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Monoclonal Antibodies to Carbohydrate Antigens in Autologous Bone Marrow Transplantation

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Normal and malignant myeloid cells express a highly immunogenic oligosaccharide, lacto-n-fucopentaose-III (LNF-III), that has been identified by numerous monoclonal antibodies (MoAb). We have been interested in the use of a particular monoclonal antibody to LNF-III, PM-81, in the treatment of patients with acute myelogenous leukemia using the antibody to treat bone marrow in vitro. Following in vitro treatment of bone marrow with PM-81 and another MoAb, AML-2-23, the remaining cells are used as an autograft in a patient treated with high-dose chemotherapy and radiotherapy. In order to enhance the ability of the MoAb to lyse leukemic cells in the remission bone marrow, we have explored the effect of neuraminidase treatment on leukemia cells. In this paper we describe that myeloid leukemia cells expressing low levels of LNF-III by immunofluorescence can be shown to have high levels of LNF-III after neuraminidase treatment. In addition, we show that normal bone marrow progenitor cells do not have cryptic LNF-III antigen, thus allowing the application of this finding to the clinical setting. Moreover, we have shown that leukemia colony-forming cells from one patient with acute myelogenous leukemia express cryptic LNF-III and that after exposure to neuraminidase there was an increased ability of PM-81 in the presence of complement to eliminate these colony forming cells. These data indicate that the LNF-III moiety is almost universally expressed on myeloid leukemia cells and their progenitors but not expressed on normal progenitors. Thus, it may be possible to enhance leukemia cell kill in vitro by neuraminidase treatment of bone marrow.

Key words: glycoconjugates, bone marrow transplantation, myeloid cells, acute myeloid leukemia

During efforts to develop monoclonal antibodies (MoAbs) to tumor-specific antigens, we and others found that a highly immunogenic oligosaccharide, previously known as stage-specific embryonic antigen (SSEA-1) [1], was commonly found on certain human neoplasms [2–5]. We developed three different hybridomas secreting IgM immunoglobulins, PMN 6 [2], PMN 29 [2], and PM-81 [3], that were selected

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for binding to normal myeloid cells and blast cells from acute myelogenous leukemia (AML) patients and that later were found to be specific for the lacto-N-fucopentaose III (LNF-III) moiety [6] expressed on glycolipids and glycoproteins. Although a disproportionate number of MoAbs developed against myeloid cells were found to react with this oligosaccharide sequence, the existence of several other polypeptide antigens expressed specifically on myeloid cells was also discovered [3,7–11].

The various protein and glycolipid antigens defined by MoAbs have been categorized into clusters by International Workshops for convenience of reference [reviewed in 12]. We have focused in this laboratory on the use of MoAbs to several myeloid-cell-associated antigens including those to LNF-III (above), one to a 55,000 kD protein, AML-2-23 [13], and one to a 124-kD protein, AML-1-99 [14]. The workshop designations for these MoAbs are CD 15 (LNF-III) and CD 14 (AML-2-23). No cluster has been designated for AML-1-99 at this time, and no other MoAbs are reported that share its specificity in binding to cells and to the same size polypeptide.

We have been investigating methods by which these MoAbs could be used in the therapy of the myeloid leukemias. Since blast cells from patients with AML usually express one or more of the antigens mentioned above, we are taking advantage of the ability of MoAbs to lyse cells to which they bind when presented with a source of complement that can be activated, cause membrane pore formation, and cytotoxicity.

MoAbs to the LNF-III molecule are ideally suited for this purpose since they are almost always IgM, a class of antibody capable of activating complement. AML-2-23 and AML-1-99 are of the IgG2b and IgM classes, respectively, and are also efficient at fixing complement [14,15].

