A Novel Anionic Modification of *N*-Glycans on Mammalian Endothelial Cells Is Recognized by Activated Neutrophils and Modulates Acute Inflammatory Responses[1]

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We previously reported an unusual carboxylated modification on *N*-glycans isolated from whole bovine lung. We have now raised IgG mAbs against the modification by immunization with biotinylated aminopyridine-derivatized glycans enriched for the anionic species and screening for Abs whose reactivities were abrogated by carboxylate neutralization of bovine lung glycopeptides. One such Ab (mAb GB3.1) was inhibited by carboxylated bovine lung glycopeptides and other multicarboxylated molecules, but not by glycopeptides in which the carboxylate groups were modified. The Ab recognized an epitope constitutively expressed on bovine, human, and other mammalian endothelial cells. Stimulated, but not resting, neutrophils bound to immobilized bovine lung glycopeptides in a carboxylate-dependent manner. The binding of activated neutrophils to immobilized bovine lung glycopeptides was inhibited both by mAb GB3.1 and by soluble glycopeptides in a carboxylate-dependent manner. The Ab also inhibited extravasation of neutrophils and monocytes in a murine model of peritoneal inflammation. This inhibition of cell trafficking correlated with the increased sequestration but reduced transmigration of leukocytes that were found to be adherent to the endothelium of the mesenteric microvasculature. Taken together, these results indicate that these novel carboxylated *N*-glycans are constitutively expressed on vascular endothelium and participate in acute inflammatory responses by interaction with activated neutrophils. *The Journal of Immunology,* **2001, 166: 624–632.**

In an earlier report (1) we presented several lines of evidence for the presence of a novel carboxylate-containing group on *N*-linked oligosaccharides (*N*-glycans) from bovine lung. The elucidation of the precise structure of this modification has proven difficult and is the subject of ongoing efforts. Meanwhile, to explore the distribution of this novel modification and its biological roles, we attempted to generate mAbs directed against it. Carbohydrate-specific mAbs often tend to be low affinity IgM reagents that can show significant cross-reactivities among various glycans (2–4) and are technically difficult to purify and stabilize. IgG mAbs with increased specificity and sensitivity would be more advantageous for in situ localization in tissues and for use in a variety of assays. We previously noted that glycans conjugated to biotinylated diaminopyridine (BAP)[5] and presented in multivalent arrays on streptavidin can evoke an IgG immune response in mice (5, 6). Taking advantage of this concept, we have now been able to generate high affinity IgG mAbs directed against the carboxylate-containing epitope from bovine lung glycans. Using one of these Abs we demonstrate that this anionic modification is highly enriched in vascular endothelial cells, and participates in acute inflammatory responses by interacting specifically with activated neutrophils.

Materials and Methods

Materials

BAP was prepared as described previously (5, 6). Recombinant streptavidin, bovine lung acetone powder, glutaraldehyde, Ig isotyping kit, protein G-Sepharose, platelet-activating factor (PAF), PMA, TNF-α, anti-human platelet-endothelial cell adhesion molecule (PECAM; CD31), monoclonal anti-BSA, R-PE-conjugated anti-mouse IgG, exoglycosidases, and zymosan were obtained from Sigma (St. Louis, MO). The following materials were obtained from the indicated sources: ionomycin, Calbiochem (La Jolla, CA); peptide-*N*-glycosidase (PNGase) F, New England Biolabs (Beverly, MA); PNGase A, a gift from Seikagaku America (Falmouth, MA); proteinase K, Roche (Indianapolis, IN); Bio-Gel P2 and peroxidase-linked goat anti-mouse IgG, Bio-Rad (Richmond, CA); 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), Pierce (Rockford, IL); alkaline phosphatase-conjugated goat anti-mouse IgG, Promega (Madison, WI); peroxidase-linked streptavidin, The Binding Site (San Diego, CA); Cy3 mAb labeling kit, Amersham Pharmacia (Arlington Heights, IL); Monopoly Resolving Medium, ICN Biomedicals (Aurora, OH); Nycoprep, Nycomed Pharma (Oslo, Norway); FITC anti-mouse Ly-6G (Gr-1) and PE anti-mouse CD11b (Mac-1), PharMingen (San Diego, CA); covalent binding plates, Xenopore (Hawthorne, NJ); bovine tissues, Pel-Freeze (Rogers, AR); and human tissue sections, Histology Core Facility of the Cancer Center, University of California (San Diego, CA). HUVECs were obtained from Cell Applications System (San Diego, CA), and calf pulmonary artery endothelial (CPAE) cells were obtained from American Type Culture Collection (Manassas, VA).

Preparation of neoglycoproteins for immunization and screening

Anionic bovine lung glycans of moderate negative charge coupled to BAP (D. K. Toomre et al., unpublished observations) were treated with *Arthrobacter ureafaciens* sialidase (10 mU), jack bean β-*N*-acetylhexosaminidase (53 mU), bovine testicular β-galactosidase (2 mU), coffee

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[5] Abbreviations used in this paper: BAP, biotinylated diaminopyridine; CPAE, calf pulmonary artery endothelial; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; PAF, platelet-activating factor; PNGase, peptide-*N*-glycosidase; PECAM, platelet-endothelial cell adhesion molecule.

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bean α-galactosidase (5 mU), and jack bean α-mannosidase (2–5 mU) in ~20 μl of sodium citrate buffer (pH 4.5). These multiple exoglycosidases were inactivated by heating at 100°C for 5–10 min, and the sample was fractionated on a DEAE-2SW HPLC column as previously described (6). Specific subfractions from the DEAE column were also analyzed by reverse phase HPLC as previously described (6). The mixed BAP-coupled oligosaccharides enriched for the carboxylate residue (see Fig. 1) were mixed with streptavidin in PBS at a ratio of 1:3, and the mixture was kept at 4°C for 1 h before immunization of mice. To generate BSA neoglycoproteins for screening, glycopeptides were prepared from bovine lung acetone powder by proteinase K digestion followed by purification on a Bio-Gel P2 column run in 0.1 M ammonium formate, pH 6.5. Glycopeptides eluting in the void volume were conjugated to BSA using either glutaraldehyde or EDC following standard protocols. The BSA conjugates (henceforth referred to as BSA neoglycoproteins for the glutaraldehyde-coupled glycopeptides, unless otherwise stated) were analyzed by PAGE gels and by neutral sugar estimation (phenol sulfuric acid assay), which indicated an average of 2.5 mol of *N*-glycans coupled/mol of protein (assuming a typical biantennary *N*-glycan; data not shown).

