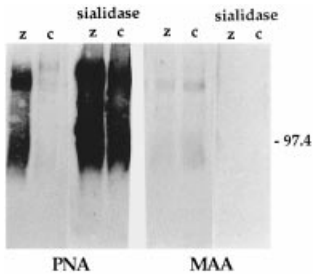

Figure 6 PG of macrosialin shows enhanced PNA reactivity even after sialidase digestion

Western blot analysis of immunoprecipitated macrosialin 16 h after zymosan (z) phagocytosis. c indicates macrosialin from control BMØ (no phagocytosis). Protein was digested with *Vibrio cholerae* sialidase and probed with lectins PNA and MAA as indicated. Positions of molecular mass markers are shown (kDa). The blot shown is representative of three independent experiments.

The small difference in PNA reactivity between the CG and the PG after sialidase digestion is in apparent contradiction with the magnitude of the difference found in radiolabelling experiments (Figures 3 and 5). This might be explained by the fact that, in order to reveal all bands probed with PNA, the sialidase-digested bands (which contain much more PNA reactivity) were always overdeveloped; this might have obscured some of the difference between the glycoforms. Since radiolabelling experiments are more reliable for obtaining quantitative information, and given the increase in ACA reactivity in the PG before sialidase digestion, our results are compatible with increased galactosylation in the PG.

These data, taken together with the increased incorporation of [^{3}H]GlcNAc and diminished sialylation of the O-linked glycans of the PG, argue for a switch to core II O-linked oligosaccharide, whereby Galβ(1-3)GalNAc would be exposed for PNA binding and GlcNAc would be incorporated in the glycans, thereby diminishing sialic acid capping.

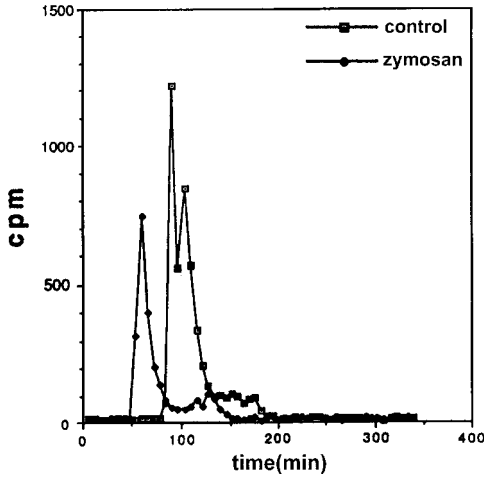

Figure 7 β-Elimination of O-linked glycans shows that the PG chains are elongated relative to those of the CG

Shown is the radioactivity in [^{3}H]galactose-labelled O-linked glycans in fractions eluted from a P4 Biogel fractionation column. O-linked glycans were hydrolysed by β-elimination (see the Materials and methods section). The void column volume is not shown. The figure is representative of two independent experiments.

We also probed O-linked chain extension by sizing CG and PG O-linked glycans on a P4 Biogel fractionation column. Glycans were hydrolysed by β-elimination (see the Materials and methods section), since our attempts to use endo-α-*N*-acetylgalactosaminidase digestion were not successful even after prior removal of sialic acids by sialidases. Interestingly, we found that PG O-glycans were eluted considerably faster, with a higher proportion of the material running as a single, more homogeneous peak. CG O-linked glycans ran more slowly and were eluted in several peaks. The results of a representative experiment are shown in Figure 7, and suggest that phagocytic activation leads to further elongation of O-linked chains, which in turn become more homogeneous in size.

DISCUSSION

The results presented here show that uptake via phagocytic receptors is a potent stimulus for alternative glycosylation of macrosialin in MØ. Other forms of endocytosis, receptor-mediated or not, fail to produce any changes in glycan acquisition. Phagocytosis of latex beads was also a relatively inefficient stimulant, which could be due to the lack of a specific receptor engagement or the engagement of a non-activating receptor. We have now tested all known MØ phagocytic receptors and found striking differences in their ability to mediate activation of alternative glycosylation (R. P. da Silva and S. Gordon, unpublished work). Not surprisingly, PMA, which produces an activation pattern similar to that of phagocytosis, was also a potent inducer.

