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Monoclonal Antibodies PMN 6, PMN 29, and PM-81 Bind Differently to Glycolipids Containing a Sugar Sequence Occurring in Lacto-N-Fucopentaose III

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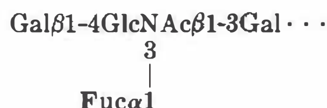
Three monoclonal antibodies, PMN 6, PMN 29, and PM-81, bind myeloid cells. Antibodies PMN 6 and PMN 29 bind specifically to granulocytes but differ in their ability to bind some other cell lines [E. D. Ball, R. F. Graziano, L. Shen, and M. W. Fanger (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5374–5378]. Antibody PM-81, in addition to granulocytes, also binds to eosinophils, monocytes, and most acute myelocytic leukemia cells [E. D. Ball, R. F. Graziano, and M. W. Fanger (1983) *J. Immunol.* **130**, 2937–2941]. Despite these differences, the binding of all three antibodies to cells was inhibited by the oligosaccharide, lacto-*N*-fucopentaose III [Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc]. Solid-phase radioimmunoassays using purified glycolipids containing sugar sequences found in lacto-*N*-fucopentaose III demonstrated different binding characteristics for each antibody. PM-81 bound lower concentrations of glycolipids than PMN 29, while PMN 6 required the highest concentration of glycolipids for binding. Autoradiography of thin-layer chromatograms of glycolipid antigens supported these results. The binding of these monoclonal antibodies to cells probably depends on the density of antigens on the cell surface, each antibody requiring a different density. Thus, cells containing antigen below a certain threshold concentration may not bind low-affinity antibodies.

Many monoclonal antibodies with apparent specificities for various cells are directed against the sugar sequence



which occurs in the human milk oligosaccharide, lacto-*N*-fucopentaose III (1). This sequence is very immunogenic in mice, and is a marker for human adenocarcinoma of the colon, stomach (2, 3), and lung (4), as well as granulocytes and granulocyte precursors (5–8). It is also the murine embryonic antigen known as SSEA-1 (9, 10).

Although the antigen is restricted to myeloid cells among hemopoetic cells as evidenced by immunofluorescence studies (5–8), small amounts of antigen were detected in glycolipids from erythrocytes by immunostaining of thin-layer chromatograms (2). Recently, three monoclonal antibodies, PMN 6, PMN 29, and PM-81, have been described which bind differently to cells of the myeloid series, including granulocytes, monocytes, and blasts from patients with acute myelogenous leukemia (11, 12). Despite these differences, the binding of all three antibodies is inhibited by lacto-*N*-fucopentaose III. The data in the present paper suggest that differences

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in the binding affinity of these antibodies for their antigen may explain their differential reactivities.

EXPERIMENTAL PROCEDURES

Materials. Monoclonal antibodies PMN 6 and PMN 29 are produced by hybridomas prepared from spleen cells obtained from mice immunized with neutrophils from normal donors (11); PM-81 is produced by a hybridoma prepared from spleen cells obtained from a mouse immunized with the promyelocytic leukemia cell line, HL-60 (12). Monoclonal antibody AML-1-201 binds β-2 microglobulin (12), and was used as a control antibody for these studies. All four antibodies are of the IgM isotype.

Lacto-*N*-fucopentaosyl(III)ceramide ($III^3FucnLcOse_4Cer$) was prepared from human colonic adenocarcinoma as previously described (3). The glycolipid was further purified by rechromatography on HPLC and was freed from lacto-*N*-fucopentaosyl(II)ceramide (Le^a glycolipid; III^4 $FucLcOse_4Cer$). The y_2 glycolipid ($V^3FucnLcOse_6Cer$) was prepared from human erythrocytes as previously described (13). Difucosyl lacto-*N*-*nor*hexaosylceramide (bands 4a–e; $III^3V^3Fuc_2nLcOse_6Cer$) was prepared from a human colonic cancer metastasis in the liver (14).