Modification of carboxylate groups by carbodiimide activation and reaction with methylamine

Glycopeptides generated from bovine lung acetone powder as described above were desialylated by mild acid treatment (10 mM HCl, 30 min at 100°C) and lyophilized. (These glycopeptides are henceforth referred to as asialo-COO^--glycopeptides). Five hundred nanomoles of such glycopeptides (by neutral sugar estimation) were dissolved in 50 μl of 50 mM MES (2-[*N*-morpholino]ethanesulfonic acid) buffer, pH 5.5, followed by addition of 100 μl of 1 M methylamine. Fifty microliters of EDC/NHS from a freshly prepared stock solution of 100 mg of EDC and 50 mg of NHS/ml water was added and incubated at 37°C. After 1 h, another 50 μl of fresh EDC/NHS was added, and the incubation was continued for another 2 h. Control glycopeptides were treated identically, except that EDC/NHS solution was replaced with water. These carboxylate-neutralized glycopeptides (henceforth referred to as asialo-CONHMe-glycopeptides) were then dialyzed against water overnight to remove excess methylamine, and coupling to BSA was conducted as described above. Carboxylate neutralization using EDC-NHS/methylamine caused about 70–80% of the negative charges to be masked as determined by QAE-Sephadex chromatography (data not shown).

Immunization procedures and hybridoma establishment

Two- to 4-wk-old female BALB/c mice were primed by s.c. injection of 10 μg of native streptavidin in CFA. Seven days later they were immunized with 10 μg of streptavidin neoglycoconjugates in IFA and then boosted i.p. with PBS at 2-wk intervals until significant titers of serum Abs against BSA neoglycoproteins were generated. Splenocytes from the mice were isolated and fused with Ag8.653 mouse myeloma cells using standard protocols. Hybridomas were cultured in DMEM (high glucose; Life Technologies, Gaithersburg, MD) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 20% FBS, and 100 μM hypoxanthine/16 μM thymidine. Screening was performed by ELISAs against bovine lung glycopeptides coupled to BSA, thus selecting for Abs directed against glycans and avoiding any against streptavidin and BAP. Seven highly reactive and 18 moderately reactive hybridomas were cloned by limiting dilution and subcloned as necessary. They were further screened for differential reactivity against BSA-coupled asialo-COO^--glycopeptides and asialo-CONHMe-glycopeptides. Selected hybridoma clones/subclones were injected i.p. into BALB/c mice for the production of ascitic fluid, and IgGs were purified from culture supernatants or ascitic fluid on protein G-Sepharose. One of the clones (mAb GB3.1, Ig subclass IgG2b) was chosen for further characterization, because it showed a marked difference in reactivity between asialo-COO^--glycopeptides and asialo-CONHMe-glycopeptides, and functioned well in both ELISAs and immunoblots.

ELISAs

Ninety-six-well plates were coated with 250 ng of BSA neoglycoproteins for 6 h at room temperature or 4 h at 37°C. Plates were washed and blocked with 3% BSA in PBS overnight and incubated with hybridoma culture supernatants or purified IgG diluted in PBS containing 1% BSA and 0.05% Tween-20. Incubations were performed for 1–2 h at room temperature or overnight at 4°C. The plates were then washed and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG, followed by development with *p*-nitrophenyl phosphate substrate. Absorbance was measured at 405 nm in an ELISA plate reader.

Deglycosylation of papain-released bovine lung glycopeptides with PNGase A/PNGase F

Bovine lung acetone powder was digested with papain in 0.1 M sodium acetate, pH 5.5, in the presence of 5 mM L-cysteine and 1 mM EDTA at 60°C for 48 h. The mixture was heat inactivated, centrifuged at 10,000 × *g* to remove undigested residue, and dialyzed using 3000 m.w. cut-off tubing. Glycopeptides (~100 nmol, as determined by neutral sugar quantitation using phenol-sulfuric acid) were digested with PNGase A in 0.1 M sodium acetate or 0.1 M citrate phosphate, pH 5.5, in the ratio of 2 μU/nmol of glycopeptide or with PNGase F in 50 mM Tris, pH 8.0, for 16 h at 37°C, both in duplicate. Control tubes had no added enzyme. After digestion, the mixtures were heat inactivated. The glycopeptides with or without treatments were coated onto covalent binding plates, and an ELISA against mAb GB3.1 was conducted as described above.

Immunostaining of cells and tissues

Tissue sections were fixed in 10% buffered formalin for 20 min, followed by blocking of nonspecific binding sites with 10% normal goat serum in PBS containing 1% BSA. After washing, Abs were overlaid onto serial tissue sections at predetermined dilutions (usually between 1 and 10 μg/ml), and the slides were incubated in a humid atmosphere for 30 min at room temperature or overnight at 4°C. The labeled streptavidin-biotin kit (Dako, Carpenteria, CA) was used following the manufacturer's instructions with PBS or Tris-buffered saline washes between every step. Biotinylated anti-mouse IgG was applied for 10 min, followed by either alkaline phosphatase or peroxidase-linked streptavidin for 10 min. After another wash, the appropriate substrate was added, and the slides were incubated in the dark for 20 min. After a wash in buffer, slides were counterstained with hematoxylin, mounted, and viewed using an Olympus BH2 microscope (New Hyde Park, NY).

CPAE or HUVEC cells for cell surface staining were grown on Lab-Tek Chamber glass slides (Nunc, Naperville, IL), washed with PBS, and fixed in 10% buffered formalin for 20 min. The slides were blocked with 3% BSA in PBS for 20 min and then incubated with 10 μg/ml of one of the following Cy3-labeled Abs, labeled according to the manufacturer's instructions: purified mAb GB3.1 or anti-human CD31 (positive control for endothelial cell staining) or CAB4 (negative control) (7). After overnight incubation at 4°C, the slides were washed and observed using epifluorescence microscopy.

Western blot analysis

Tissue or cell proteins were subjected to SDS-PAGE in 12% polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked overnight with 10% skimmed milk or 3% BSA in PBS, washed with TBS containing 0.05% Tween 20, and incubated for 1–2 h at room temperature with Ab from hybridoma culture supernatants (or purified IgG) diluted in PBS containing 1% BSA and 0.05% Tween 20. Membranes were then reacted with alkaline phosphatase-conjugated goat anti-mouse IgG, and reactive proteins were visualized by incubation with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate.

Isolation and activation of human peripheral blood leukocytes

Human neutrophils were isolated from fresh peripheral (EDTA-anticoagulated) blood of healthy volunteers by differential migration through monopoly resolving medium (Ficoll-Hypaque; density, 1.114) followed by hypotonic lysis of residual erythrocytes. The preparations were >98% granulocytes as confirmed using Turk's staining (0.01% crystal violet in 3% acetic acid). Cells were resuspended at 5×10^6 neutrophils/ml in HBSS buffer containing 0.2% BSA and were used within 30 min after isolation for adhesion assays. For flow cytometric assays, mononuclear cells from the above separation were further fractionated into monocytes and lymphocytes using Nycoprep medium (cells ~80% pure). For activation, cells were resuspended at 3×10^6/ml in 10 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, and 5.5 mM glucose and were incubated in the presence or the absence of 1 μM PAF (neutrophils) or 100 ng/ml PMA/1 μM ionomycin (neutrophils and mononuclear cells) for 15 min at 37°C. Cells were pelleted at 300 × *g* for 10 min and analyzed as described below.