Phagocytosis involves a complex series of events resulting in particle internalization, and formation and maturation of the phagosome. Cytochalasin B, an inhibitor of phagocytosis [26], but not of receptor binding or clathrin-mediated endocytosis, blocked the stimulatory signal resulting in glycoform modification (results not shown). Cytochalasin B disrupts actin-based filaments that are necessary to form the phagocytic cup, and subsequent engulfment [26,27], suggesting that processes beyond receptor binding, involving elements present in the phagosomal coat, are required to trigger the full-magnitude signal. These elements could be bound by sequences in the cytoplasmic tails of MØ receptors specifically engaged during phagocytic uptake, which differ from those involved in the endocytosis of soluble ligands [28,29]. This could explain the unique properties of phagocytic activation as a major stimulant of alternative glycosylation in MØ.

This study explores structural differences between the CG and the PG of macrosialin. Taken together, our data indicate that changes occurring after phagocytosis include an increase in newly transferred GlcNAc residues and greater expression of, or the presence of more accessible, Galβ(1-3)GalNAc structures (demonstrated by increased binding of the core I-specific lectins PNA and ACA) in the O-linked glycans of the PG. Additionally, our results reveal that, in either glycoform, sialic acid is transferred mostly in the α(2-3) configuration, as demonstrated by the strong binding of MAA, but not of SNA, to the proteins. After phagocytosis, however, sialylation of the PG is decreased, since we detected less MAA binding to this glycoform, showing that the PG has a higher proportion of glycans uncapped by sialic acid. These findings are suggestive of a switch to the expression of core II O-linked oligosaccharides, similar to the structural changes described for leukosialin after T-cell activation [10] (Figure 8).

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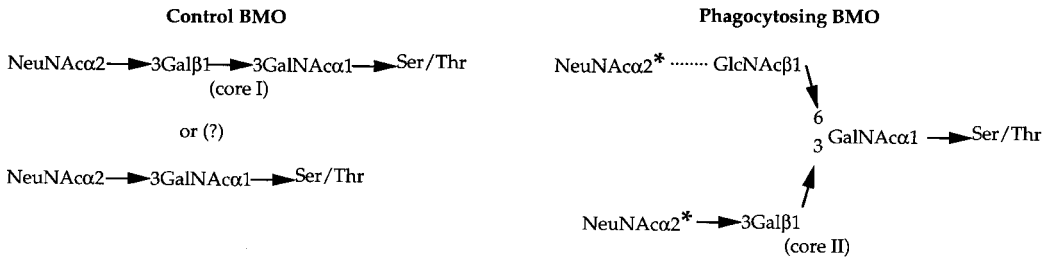

Figure 8 Changes in O-linked core glycans on macrosialin after phagocytosis

Alterations in O-linked glycans on macrosialin*, as described in the text, are consistent with a switch from core I to core II oligosaccharides after phagocytosis, promoting increased complexity and elongation of O-linked chains in the PG. Sialic acid capping in the PG is decreased, as shown by the levels of PNA and MAA binding to this glycoform.

The precise mechanisms responsible for these changes are at present unclear. Our experiments point to changes in the biosynthetic pathway rather than intra-phagosomal degradation, since we detected increases in the metabolic incorporation of several labelled sugars for the mature and precursor proteins. Moreover, our β-elimination experiments showed that the PG O-linked chains had been elongated. Only one transcript and one precursor protein have ever been detected during the synthesis of macrosialin, thus excluding differential splicing of RNA transcripts as the underlying mechanism. Changes in the rate of protein transport through the Golgi apparatus, which have been implicated in the differential glycosylation of LAMPs in cell lines [30], or the specific activation of glycosyltransferases are likely mechanisms.