In order to determine whether any of the above MoAbs could be useful therapeutically, we initially injected unmodified MoAbs into patients with AML [16]. As only transient effects on blast cells were found [16], we redirected our approach. We decided to treat bone marrow from patients with AML at high risk for relapse owing to residual occult disease in remission in vitro, where variables affecting the ability of the MoAbs to kill cells could be better controlled. This in vitro treatment of bone marrow is coupled with the delivery of high-dose chemotherapy and total body irradiation (TBI) to the patient so that the "treated" bone marrow is used as an autologous bone marrow transplant (ABMT). The success of this approach depends on both the ability of the systemic chemotherapy/total body irradiation (TBI) and the MoAb treatment to kill tumor cells. Since transplantation of normal allogeneic marrow into patients treated with the high-dose chemotherapy and TBI can fail to prevent recurrence of the disease [17], it is clear that the success of autologous BMT will have the same limitations. We have recently reported the preliminary results of treating patients in this manner [18]. Although the results of treating these patients have been encouraging, and long-term disease-free survival has been achievable in some patients, relapses have occurred. Unfortunately, it is not possible to determine if relapse was due to failure of the chemoradiotherapy to clear the patient of tumor cells, contamination of the bone marrow with residual malignant blasts, or other reasons. In this paper we will present studies that focus on enhancing the elimination of AML cells from bone marrow in vitro. We will discuss the use of neuraminidase in exposing cryptic LNF-III antigen on AML cells.

MATERIALS AND METHODS

Cells

Peripheral blood (PB) and bone marrow (BM) samples from newly diagnosed patients with AML at the Dartmouth-Hitchcock Medical Center were obtained for study with informed consent. Mononuclear cells were isolated by ficoll-hypaque gradient centrifugation (Histopaque, Sigma, St. Louis, MO). The interface layer was washed in RPMI 1640 (GIBCO, Grand Island, NY). Normal PB and BM were obtained from healthy donors and a mononuclear cell layer obtained in an identical manner.

Monoclonal Antibodies

The PM-81 hybridoma was obtained as a fusion product of splenocytes from a BALB/c mouse immunized with HL-60 promyelocyte leukemic cells and NS-1 cells as described [3]. After selection for binding to both normal neutrophils and AML blast cells, PM-81 was characterized by Ouchterlony analysis to be of the IgM class. Antigenic specificity to the LNF-III molecule was determined by thin layer chromatography [6]. The AML-1-99 and AML-2-23 MoAbs were produced by fusion using cells from patients with AML classified as M1 and M4, respectively, by the French-American-British (FAB) system as the immunogen. AML-2-23 was selected for binding to normal monocytes and to AML blast cells [2]. This MoAb was determined to be of the IgG2b subclass and is specific for My23, a 55-kD glycoprotein on monocytes [13]. The AML-1-99 MoAb was reactive to the majority of AML blast cells but displayed minimal reactivity to peripheral blood monocytes (20%) and neutrophils (2%). AML-1-99 is of the IgM subclass and reacts with a 124-kD glycoprotein on U937 cells (manuscript submitted).

Treatment With Neuraminidase

PB and BM mononuclear cells were washed once with RPMI 1640 supplemented with 3 mM $CaCl_2$, pH 7.25. The cells were divided equally among two tubes; to one, the cell pellet was resuspended in RPMI 1640 + 3 mM $CaCl_2$ at a cell concentration of 10^7/ml, and the other cell pellet was resuspended in 0.025 units of neuraminidase (N-2876, Sigma Chemical Co.) at a final cell concentration of 10^7/ml. Both control and neuraminidase-treated cells were then incubated at 37°C for 10 min, washed, and resuspended in RPMI 1640 for cell counts and viability.

Monoclonal Antibody-Mediated Cytotoxicity

Two million cells were treated at a final cell concentration of 4×10^6/ml in 5 ml sterile capped tubes (Falcon, Lincoln Park, NJ). MoAbs diluted to 50 μg/ml in RPM1 1640 plus 10% fetal bovine serum (FBS) were incubated with the cells for 15 min at room temperature with continuous shaking. Baby rabbit complement (Pel Freeze, Brown Deer, WI) was added to a final dilution of 1:6. The cells were incubated for an additional 60 min at room temperature with shaking. The cells were washed with RPMI 1640 and resuspended to 2×10^6/ml for determination of colony growth in methylcellulose.