Flow cytometric assays

Activated and unactivated peripheral blood leukocyte populations were incubated with 50 μg/ml BSA-conjugated asialo-COO^--glycopeptides or asialo-CONHMe-glycopeptides in HBSS buffer for 30 min on ice, followed by mouse monoclonal anti-BSA and R-PE-conjugated anti-mouse

IgG, each for similar lengths of time on ice and with two washes in between. Cells stained with second- and third-stage reagents only or with BSA followed by second- and third-stage reagents, were used as negative controls. They were analyzed by flow cytometry employing a FACScan (Becton Dickinson, Mountain View, CA) equipped with CellQuest software and were gated by side scatter and forward scatter filters.

Neutrophil adhesion assays with immobilized carboxylated glycans

Ninety-six-well microtiter plates were coated with 250 ng/well of BSA-coupled bovine lung asialo-COO^--glycopeptides for 4 h at 37°C. Plates were washed and blocked with 3% BSA in PBS overnight. Control wells were coated with BSA alone. Neutrophils (0.5×10^6) in 100 μl of HBSS buffer containing 0.2% BSA and 1 μM PAF were added to each well and incubated for 30 min at 37°C in the presence or the absence of varying concentrations of mAb GB3.1, control Ab, asialo-COO^-, or asialo-CONHMe-glycopeptides in a total volume of 200 μl. PAF was excluded from the incubation mixture in some wells. Unbound cells were removed by flicking out the plates and washing five times with HBSS. Bound cells were quantitated by assaying myeloperoxidase. Briefly, adherent cells were solubilized in 100 μl of 1% Triton X-100 and incubated with 25 μl of 0.5% hydrogen peroxide and 25 μl of 4.7 mM orthodianisidine for 30 min at room temperature. The reaction was stopped with 0.4% sodium azide, and absorbance was read at 450 nm. Assays were performed in duplicate.

Neutrophil adhesion to endothelial cells

HUVECs were grown to confluence in endothelial cell growth medium (Cell Applications, San Diego, CA) at 37°C in 5% CO_2. Cells at passages 2–3 that were positive for cell staining and Western blotting with mAb GB3.1 were used in the adhesion assays. At confluence, HUVECs were detached and seeded on fibronectin (1 $\mu g/cm^2$)-coated 96-well culture wells. Confluent EC monolayers were treated with medium containing 1 μM PAF for 4 h at 37°C and washed. In some experiments unstimulated endothelial cells were also tested. Neutrophils isolated and suspended in adhesion buffer as described above were added and incubated under static conditions in the presence of PAF for 30 min at 37°C in 5% CO_2 in the presence or the absence of modifiers. Nonadherent cells were removed by washing, and adherent cells were quantitated using myeloperoxidase assay as described above.

Zymosan-induced acute peritoneal inflammation

Female (BALB/c) mice (Harlan Sprague Dawley, Indianapolis, IN; ~16–18 g body weight) were maintained on a standard chow pellet diet with tap water ad libitum and were used 2–3 days after arrival. The experimental protocols followed the criteria of the institutional animal care and use committee at the Burnham Institute (La Jolla, CA). Peritonitis was induced by injection of 1 mg of zymosan i.p. in 0.5 ml of PBS. Animals were injected i.v. with saline, mAb GB3.1, or isotype control mouse IgG at the time of zymosan injection. Mice were euthanized at different time points, and peritoneal cavities were lavaged with RPMI medium containing 2% FBS, 3 mM EDTA, and 25 U/ml heparin. Lavage fluids were centrifuged, aliquots of cells were stained with Turk's solution, and differential cell counts were performed with a Neubauer hemocytometer. In parallel experiments, the peritoneal exudate cells were also immunostained with either FITC-labeled anti mouse Gr-1 (Ly6G) or PE-labeled anti-mouse Mac-1 for 20–30 min on ice, washed, and analyzed by flow cytometry. The mesenteries obtained from these animals were fixed in 10% buffered formalin, embedded in paraffin, stained with hematoxylin and eosin, and evaluated by brightfield microscopy using a Nikon microscope (Melville, NY). Digital images were captured on a CCD camera at different magnifications and rendered with Adobe Photoshop (Abacus Concepts, Berkeley, CA).

Results and Discussion

Preparation of the immunogen

We had previously shown that BAP-conjugated glycans presented in multivalent arrays on streptavidin can evoke an IgG serum immune response in mice (5, 6). To generate Abs directed against the novel carboxylate-associated negative charge, a mixture of anionic hydrazine-released bovine lung glycans was coupled to BAP (D. K. Toomre and A. Varki, unpublished observations, details to be reported elsewhere). To enrich for coupled glycans bearing the novel carboxylate, we applied multiple exoglycosidase treatment to molecules with more than two negative charges that had been obtained by anion exchange chromatography on DEAE-HPLC. The exoglycosidases chosen (see *Materials and Methods*) were highly specific and did not affect the negative charge of the carboxylate (data not shown). Following the mixed exoglycosidase treatment, we collected the fractions that shifted to an elution position on DEAE-HPLC that is typical for glycans with a single negative charge (Fig. 1*A*, pool II). Because sialidase was included in the digestions, the mixture was expected to be enriched for molecules carrying one copy each of the novel carboxylate modification. Indeed, this material eluted from reverse phase HPLC (Fig. 1*B*) in the general region expected for BAP-coupled *N*-glycans with one or two negative charges, such as mono- or bisialylated biantennary *N*-glycans. This mixture of BAP-coupled glycans was bound to streptavidin to form multivalent pseudoneoglycoproteins. Injection of these complexes into mice elicited a serum IgG response that was at least partly directed against the oligosaccharides, as detected by reactivity in ELISA with BSA-coupled bovine lung glycopeptides or whole bovine lung homogenates (data not shown).