One possibility is that, in the non-phagocytic cells, β-1,3-galactosyltransferase activity is limited, and some sialic acid might therefore be transferred directly to GalNAc serine/threonine residues. NeuAcα2-6GalNAcαSer/Thr is a common structure in colon carcinoma cells, for example [31]; MØ may contain an α-2,3-sialyltransferase with similar capacity. After phagocytosis, β-1,3-galactosyltransferase may be fully activated and out-compete the α-2,3-sialyltransferase in using GalNAc as a substrate. Activation of other glycosyltransferases might also use the core I oligosaccharide, giving rise to core II structures and further chain elongation by addition of GlcNAc to O-linked glycans (Figure 8). Diminution of α-2,3-sialyltransferase activity could lead to similar changes. Uncapped chains would expose suitable substrates to other glycosyltransferases. Decreased or no acquisition of sialic acid capping might also promote extension and increased heterogeneity in the backbone of PG O-linked chains.

Differential glycosylation of macrosialin has already been described during the inflammatory recruitment of peritoneal cells [1,3]. In TMØ, as opposed to resident MØ, macrosialin is differentially sialylated and decorated by poly-*N*-acetyl-lactosaminoglycan repeats [1,3]. The structural alterations described in the present study are associated exclusively with phagocytosis. Glycosylation of the CG in the BMØ, also an inflammatory cell, resembles that in the TMØ glycoform: the BMØ CG binds WGA strongly, is already sialylated and, as indicated by its susceptibility to endo-β-galactosidase digestion, contains lactosaminoglycan repeats as well. On the other hand, phagocytosis of zymosan by TMØ results in the same glycosylation pattern as we describe here for the BMØ PG (results not shown). We chose to study BMØ since these cells, unlike TMØ, have undergone no previous endocytic activation during inflammatory recruitment, as judged by the absence of lysosomal residues.

In vivo, we have also detected considerable variation in glycoform expression by MØ in different tissues. MØ in spleen, bone marrow and thymus, which are known to be actively phagocytic, express the fully altered, heavily sialylated glycoform corresponding to the PG described here (R. P. da Silva and S. Gordon, unpublished work). These observations support the view that phagocytic uptake is fundamental in regulating glycosylation of macrosialin *in vivo* as well.

Macrosialin antigen, which is expressed mainly in early and late endosomes [3], has been detected in all phagosomes analysed to date (R. P. da Silva, unpublished work). Glycan remodelling might provide a possible mechanism to protect the phagosomal membrane from the highly degradative intravacuolar milieu. While endocytosis of soluble, single-component ligands will activate endocytic function to a certain extent, engulfment of large, complex particles will require greater amounts of various lysosomal enzymes to be delivered to the phagosome for efficient digestion. Macrosialin, with its novel mucin domain, might provide such a protective function. Mucins have O-linked glycans attached in high density, as clusters, and are very heterogeneous, characteristics which make them difficult to digest [32,33]. Previous work by other groups has shown that LAMP glycans provide a barrier against parasitic proteases needed for degradation of the vacuole wall and escape into the cytosol [34].

Macrosialin has been reported to specifically bind oxidized low-density lipoproteins [6,7]. Interpretation of these data is complicated by the fact that macrosialin is predominantly expressed intracellularly [3], and thus a plasma membrane receptor function is unlikely. It is also unclear whether differential glycosylation is related to its putative receptor functions.

Our future experiments will include sequencing of oligosaccharide structures and assaying various glycosyltransferase activities in order to understand the processes involved in the differential glycosylation of macrosialin. We also aim to explore a possible role for this protein in maintaining phagosomal integrity or as a receptor by genetic deletion of the expression of macrosialin in cell lines or in the whole animal.

This work was supported by grants from The Wellcome Trust and the Medical Research Council. We thank Jim Mahoney, Fran Platt and Terry Butters for critical reading of the manuscript, and Nick Platt for helpful discussion of the data. We also thank Dr. A. Bernard (Institute of Molecular Biology, Glaxo Wellcome Laboratories, Geneva, Switzerland) for production of the FA-11 monoclonal antibody.

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Received 1 July 1998/11 November 1998; accepted 22 December 1998

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