Colony-Forming Assays

Neuraminidase or control-treated cells treated with MoAb and C′ were plated in methylcellulose to assess colony formation. Methylcellulose (0.8%) was supple-

mented with 30% FBS, 10% deionized bovine serum albumin (BSA), 10% GCT medium (GIBCO) as a source of colony-stimulating activity, 2 mM glutamine, and 5 $\times$ 10^{-5} 2-mercaptoethanol. To a 2.7-ml suspension of these ingredients, 0.3 ml of cells at 2 $\times$ 10^6/ml were added, mixed, and plated in 35-mm grided petri dishes (Lux, Miles Scientific, Naperville, IL) in 1-ml volumes. Two units of erythropoietin (Amgen Biologicals, Thousand Oaks, CA) were added to each dish and colonies enumerated by microscopy after 14 days in a humidified, 5% CO_2, 37°C incubator. Erythropoietin was deleted in the samples from AML patients.

Indirect Immunofluorescence and Flow Cytometry

Cells treated with medium or neuraminidase were analyzed for surface antigen expression by indirect immunofluorescence (IF). One million cells were washed once with phosphate buffered saline (PBS) containing 0.1% BSA and 0.05% sodium azide (P/B/A). The cell pellet was resuspended in saturating amounts of MoAb (50 μl of 20 μg/ml solution) or 300 μl of supernatant from the P3X63Ag8 (IgG1) parent myeloma line (negative control). After 30 min at 4°C, the cells were washed with P/B/A and resuspended in 25 μl of fluorescein-isothiocyanate (FITC)-conjugated goat antimouse IgG and IgM. The cells were incubated for an additional 30 min at 4°C, washed twice with P/B/A, and resuspended in 1% paraformaldehyde (Kodak, Rochester, NY) until analysis by flow cytometry. Cells were analyzed for fluorescence using an Ortho Systems 50H cytofluorograph equipped with a 2150 computer using a linear amplification on a scale of 0–1000. The percentage of cells positive for a particular antibody was determined within a region chosen to contain no more than 5–10% cells fluorescent after treatment with a control MoAb. The mean fluorescence intensity (MFI) was determined from the mean channel number of the entire cell population.

RESULTS

Effect of Neuraminidase on Binding of MoAb PM-81 to AML Blast Cells

During our characterizations of blast cells from newly diagnosed AML patients, we observed that a small percentage of the patients did not exhibit binding with the PM-81 MoAb by IF. To determine whether the lack of binding was due to the masking of the LNF-III molecule by sialic acid, we treated the cells with neuraminidase and reexamined the cells for PM-81 binding. Of four patients studied, three demonstrated a marked increase in both the percentage of cells reactive with PM-81, as well as an increase in the mean fluorescence intensity (Table I). Binding of another IgM MoAb, AML-1-99, did not change after neuraminidase treatment (data not shown).

Effect of Neuraminidase on the Antigen Expression on Normal Bone Marrow Progenitor Cells

Since in our clinical studies, bone marrow is treated with MoAbs PM-81 and AML-2-23 and C′ to eliminate occult leukemia cells, we were concerned that the treatment of remission marrow with neuraminidase (to enhance PM-81 binding) may result in the exposure of LNF-III molecules on normal hematopoietic progenitor cells. This exposure might therefore lead to the lysis of this population of cells required for the engraftment of the bone marrow. To determine if neuraminidase treatment followed by PM-81 and AML-2-23 and C′ treatment was cytotoxic to normal colony-forming units (CFU) and burst-forming units-erythroid (BFU-E), we examined nor-

TABLE I. Expression of LNF-III Molecules on AML Blast Cells After Neuraminidase Treatment*

Patient No.	Source of blast cells	Treatment	Immunofluorescence[a] Percent positive	MFI
1	PB	Untreated	17	52
		Neuraminidase	87	625
2	PB	Untreated	10	15
		Neuraminidase	29	19
3	BM	Untreated	38	50
		Neuraminidase	100	859
4	PB	Untreated	38	107
		Neuraminidase	99	543

*Blast cells from peripheral blood (PB) or bone marrow (BM) from patients with AML were treated with buffer alone (untreated) or 0.025 units of neuraminidase as described in the Materials and Methods section. After treatment, both groups were analyzed by immunofluorescence and flow cytometry.
[a]The percentage of cells positive with a negative control MoAb were subtracted from each value. Similarly, the mean fluorescence intensity (MFI) of the whole cell population of the negative isotype-matched MoAb control was subtracted from each value.

