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Anti-MY9-Blocked-Ricin: An Immunotoxin for Selective Targeting of Acute Myeloid Leukemia Cells

By Denis C. Roy, James D. Griffin, Marcia Belvin, Walter A. Blättler, John M. Lambert, and Jerome Ritz

The use of immunotoxins (IT) to selectively destroy acute myeloid leukemia (AML) cells in vivo or in vitro is complicated by both the antigenic similarity of AML cells to normal progenitor cells and the difficulty of producing a sufficiently toxic conjugate. The monoclonal antibody (MoAb) anti-MY9 is potentially ideal for selective recognition of AML cells because it reacts with an antigen (CD33) found on clonogenic AML cells from greater than 80% of cases and does not react with normal pluripotent stem cells. In this study, we describe an immunotoxin that is selectively active against CD33+ AML cells: Anti-MY9-blocked-Ricin (Anti-MY9-bR), comprised of anti-MY9 conjugated to a modified whole ricin that has its nonspecific binding eliminated by chemical blockage of the galactose binding domains of the B-chain. A limiting dilution assay was used to measure elimination of HL-60 leukemic cells from a 20-fold excess of normal bone marrow cells. Depletion of CD33+ HL-60 cells was found to be dependent on the concentration of Anti-MY9-bR and on the duration of incubation with IT at 37°C. More than 4 logs of these leukemic cells were specifically depleted following short exposure to high concentrations (10^{-8} mol/L) of Anti-MY9-bR. Incubation with much lower concentrations of Anti-MY9-bR (10^{-10} mol/L), as compatible with in vivo administration, resulted in 2 logs of depletion of HL-60 cells, but 48 to 72 hours of continuous exposure were required. Anti-MY9-bR was also shown to be toxic to primary AML cells, with depletion of greater than 2 logs of clonogenic cells following incubation with Anti-MY9-bR 10^{-8} mol/L at 37°C for 5 hours. Activity of Anti-MY9-bR could be blocked by unconjugated Anti-MY9 but not by galactose. As expected, Anti-MY9-bR was toxic to normal colony-forming unit granulocyte-monocyte (CFU-GM), which expresses CD33, in a concentration- and time-dependent manner, and also to burst-forming unit-erythroid and CFU-granulocyte, erythroid, monocyte, megakaryocyte, although to a lesser extent. When compared with anti-MY9 and complement (C'), Anti-MY9-bR could be used in conditions that provided more effective depletion of AML cells with substantially less depletion of normal CFU-GM. Therefore, Anti-MY9-bR may have clinical utility for in vitro purging of AML cells from autologous marrow when used at high IT concentrations for short incubation periods. Much lower concentrations of Anti-MY9-bR that can be maintained for longer periods may be useful for elimination of AML cells in vivo.

IMMUNOTOXINS (IT) are hybrid molecules consisting of a monoclonal antibody (MoAb) covalently linked to a toxin. IT have several theoretical advantages over conventional therapeutic agents including selectivity for tumor cells[1] and potential delivery of extremely potent toxins.[2,3] However, there have been several obstacles to the use of IT in leukemia therapy. First, it has been difficult to find MoAbs directed specifically against antigens found on clonogenic leukemia cells and absent from most normal hematopoietic precursor cells. Second, it has been difficult to find a highly potent toxin without significant nonspecific toxicity. Most single chain toxins, like pokeweed antiviral protein (PAP), saporin, and gelonin, have shown little toxicity against leukemic targets when linked to MoAbs.[4] Similar results were obtained with immunotoxins containing ricin A-chain alone.