Globoside was purchased from Supelco Inc., Bellefonte, Pennsylvania. Sialylated lacto-*N*-fucopentaosyl(III)ceramide was kindly provided by Dr. H. Rauvala (University of Helsinki, Helsinki, Finland). Oligosaccharides, lacto-*N*-fucopentaose III, and lato-*N*-fucopentaose I were isolated from human milk as previously described (15).

Affinity-purified goat anti-mouse IgM (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was iodinated with $Na^{125}I$ (ICN Biochemicals, Irvine, Calif.) to a specific activity of about 40 μCi/μg using Iodogen (16) (Pierce Chemical Co., Rockford, Ill.).

Total lipid extracts were prepared by homogenization of cells in chloroform/methanol/H_2O (30/60/4, final ratio) (17).

Inhibition of binding of antibodies to cells by oligosaccharides. Monoclonal antibodies PMN 6, PMN 29, PM-81, and AML-1-201 (5 μg/ml) were preincubated with 5.4 mM lacto-*N*-fucopentaose I or lacto-*N*-fucopentaose III for 30 min at room temperature. This mixture was added to 10^6 neutrophils previously washed with phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin and 0.05% sodium azide, and incubated for 30 min at 4°C. After washing with the same buffer, fluorescein isothiocyanate-conjugated goat F(ab')2 antibody directed to mouse immunoglobulin (Boehringer-Mannheim, Indianapolis, Ind.) was added and incubated for 30 min at 4°C. Controls in which each monoclonal antibody was incubated with neutrophils in the absence of oligosaccharides were run in parallel. Cells treated in this manner were analyzed for fluorescence on the Ortho Cytofluorograph System 50H.

Solid-Phase radioimmunoassay. The binding of antibody to purified glycolipids was measured by solid-phase radioimmunoassay as previously described (18, 19) with minor modifications. Glycolipids in 30 μl methanol were added to the wells of a round-bottom polyvinylchloride microtiter plate (Dynatech, Alexandria, Va.), and the solutions were dried by evaporation. The wells were then filled with 0.05 M Tris-HCl, pH 7.8, containing 0.15 M NaCl, 1% bovine serum albumin, and 0.1% NaN_3 (Buffer A). After 30 min, the wells were emptied and to each was added 30 μl buffer A containing 5 μg/ml monoclonal antibody. The wells were covered with parafilm, incubated for 3 h at 22°C, washed once with buffer A, and then to each was added 100,000 cpm of ^{125}I-labeled goal anti-mouse IgM (40–50 μCi/μg) in 30 μl buffer A. After 3 h, the wells were washed six times with cold phosphate-buffered saline (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4), cut from the plate, and assayed for ^{125}I in an Auto-Gamma spectrometer.

Autoradiography of glycolipid antigens. Glycolipid antigens were detected on thin-layer chromatograms by autoradiography as previously described (19) with minor modifications. Glycolipids were chromatographed on aluminum-backed high-performance thin-layer chromatography plates (silica gel 60, E. Merck, Darmstadt, West Germany) in chloroform/methanol/0.25% KCl (50/50/12, by volume). The dried chromatogram was soaked for 1 min in a 0.1% solution of polyisobutylmethacrylate beads (Polysciences, Inc., Warrington, Pa.) dissolved in hexane. After drying in air, the chromatogram was sprayed with phosphate-buffered saline (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4) and immediately soaked in buffer A until all of the silica gel was wet (about 15 min). The plate was then removed and laid horizontally on a slightly smaller glass plate in a large Petri dish. Monoclonal antibody (5 μg/ml) diluted in buffer A was layered on the plate (60 μl/cm^2 chromatogram surface). After incubation at 22° for 2 h, the chromatogram was washed by dipping in four successive changes of cold phosphate-buffered saline at 1-min intervals, and overlayed with buffer A containing 2 × 10^6 cpm/ml ^{125}I-labeled goat anti-mouse IgM. After 1 h at 22°C, the chromatogram was washed as before in cold phosphate-buffered saline, dried, and exposed to Xar-5 X-ray film (Eastman-Kodak, Rochester, N. Y.) for 10 h at 22°C.