mal colony formation after such treatments. Normal peripheral blood or bone marrow mononuclear cell populations were treated with either medium alone, or neuraminidase, and both groups treated with the combination of PM-81 and AML-2-23 plus C′. The treated cells were plated in methylcellulose to evaluate colony formation in the presence of colony-stimulating activity (CSA) and erythropoietin. As shown in Table II, there was no reduction in normal CFU of granulocyte, monocyte, (CFU-GM), mixed cell (CFU-GEMM), or BFU-E after neuraminidase treatment. In fact, there were generally greater numbers of colonies after neuraminidase and MoAb + C′ treatment. Although not shown, we also did not observe any reduction in cell recovery or viability when comparing neuraminidase-treated to control-treated cells.

Effect of Neuraminidase on Antigen Expression of Leukemia Colony-Forming Cells (L-CFC)

Of the four AML patients analyzed for PM-81 binding, one patient's cells formed leukemia colonies in methycellulose. We were therefore able to evaluate the effect of neuraminidase treatment and MoAb and C′ purging on the L-CFC population. As depicted in Figure 1, we obtained a marked reduction in L-CFC in those samples treated both with neuraminidase and a mixture of PM-81 and AML-2-23 MoAbs plus C′. Similar results were obtained when PM-81 alone was used (data not shown).

DISCUSSION

We have described here several observations that have immediate therapeutic implications. We have shown that AML blast cells from patients negative by IF for the LNF-III antigen as defined by PM-81 binding can be rendered strongly positive after removal of sialic acid with neuraminidase. Importantly, we have shown for the first time that clonogenic leukemia cells are also rendered more sensitive to C′-mediated lysis using MoAb PM-81. It follows that more effective killing of AML cells in autografts could be achieved by neuraminidase treatment prior to PM-81 and

TABLE II. Normal Progenitor Cell Colony Formation After Neuraminidase and MoAb and C Treatment*

Donor No.	Cell source	Treatment	Monoclonal antibody	CFU-GM[a]	BFU-E	CFU-GEMM
1	PB	Medium	Control	57.5 ± 13.5		
			PM-81+AML-2-23	90.5 ± 5.5		
		Neuraminidase	Control	52.0 ± 6.0		
			PM-81+AML-2-23	63.5 ± 3.5		
2	BM	Medium	Control	42.0 ± 7.0	41.0 ± 19.0	0.5 ± 0.5
			PM-81+AML-2-23	40.5 ± 5.5	102.0 ± 2.0	0
		Neuraminidase	Control	61.0 ± 10.0	20.5 ± 1.5	0
			PM-81+AML-2-23	49.5 ± 6.5	53.5 ± 4.5	0
3	PB	Medium	Control	108.5 ± 11.5		
			PM-81+AML-2-23	112.5 ± 25.5		
		Neuraminidase	Control	120.0 ± 45.0		
			PM-81+AML-2-23	94.5 ± 14.5		
4	BM	Medium	Control	196.5 ± 24.5	84.0 ± 0	1.5 ± 0.5
			PM-81+AML-2-23 10 μg/ml	423.5 ± 9.5	132.5 ± 16.5	0
			PM-81+AML-2-23 50 μg/ml	368.0 ± 25.0	65.0 ± 17.0	0.5 ± 0.5
		Neuraminidase	Control	379.0 ± 63.0	11.0 ± 7.0	0
			PM-81+AML-2-23 10 μg/ml	683.0 ± 128.0	240.0 ± 28.0	0
			PM-81+AML-2-23 50 μg/ml	609.0 ± 263.0	212.0 ± 20.0	0

*Peripheral blood (PB) or bone marrow (BM) mononuclear cells from normal donors were treated with medium alone, or medium containing 0.025 units of neuraminidase (see Materials and Methods). After treatment, both cell groups were washed and treated with control MoAb and C′ treatment, or a combination of PM-81 + AML-2-23 + C′ at 50 μg/ml.

[a]Progenitor cells were determined after 14 days in methycellulose culture. BFU-E were enumerated in bone marrow cultures only. Numbers shown are the mean ± standard deviations of replicate cultures of 2×10^5 cells.