Monoclonal Abs that recognize the novel carboxylated epitope on bovine lung glycans

We screened specifically for IgG secreting hybridomas that reacted with total bovine lung glycopeptides coupled to BSA. Because the original immunogen contained only bovine glycans and no peptides, this strategy selectively detected only Abs directed against the oligosaccharides, while avoiding detection of Abs directed against streptavidin or BAP. Early on, we noted that some of the

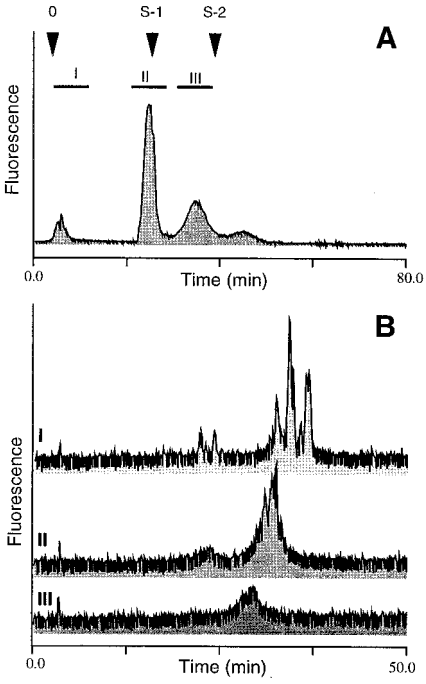

FIGURE 1. Preparation of a mixed BAP-coupled oligosaccharide Ag enriched for the novel carboxylate residue. BAP-coupled anionic bovine lung glycans of moderate negative charge (D. K. Toomre et al., unpublished observations) were treated with multiple exoglycosidases, the enzymes were inactivated as described in *Materials and Methods*, and the sample was refractionated on a DEAE-2SW HPLC column (*A*). Arrowheads indicate the elution positions of standard neutral glycans (0) and glycans containing one sulfate (S-1) or two sulfates (S-2). Pools I, II, and II were collected, and aliquots were analyzed by reverse phase HPLC (*B*). Pool II was used as the Ag for immunization.

Abs reacted differentially depending on whether the original coupling of the glycopeptides to BSA was performed with glutaraldehyde (which reacts with amino groups) or carbodiimide (which reacts with carboxyl groups). We reasoned that the decreased reactivity with carbodiimide-coupled glycopeptides might result from a carbodiimide-induced modification of the novel carboxylate on the glycans. In fact, we found that binding of four of the most reactive Abs was also substantially abrogated by direct methylamidation of the carboxylates on the target glycopeptides (data not shown). Of these, we chose to focus on mAb GB3.1, of the IgG2b subclass, because it gave the best reactivity in blots and ELISAs.

Characterization of mAb GB3.1 specificity

mAb GB3.1 binding to immobilized BSA neoglycoproteins could be blocked by asialo-COO^--glycopeptides in solution, but not by asialo-CONHMe-glycopeptides (Fig. 2*A*). We further checked the consequences of protonation of negative charges on binding by studying reactivity at different pH values. As shown in Fig. 2*B*, there was marked loss of reactivity below pH 6.0, whereas the reactivities of two control mAbs against their respective Ags were unaffected. Therefore, the pH dependence of mAb GB3.1 reactivity suggests either that the target carboxyl group has a pKa of ~5.8, or that the Ab binding site has a critical residue with a pKa in this range. Regardless, we pursued the possibility that the Ab reactivity might be blocked by other carboxylic acids. Indeed, as shown in Fig. 2*C*, reactivity is inhibited by low millimolar concentrations of several aliphatic carboxylate-containing compounds, such as acetate, succinate, citrate, isocitrate, and EDTA, roughly in proportion to the number of carboxyl groups on each (all compounds were tested at pH 7.5 where the respective carboxyl groups are ionized). In contrast, similar concentrations of glucuronic and galacturonic acids (Fig. 2*C*) or sialic, lactic, or pyruvic acids (data not shown) did not inhibit binding. Overall, it appears that molecules carrying multiple aliphatic carboxylate groups were the most effective inhibitors.

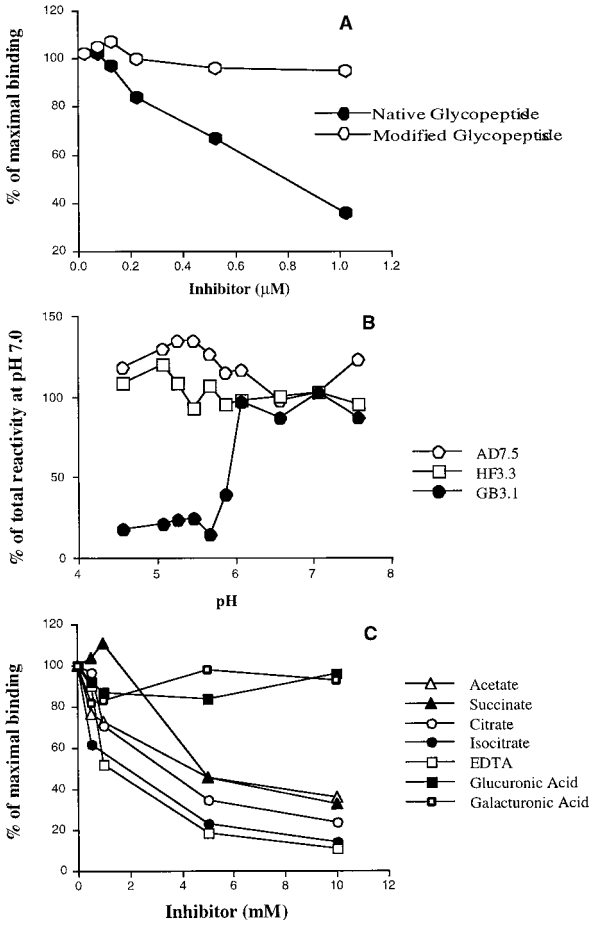

FIGURE 2. Characterization of mAb GB3.1 reactivity. Proteins were coated onto microtiter plates (5 μg/ml) and blocked with 3% BSA in PBS, and the binding of various Abs was studied. *A*, Wells with bovine lung proteins probed with mAb GB3.1 hybridoma supernatant (20 ng IgG/ml) in the presence or the absence of varying concentrations of bovine lung asialo-COO^- and asialo-CONHMe-glycopeptides. Binding in the absence of inhibitor was defined as 100%. *B*, Bovine lung proteins or *Dictyostelium discoideum* lysates probed with mAb GB3.1, AD7.5, or HF3.3 at different pH values as indicated. AD7.5 recognizes GlcNAc-1-P residues on *Dictyoselium* cysteine proteinases (9). HF3.3 is another Ab from the group of hybridomas isolated in the present study. Its reactivity with bovine lung proteins is unaffected by carboxylate neutralization. Binding at pH 7.0 was defined as 100% for each. *C*, Bovine lung proteins were probed with mAb GB3.1 hybridoma supernatant (20 ng IgG/ml) in the presence or the absence of varying concentrations of seven different carboxylate-containing compounds. Binding in the absence of inhibitor was defined as 100%.