RESULTS

Effects of Oligosaccharides on Cell Binding

Monoclonal antibodies PMN 6, PMN 29, and PM-81 bound to most neutrophils, and

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this binding was completely inhibited by 5.4 mM lacto-*N*-fucopentaose III but not by 5.4 mM lacto-*N*-fucopentaose I (Fig. 1). Neither oligosaccharide inhibited the binding of monoclonal antibody AML-1-201, an IgM which binds β-2 microglobulin (12).

Autoradiography of Glycolipid Antigens

Glycolipid antigens were detected by autoradiography of thin-layer chromatograms as described under Experimental Procedures. Purified glycolipids Y_2 and 4c, which contain a carbohydrate sequence found in lacto-*N*-fucopentaose III (see Table I), bound PMN 6, PMN 29, and PM-81 (Figs. 2A, B, C, lanes 1). The smaller glycolipid, lacto-*N*-fucopentaosyl(III)ceramide, bound only PMN 29 and PM-81 under these conditions.

The reactivity of these antibodies, particularly PMN6, resembled that of SSEA-1 (9, 10); ZWG 13, ZWG 14, and ZWG 111 (2); and FH-1 and FH-5 (20); which do not bind as well to lacto-*N*-fucopentaosyl-(III)ceramide as to glycolipids with longer carbohydrate chains, including di- and trifucosylated derivatives.

All three antibodies detected glycolipid antigens from total lipid extracts of granulocytes and HL-60 cells (Figs. 2A, B, and C; lanes 6 and 7). Both of these cell types have high concentrations of glycolipids containing a carbohydrate sequence found in lacto-*N*-fucopentaose III (5). Antigen comigrating with lacto-*N*-fucopentaosyl-(III)ceramide, however, was not detected