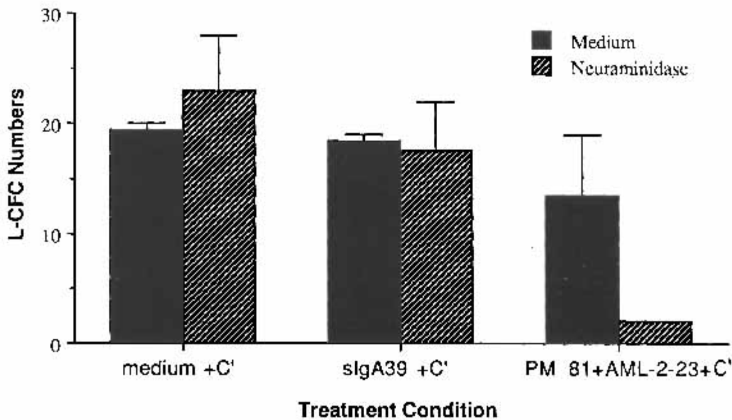

Fig. 1. Exposure of LNF-III molecules on L-CFC by neuraminidase. Cells from a patient with AML were either exposed to neuraminidase (black bar) or medium alone (striped bar) and then treated with MoAbs PM-81 and AML-2-23 + C′ and placed in methylcellulose culture. Colonies of >40 cells were enumerated 10 days later. Numbers shown are colony numbers per 10^5 cells cultured.

C′ treatment. However, exposure of cryptic antigen on normal hematopoietic progenitor cells could occur as well, leading to increased killing of these cells and adverse effects on engraftment of PM-81-treated bone marrow. We have addressed these questions by measuring the effect of PM-81 and C′ on normal bone marrow cells treated with neuraminidase. The negative results we obtained—ie, there was no excess toxicity to progenitor cells—indicate that we probably can safely use neuraminidase to enhance the effects of PM-81 binding on residual AML cells without excess toxicity. Admittedly, small numbers of CFU-GEM were observed in these experiments, thus perhaps underestimating the effects of exposure of LNF-III on these cells. Long-term marrow culture might allow a more accurate assessment of the effects of neuraminidase on more primitive progenitor cells. However, the final test of whether the cell truly responsible for engraftment is affected by neuraminidase and PM-81 plus C′ will be in the clinical setting since it is not possible to assay the pluripotent human stem cell at the present time in vitro.

The possible mechanisms by which neuraminidase treatment leads to enhanced binding of PM-81 are either that sialic acid is cleaved from the LNF-III moiety and/or that removal of sialic acid from adjacent molecules results in electrostatic or steric changes that make LNF-III more accessible. In support of the first mechanism is the observation of Spitalnik et al [19] that the presence of sialylated LNF-III correlated with enhanced MoAb binding to myeloid leukemia cell lines after neuraminidase treatment. A direct test of the presence of sialo-LNF III could be performed with MoAbs that are specific to sialo-LFN III, such as FH6 described by Hakomori et al [20]. We would expect reciprocal changes in PM-81 and FH6 binding before and after neuraminidase treatment of AML cells. Moreover, a MoAb to sialo-LNF-III could have therapeutic value in conjunction with PM-81. Such a combination could possibly circumvent the need to use neuraminidase to expose cryptic LNF-III.

Although AML-2-23 has been combined with PM-81 for marrow treatment, we have not yet done so with AML-1-99 because this MoAb kills normal bone marrow (BM) progenitor cells. Since regrowth of progenitor cells has been reported after treatment of BM with a MoAb to an antigen also expressed on progenitor cells, L4F3 (gp 67) [21], it is possible that we will find the same results with AML-1-99. These studies are in progress and will form the basis of whether to begin to use the AML-1-99 MoAb clinically.

It seems likely that residual marrow disease can be eliminated by MoAb plus C′ treatment in vitro [22]. Another problem that needs to be addressed is the method of treating the patient in preparation for the transplant. Since relapses occur after transplantation of normal allogeneic marrow using the regimens employed to date, it is clear that better approaches to eradication of the disease in vivo are required. These might include different chemotherapeutic drugs or schedules [23] and possibly the use of biological response modifiers such as gamma interferon [24] or tumor necrosis factor [25], both of which have been shown to be toxic to AML cells.

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