Monoclonal Ab GB3.1 does not recognize other known anionic glycans

In other studies we had noted that a significant part of the sialidase-resistant negative charge in the bovine lung glycans can be explained by sulfate esters (D. K. Toomre and A. Varki, unpublished observations). These would not be neutralized by the methylamidation procedure. However, to verify that mAb GB3.1 did not cross-react with sulfated glycans, Western blots were conducted on thyroglobulin, which carries terminal Gal-3-sulfate and internal GlcNAc-6-sulfate, and on N-CAM, which is known to express the HNK-1 epitope carrying a terminal glucuronic acid-3-sulfate. mAb GB3.1 did not react with either of these glycoproteins. Chondrosamine, chondroitin sulfate (tetramer and octamer), and hyaluronic acid (tetramer and octamer) at 1–5 mM also do not inhibit binding, suggesting that glycosaminoglycan-like epitopes do not cross-react. Reactivity in the ELISA was unaffected by sulfated or phosphorylated sugars such as Glc-1-phosphate, Glc-6-phosphate, Man-6-phosphate, Gal-6-phosphate, GlcNAc-1-and GlcNAc-6-phosphates, Glc-6-sulfate, and Gal-6-sulfate (when tested at or below 5 mM; data not shown).

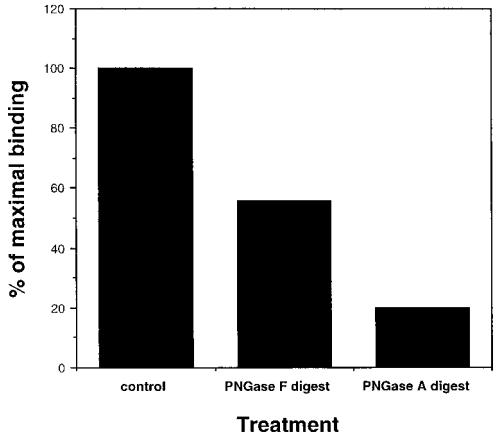

FIGURE 3. A major fraction of the mAb GB3.1-reactive epitope is carried on *N*-linked glycans, including PNGase F-resistant ones. Bovine lung glycopeptides were digested individually with PNGase F or PNGase A as described under *Materials and Methods*. Control glycopeptides (incubated in the absence of enzymes) and deglycosylated peptides were tested for their reactivity in ELISA against mAb GB3.1. Reactivity in control wells was defined as 100%.

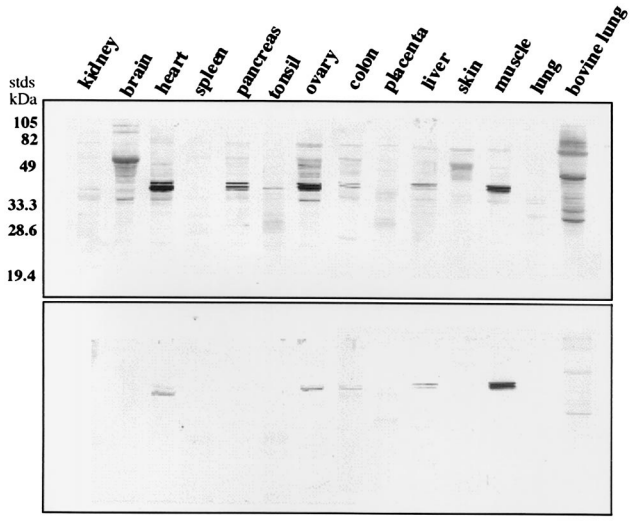

FIGURE 4. mAb GB3.1 recognizes proteins from several human tissues. Human tissues were homogenized, and 50 μg of protein from various extracts was separated on SDS-PAGE gels, blotted onto nitrocellulose membranes, blocked, and probed with mAb GB3.1 (400 ng/ml) as described in *Materials and Methods*. Ab incubation was performed in the absence (*top*) or the presence (*bottom*) of 100 μM total bovine lung glycopeptides. The blots were then developed with anti-mouse IgG-alkaline phosphatase conjugates and BCIP-NBT substrate.

A major fraction of the mAb GB3.1 reactive-epitope is carried on N*-linked glycans, including PNGase F-resistant ones*

Bovine lung papain-released glycopeptides were digested with PNGase F or PNGase A and tested for their reactivity in an ELISA against mAb GB3.1 before and after digestion. Intact glycoproteins are poor substrates for PNGase A, but both enzymes can act well on glycopeptides released by thermolysin, pepsin, trypsin, or papain (8). The deglycosylated peptides showed partial loss of Ab reactivity compared with undigested glycopeptides (incubated in the absence of enzymes). This loss of activity was greater with PNGase A digestion than with PNGase F (Fig. 3). This may be because some of the carboxylate epitopes are present on PNGase F-resistant oligosaccharides carrying unusual core modifications (G. Srikrishna and H. Freeze, unpublished observations).

The mAb GB3.1 epitope is found on a subset of proteins in many bovine, human, and other mammalian tissues

Western blots showed that several proteins from bovine lung and other bovine tissues reacted with mAb GB3.1. Reactivity was abrogated in the presence of lung asialo-COO^--glycopeptides, but not by asialo-CONHMe-glycopeptides (data not shown). There was also widespread reactivity in several human tissues (Fig. 4, *top*). Binding to a majority of bands, except for the 38-/40-kDa doublet bands in muscle tissue, was blocked by asialo-COO^--glycopeptides (Fig. 4, *bottom*). The somewhat lower staining seen with human lung compared with bovine lung was reproducible and may be due to relative species difference. Western blots of a variety of mouse and rat tissues were also positive, with almost all binding being blocked by asialo-COO^--glycopeptides (data not shown). This apparently widespread distribution of reactive epitopes is actually explained by the fact that they are concentrated on endothelial cells, which are, of course, present in all tissues (see below).

Ab reactivity is predominantly directed against endothelial cells

In frozen sections of bovine lung, the modification was localized mainly to the vascular endothelium, showing that the novel glycopeptides we had been studying from whole bovine lung had