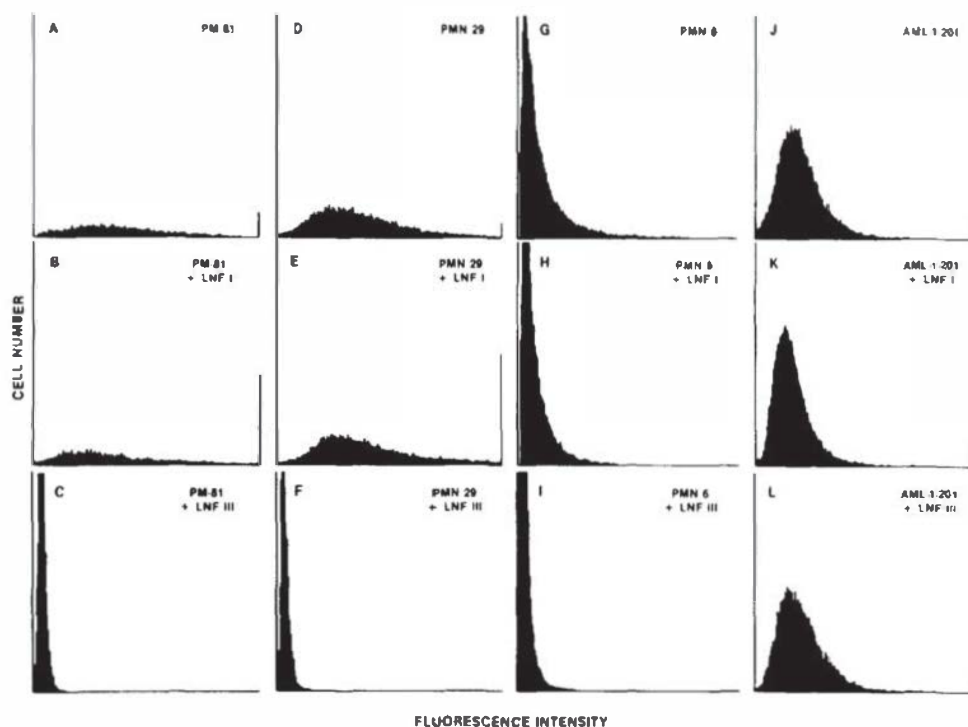

FIG. 1. The effect of lacto-*N*-fucopentaose I (LNF I) and lacto-*N*-fucopentaose III (LNF III) on the binding of monoclonal antibodies PM-81, PMN 29, PMN 6, and AML-1-201 was determined by cytofluorography as described under Experimental Procedures. The fluorescence of neutrophils stained with PM-81, PMN 29, PMN 6, and AML-1-201 is shown in panels A, D, G, and J, respectively. The effect of lacto-*N*-fucopentaose I and lacto-*N*-fucopentaose III on this fluorescence is shown in panels B, E, H, and K, and panels C, F, I, and L, respectively.

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TABLE I

STRUCTURE OF CARBOHYDRATES

Name	Structure
Glycolipids	
Lacto-*N*-fucopentaosyl(III)ceramide	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer 3 (on GlcNAc) | Fucα1
4c	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer 3 (on each GlcNAc) | Fucα1 Fucα1
Y_2	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer 3 (on first GlcNAc) | Fucα1
Globoside	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer
Oligosaccharides	
Lacto-*N*-fucopentaose III	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 (on GlcNAc) | Fucα1
Lacto-*N*-fucopentaose I	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc

by PMN 6. The same chromatographic pattern was obtained by all three antibodies; however, the intensity of staining increased from PMN 6 to PMN 29 to PM-81.

No antigens were detected in the total lipid extracts of acute myelocytic leukemia cells or monocytes by antibodies PMN 6 and PMN 29 (Figs. 2A and B, lanes 2, 3, 4, 5). Under the same conditions PM-81 detected low levels of antigen in both extracts

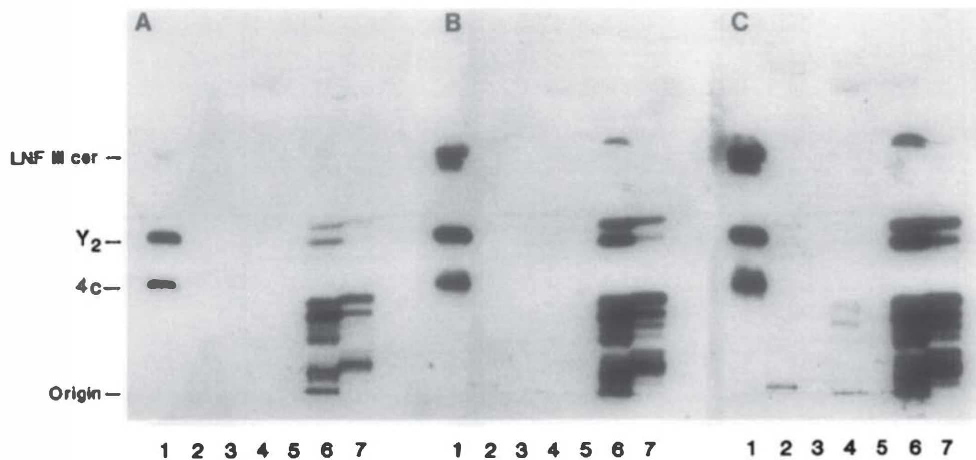

FIG. 2. Autoradiography of glycolipid antigens. Autoradiography of glycolipid antigens was performed as described under Experimental Procedures. (A) was stained with antibody PMN 6, (B) with PMN 29, and (C) with PM-81, each at 5 μg/ml. Purified glycolipids (30 ng) 4c, Y_2, and lacto-*N*-fucopentaosyl(III)ceramide (LNF III cer) were chromatographed in lane 1. The amount of extract chromatographed expressed as the volume of packed cells from which it was obtained is lane 2, 5 μl AML blasts; lane 3, 2 μl AML blasts; lane 4, 5 μl monocytes; lane 5, 2 μl monocytes; lane 6, 2 μl granulocytes; and lane 7, 2 μl HL-60 cells. The positions of the purified glycolipids are shown on the left.