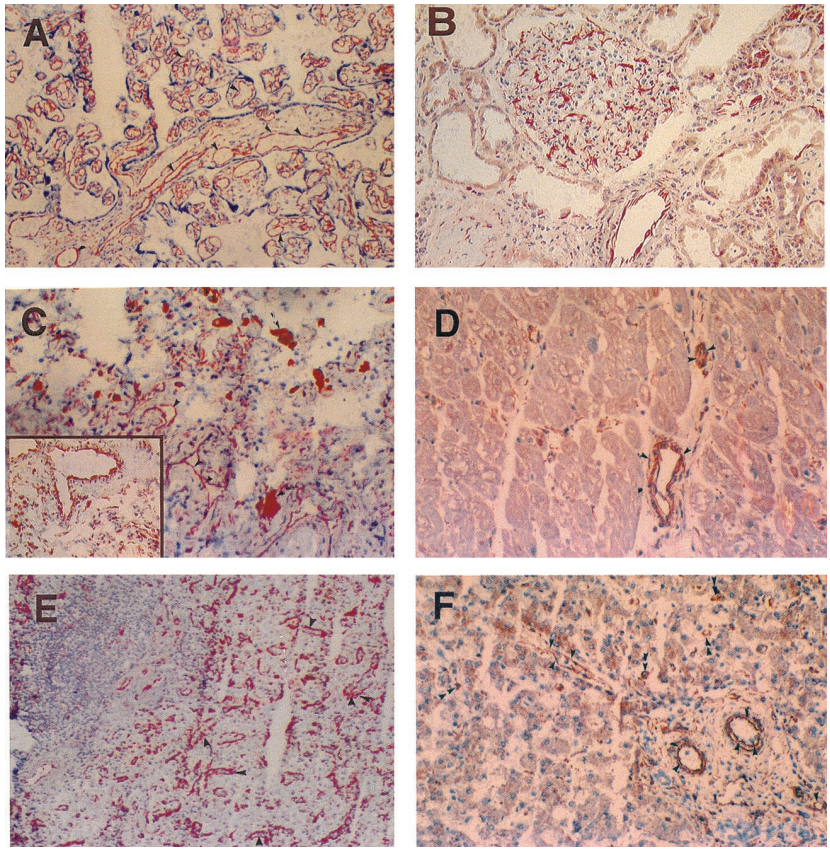

FIGURE 5. Expression of GB3.1-reactive epitopes in human tissues. Frozen sections of various human tissues were immunostained with mAb GB3.1 as described in *Materials and Methods*. The reddish-brown reaction product indicates prominent staining on vascular endothelium. *A*, Placenta; *B*, kidney; *C*, lung; *D*, heart; *E*, spleen; *F*, liver. The inset to *C* shows a higher magnification view of positively stained lung microvasculature.

primarily originated from the endothelium. Again, binding was almost completely blocked by asialo-COO$^-$-glycopeptides (data not shown), confirming the specificity of the interactions. A more extensive survey of various human tissues showed that Ab reactivity was again predominantly localized to the vascular endothelium of most blood vessels (see examples in Fig. 5). In the spleen and placenta almost every blood vessel was stained. At higher magnification of the skin vessels, it was evident that the endothelial cells were specifically stained (data not shown). The only positive nonendothelial cells were some lung macrophages. Staining of cultured human and bovine endothelial cells with Cy3-labeled mAb GB3.1 showed predominant localization of the epitope on the cell surface (see example in Fig. 6).

Neutrophils acquire binding sites for the novel endothelial glycans upon short term activation

Because most of the Ab reactivity was localized to endothelial cells, we reasoned that cognate lectins for these molecules might be found on peripheral blood leukocytes. Therefore, we probed freshly isolated human leukocyte populations (neutrophils, monocytes, and lymphocytes) with the BSA-coupled asialo-COO$^-$-glycopeptides (to enhance multivalency, which is frequently required for lectin-carbohydrate interactions). Desialylated glycopeptides were used to eliminate any sialic acid-dependent binding. We found a strong expression of cell surface binding sites for the novel glycans on the entire population of activated, but not resting, neutrophils within 15 min of activation with PAF or PMA/ionomycin (Fig. 7). The specificity of this binding was proven by selective binding of BSA-coupled asialo-COO$^-$-glycopeptides, but >80% reduction in the binding of BSA-coupled asialo-CONHMe-glycopeptides (Fig. 7). The residual binding of neutralized glycopeptides is probably explained by the fact that carboxylate neutralization by methylamidation does not achieve 100% completion even after two rounds of treatments. In addition to these surface binding sites, secretions elicited upon activation with other physiologically relevant inflammatory mediators such as TNF-α or fMLP contained proteins that bound to the novel glycans in a carboxylate-dependent manner (data not shown). Also, induction of binding sites on monocytes was observed under the same conditions, albeit to a lesser extent (data not shown).

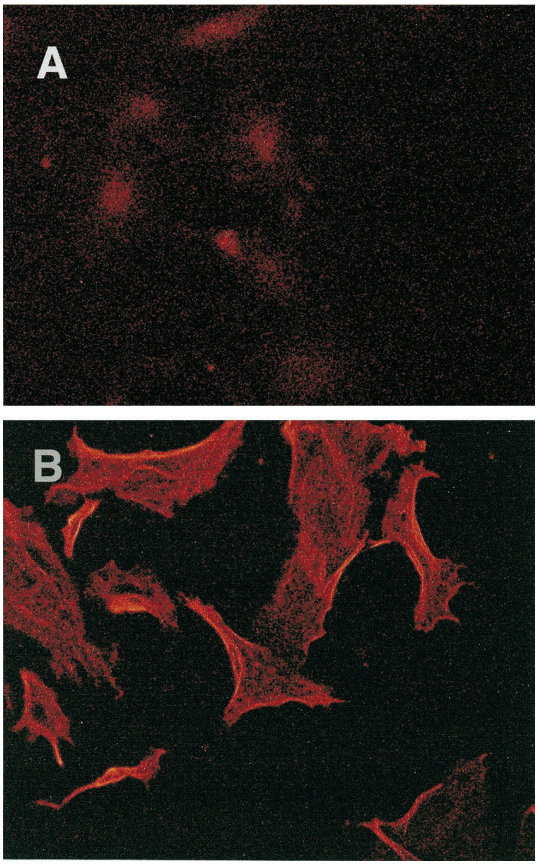

FIGURE 6. Expression of GB3.1-reactive epitopes on cultured endothelial cells. CPAE cells were grown on chamber glass slides, washed with PBS, and fixed in 10% buffered formalin for 20 min. The slides were blocked with 3% BSA in PBS for 20 min and then incubated with 10 μg/ml of one of the following Cy3 primary-labeled Abs as described in *Materials and Methods*: CAB4 (negative control; *A*) or purified mAb GB3.1 IgG (*B*). After overnight incubation at 4°C the slides were washed and observed using epifluorescence microscopy.

Immobilized glycans directly support the binding of activated human neutrophils

Because a mouse mAb had to be used to detect the BSA to which the glycopeptides were coupled, we could not test the effects of the mouse mAb GB3.1 in the above flow cytometric assay. To address this issue and to obtain further evidence for specific binding, we incubated freshly isolated neutrophils with BSA-conjugated asialo-COO$^-$-glycopeptides coated on plastic. As shown in Fig. 8, activated human neutrophils bound to the carboxylated glycans, while minimal binding was observed to BSA itself. Adhesion was completely inhibited by mAb GB3.1 at various concentrations, whereas a control anti-carbohydrate Ab did not have any effect. Binding was also progressively and completely blocked by adding soluble bovine lung asialo-COO$^-$-glycopeptides, but not by asialo-CONHMe-glycopeptides.

Effect of mAb GB3.1 on neutrophil interactions with endothelial monolayers

To explore the potential role of the novel glycans in neutrophil adherence to vascular endothelium, we measured neutrophil adhesion to HUVECs in the presence of various modulators. An isotype-specific control Ab did not have any effect. In contrast, increasing amounts of mAb GB3.1 enhanced adhesion by 2- to

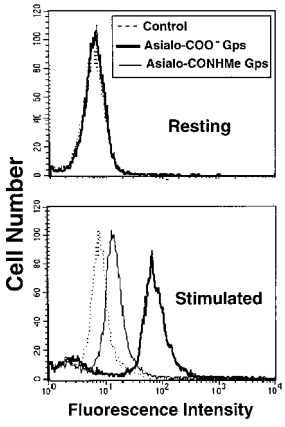

FIGURE 7. Analysis of neutrophils for carboxylated glycan binding sites. Freshly isolated neutrophils were analyzed with or without activation for 15 min with 100 ng of PMA and 1 μM ionomycin/well as described in *Materials and Methods*. The cells were probed with asialo-COO$^-$-glycopeptides or asialo-CONHMe-glycopeptides conjugated to BSA. Binding was detected by flow cytometry after staining with anti-BSA mAb followed by PE-conjugated secondary goat anti-mouse IgG mAb. Cells stained with second- and third-stage reagents only were used as negative controls.

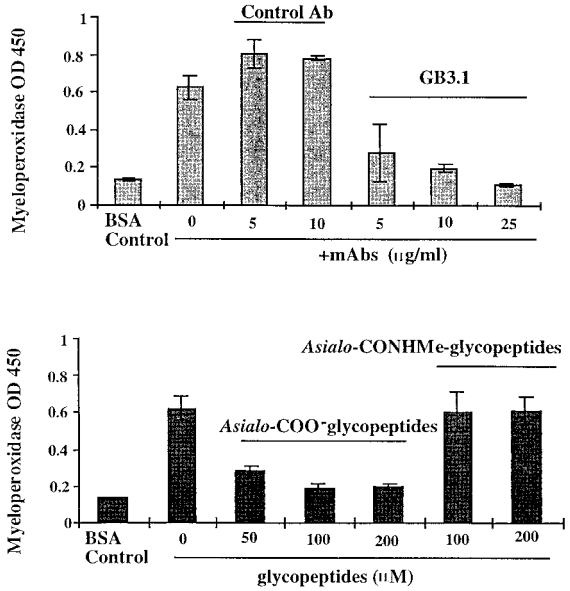

FIGURE 8. Specific adhesion of neutrophils to carboxylated glycans coated on plastic. Ninety-six-well microtiter plates were coated with 250 ng/well of BSA-coupled bovine lung asialo-COO^- -glycopeptides for 4 h at 37°C. Plates were washed and blocked with 3% BSA in PBS overnight. Control wells had BSA alone. Neutrophils (0.5×10^6) in 100 μl of HBSS buffer containing 0.2% BSA and 1 μM PAF were added to each well and incubated for 30 min at 37°C in the presence or the absence of varying amounts of modifiers in a total volume of 200 μl. Unbound cells were removed, and binding cells were quantitated by assaying myeloperoxidase. Data show the mean ± SD of duplicate assays.

3-fold (data not shown). This effect was seen regardless of whether the endothelial cells were prestimulated or the endothelial cells were incubated with the Ab before the neutrophils were allowed to adhere. Adhesion was also increased in the presence of asialo-COO^--glycopeptides, but not by asialo-CONHMe-glycopeptides. The reasons for the diametrically opposite effects of mAb GB3.1 and the soluble asialo-COO^--glycopeptides on adherence of neutrophils to plastic-immobilized glycans vs endothelial cells are unclear. Presumably, the situation involving intact endothelial cells is more biologically complex and involves other signaling circuits and/or other adhesion pathways. For example, the interaction of activated neutrophils with the endothelial glycans could trigger *cis* or *trans* signaling in either or both of these cells, eventually resulting in inhibition of the overall interactions. Regardless, the results clearly indicate that these novel glycans on endothelial cells can modulate interactions with activated neutrophils.

Monoclonal Ab GB3.1 inhibits leukocyte influx in a mouse model of acute peritonitis by reducing transmigration of cells across the endothelium

Based on the above results and to further understand the in vivo role of the novel glycans in the dynamic interaction between endothelial cells and leukocytes, we checked the effects of mAb GB3.1 on the pathophysiology of acute inflammation. Because the novel glycan is also expressed in murine tissues, we reasoned that the inflamed mouse peritoneum would be a convenient model to quantitatively examine such effects. Zymosan-induced peritonitis in mice was characterized by a time-dependent accumulation of cells in the peritoneal cavity. Neutrophil content in peritoneal cavities rose from about 0.75 million cells in saline-treated mice to about 12 million cells within 4 h after zymosan challenge. Neutrophil influx was followed by monocyte infiltration, which rose from ~10 million cells in control mice to ~50 million cells 16 h after zymosan treatment. Intravenous injection of mAb GB3.1 immediately before the induction of peritonitis resulted in a dose-dependent reduction in the extent of neutrophil and monocyte accumulation, while an isotype-matched mouse IgG or mAbAD7.5 or an unrelated anti-carbohydrate Ab (9) had minimal effect (Fig. 9). Titers of mAb GB3.1 checked at various time points after injection in control mice showed that Ab levels stabilized by 2 h and remained steady for up to 24 h in the circulation (data not shown). The dose of 20 μg/g body weight, which gave about 65% reduction in infiltration was chosen to further confirm the patterns of cells in the inflamed peritoneum by flow cytometry. Peritoneal cells from untreated and zymosan-treated mice were stained with FITC-labeled anti-mouse Gr-1 (anti-neutrophil) or PE-labeled anti-mouse Mac-1 (monocytes and neutrophils) and analyzed by flow cytometry. Four hours after zymosan injection, Gr-1^{high} neutrophils were the most abundant in the peritoneum, followed by recruitment of Gr-1^{low}/Mac-1^{high} monocytes at 16 h (data not shown). Again, injection of mAb GB3.1 caused a substantial reduction in appearance of both types of cells, while a control Ab had no effect (data not shown).

Decreased neutrophil and monocyte entry into the inflamed peritoneum upon injection of mAb GB3.1 could result from a decreased ability to adhere to the endothelium or a failure to emigrate after the cells have adhered. To directly address this issue, we histologically examined mesenteric vessels from both 4 and 16 h points for intravascular and extravascular accumulation of leukocytes. The following observations were made at both time points.