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(Fig. 2C, lanes 2, 3, 4, 5). These data support the previous findings that PMN 6 and PMN 29 bind to neutrophils (11) while PM-81 binds to neutrophils, monocytes, and acute myelocytic leukemia cells (12).

Solid-Phase Radioimmunoassay

Monoclonal antibodies PMN 6, PMN 29, and PM-81 were assayed for binding to purified glycolipids by solid-phase radioimmunoassay as described under Experimental Procedures. Differences in binding were found for each antibody as shown in Fig. 3. PM-81 bound to the lowest concentration of glycolipids containing sugar sequences found in lacto-*N*-fucopentaose III. Higher concentrations of glycolipids were required for binding antibody PMN 29. PMN 6 showed the least binding to high concentrations of glycolipids Y_2 and 4c, and did not bind to lacto-*N*-fucopentaosyl(III)ceramide at the concentrations tested. These results agree with the intensity of staining of glycolipid antigens shown in Fig. 2.

Differences in binding were also found for each purified glycolipid. All three antibodies bound to glycolipids Y_2 and 4c at lower concentrations than to lacto-*N*-fucopentaosyl(III)ceramide (Fig. 3). These results also agree with the chromatographic patterns of glycolipid antigens shown in Figure 2. None of these antibodies bound to a monosialoganglioside containing sialic acid linked α2-3 to the terminal galactose of lacto-*N*-fucopentaosyl-(III)ceramide (data not shown).

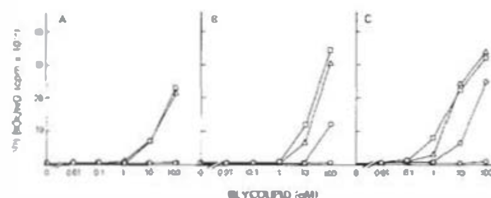
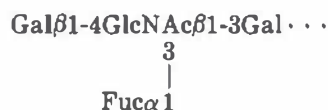


FIG. 3. Binding of antibodies to purified glycolipids. Solid-phase radioimmunoassays were performed as described under Experimental Procedures. Antibody PMN 6 was used for assays in (A), PMN 29 for (B), and PM-81 for (C). Purified glycolipids tested were 4c, △; Y_2, □; lacto-*N*-fucopentaosyl(III)ceramide, O; and globoside, ○. Structures of these glycolipids are depicted in Table I.

DISCUSSION

The carbohydrate sequence



is a potent antigen to the mouse. Out of 325 monoclonal antibodies from different laboratories that have been analyzed in our laboratory, 55 are directed against this sequence (21).

Some antibodies directed against the same antigen, as judged by hapten binding or hapten inhibition studies, have different cell specificities. For example, a rabbit antiparagloboside antibody (22) and a Waldenström cold agglutinin (cold agglutinin McC) (23) both bind to paragloboside (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer), yet react differently with cells: the rabbit antibody reacts equally well with human cord and adult erythrocytes (22) while the cold agglutinin reacts strongly with cord cells but weakly or not at all with adult cells (23). This differential reactivity might be explained in some cases by the fact that some antibodies bind to different parts or to different sides of the same sugar chain (18, 20, 24, 25). If the adult erythrocyte antigen were actually substituted paragloboside and the two antibodies bound to different parts of the paragloboside sugar chain, the antibodies would react differently with the substituted paragloboside depending on where the substitution occurred. This hypothesis, however, is not likely to explain the differential reactivity of antibodies PMN 6, PMN 29, and PM-81 with various cell types, as the three antibodies appear to bind to the same glycolipid antigens (Fig. 2). It is more likely that their differential reactivity is explained by their different affinities for antigen (Fig. 3). PM-81 has the highest affinity and binds to more cell types than do PMN 6 or PMN 29. It is the only antibody that binds monocytes (11, 12) which contain little glycolipid antigen (Fig. 2C, lane 4). PMN 29 detects intermediate concentrations of glycolipid antigen and binds to some cell lines that PMN 6 does not (11). Thus, cells that contain antigen below a

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