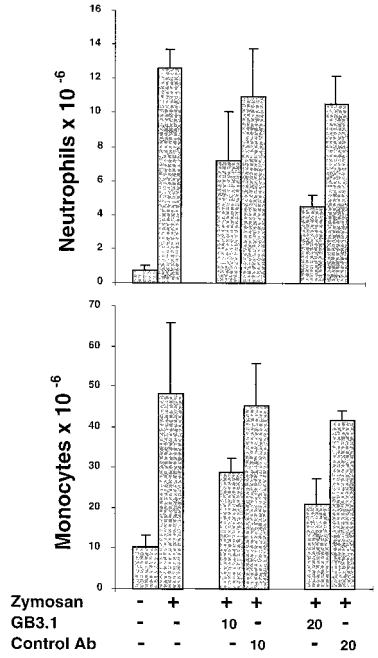

FIGURE 9. Effect of mAb GB3.1 on neutrophil and monocyte accumulation in the inflamed mouse peritoneum. Mice were administered sterile saline, mAb GB3.1, or isotype control mouse IgG i.v. immediately before i.p. administration of 1 mg of zymosan. Control mice received saline both i.v. and i.p. Neutrophil influx was measured at 4–5 h, and monocyte influx was measured at 16–18 h after zymosan challenge as described in *Materials and Methods*. The numbers indicate micrograms of Ab injected per gram of mouse body weight. Data shown are the mean ± SD of four mice per group.

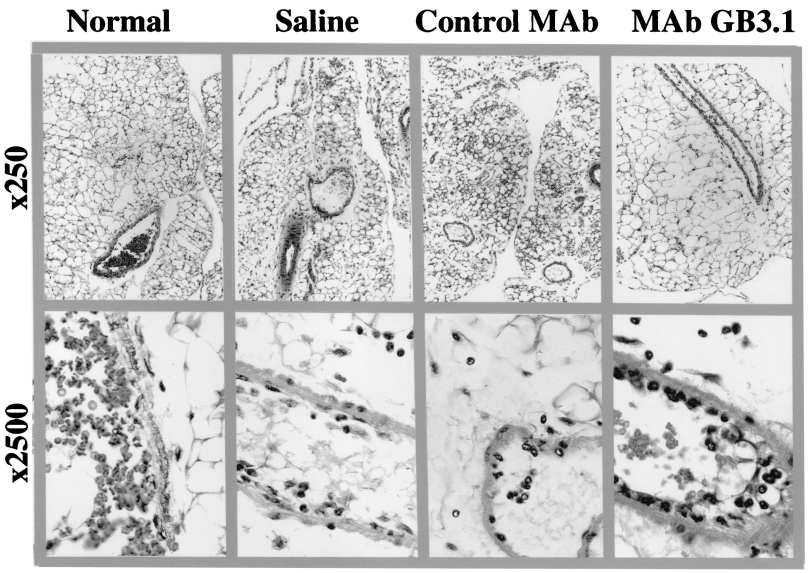

FIGURE 10. Effect of mAb GB3.1 on leukocyte sequestration in mesenteric vasculature during peritoneal inflammation. Mesenteries from untreated and zymosan-treated mice were removed, fixed in formalin, and stained histochemically by hematoxylin and eosin. The *upper panels* (×250 magnification) show extravascular infiltration of leukocytes (seen as dark dots) in inflamed tissues, which is reduced by mAb GB3.1 treatment but unaltered by treatment with a control mAb. The *lower panels*, at higher magnification (×2500), focus on the accumulation of leukocytes along inflamed vessels in saline- and control Ab-treated mice and the markedly increased numbers of adherent leukocytes along the endothelium in mAb GB3.1-treated mice.

First, zymosan injection caused extravascular migration of leukocytes, correlating with the increase in exudated cells in the peritoneal lavage fluids (see Fig. 10, showing the 16 h point). Second, this was unaffected by injection of a control Ab. Third, increased leukocyte adherence to the mesenteric microvasculature could also be detected in saline- or control mAb-treated mice; however, only mAb GB3.1-treated mice showed a clear reduction in leukocyte recruitment into the inflamed tissues. This effect of the mAb was associated with marked increase in adherence of cells to the endothelium in over half of randomly examined mesenteric vessels. This adherence was more dramatic at the 16 h point compared with the 4 h point. Thus, the reduction in cell efflux into the peritoneal cavity can be explained by a reduction in transmigration across the vessel walls. These findings suggest that the novel glycans can selectively modulate adhesion and transmigration of activated leukocytes across the vascular endothelium in the setting of acute inflammation.

Leukocyte recruitment into sites of inflammation is a multistep process of interrelated events mediated by complex and overlapping functions of multiple adhesion molecules (10, 11). Selectins mediate the initial rolling and tethering of circulating neutrophils. L-selectin is constitutively expressed by most leukocytes, and P- and E-selectins are expressed by activated endothelial cells following exposure to inflammatory conditions (12–14). Concomitant up-regulation of β_2 integrins on the neutrophils are largely responsible for stronger adhesive interactions with intercellular adhesion molecules (ICAM-1 and ICAM2). ICAM-1 is constitutively expressed at low levels by endothelial cells, but is rapidly up-regulated during inflammation (10, 11). Although rolling and firm adhesion of leukocytes are well understood, there is much less information regarding the in vivo mechanisms mediating transmigration. Several in vitro and in vivo studies have shown that PECAM-1 (CD31) is critically involved in transendothelial migration (15). Interestingly, Abs against PECAM-1 inhibit leukocyte extravasation from mesenteric vessels (16, 17), similar to the effects of mAb GB3.1 seen in our studies. However, the expression patterns of CD31 on the surface of most leukocytes, platelets, and intercellular junctions of endothelial cells (15) is different from that of the GB3.1 Ags. Collectively, our findings indicate the carboxylated glycans participate in yet another set of novel mechanisms involved in the regulation of acute inflammation.

Conclusions and perspectives

Here we have used a novel approach to generate an IgG mAb that detects unusual carboxylated *N*-glycans that are predominantly localized in the vascular endothelium of a variety of mammalian tissues. The vascular endothelium is a dynamic and complex system that possesses many secretory, synthetic, immunologic, and metabolic functions (18). The contribution of carbohydrates to endothelial function is best exemplified by the selectin family of adhesion molecules, which recognize sialyl Lewis^x- and sialyl Lewis^a-containing structures (12–14). Also, unusual anionic oligosaccharides such as sialyl Lewis$^{x/a}$ as well as sulfosialyl Lewis$^{x/a}$, Man-6-phosphate (19), polysialic acid (20), and GalNAc-4-sulfate (21) have been noted to be more involved in mediating specific biological actions than the more common neutral glycans. Therefore, the predominant localization of these novel carboxylated glycans on the vascular endothelium raised the intriguing possibility that they could participate in endothelial functions or in interactions with cells or proteins in the blood. The functional studies described here clearly indicate that they can mediate interactions with activated neutrophils and modulate inflammatory responses. Further studies of the structure and biosynthesis of the carboxylated glycans and the identification of their cognate receptors on activated neutrophils are now underway.

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