In this study, we report the development of a novel IT that is composed of the MoAb anti-MY9 and a modified whole ricin toxin. The MoAb anti-MY9 (anti-CD33) is potentially ideal for targeting of acute myeloid leukemia (AML) cells because it reacts with clonogenic AML cells from greater than 80% of cases.[5] Previous studies have shown that CD33 antigen is present on normal colony-forming unit granulocyte-monocyte (CFU-GM), on a fraction of burst-forming unit-erythroid (BFU-E) and CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM),[5,6] and absent from normal pluripotent stem cells.[7,8] One of the most powerful natural toxins, whole ricin, consists of two chains: an A-chain that inactivates 60S ribosomes and a B-chain that binds to galactose moieties found on the surface of all eukaryotic cells. We have devised a strategy to irreversibly block the binding sites of whole ricin, effectively blocking nonspecific binding to normal cells, while leaving both chains for increased intracellular toxicity.[9] This "blocked-Ricin" has been covalently linked to anti-MY9 forming an IT with increased cytotoxic activity.

We evaluated the capacity of Anti-MY9-blocked-Ricin (Anti-MY9-bR) to selectively eliminate AML cells under conditions compatible with both in vitro and in vivo use. We found that Anti-MY9-bR was highly toxic against AML cells when used under conditions simulating marrow purging (high concentrations of IT for short incubation periods) or in conditions simulating in vivo infusions (lower concentrations of IT for longer exposures). Compared with anti-MY9 and rabbit complement (C'), Anti-MY9-bR induced more depletion of leukemic cells and was less toxic to CFU-GM. These findings suggest that this IT may have clinical utility as both a purging agent for autologous bone marrow transplantation (BMT) and as a direct therapeutic agent.

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MATERIALS AND METHODS

Bone Marrow (BM) Cells

After obtaining informed consent under protocols approved by the Human Subjects Protection Committee of the Dana-Farber Cancer Institute, BM was obtained from healthy volunteer donors. BM mononuclear cells (BMMC) were isolated by Ficoll-Hypaque density gradient centrifugation. BMMC received 40 Gy of irradiation at 11.1 Gy/min (^{137}Cs; Gamma Cell, Atomic Energy of Canada, Ottawa) before they were mixed with leukemia cells in purging experiments.

Leukemic Cell Line

The cell line HL-60 is a human promyelocytic leukemia cell line expressing MY9 (CD33). Cells were grown in RPMI 1640 medium (GIBCO Labs, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone Lab, Logan, UT), 2% L-glutamine, 1% sodium pyruvate, and 1% penicillin and streptomycin.

Primary Leukemic Cells

Clonogenic AML cells were obtained from diagnostic specimens of 12 untreated patients originating from peripheral blood and, rarely, BM (two patients) samples. AML was diagnosed according to French-American-British (FAB) classification.[10] Mononuclear cells were prepared by Ficoll-Hypaque sedimentation and cryopreserved in 10% dimethyl sulfoxide (DMSO) in the vapor phase of liquid nitrogen. All leukemia samples contained greater than 75% blasts. The percentage of blasts expressing MY9 antigen varied from 60% to 91%.

MoAbs and Immunoconjugates

Both Anti-MY9-bR and Anti-B4-bR were supplied by ImmunoGen Inc (Cambridge, MA). These IT were prepared in several steps as described by Lambert et al.[9] First, a triantennary *N*-linked oligosaccharide from fetuin was modified to afford a reagent that contained a terminal residue of 6-*N*-methylamino-6-deoxy-D-galactose on one branch of the triantennary structure and terminal galactose residues on the other two branches. The ligand was activated with cyanuric chloride and the resulting dichloro-triazine derivative of the ligand reacted with ricin, forming a stable covalent linkage. Anti-MY9-bR was made by covalently linking a single molecule of this irreversibly blocked ricin to anti-MY9, an IgG2a MoAb with anti-CD33 specificity[5] developed at ImmunoGen. Anti-B4-bR (anti-CD19-bR) consisted of an anti-B4 IgG1 antibody with specificity against a 95-Kd glycoprotein absent from AML cells[11] that was linked covalently to blocked-Ricin. Each IT had less than 0.1% free ricin and was pyrogen-free. Stable linkage of the MoAb to the blocked-Ricin was maintained for a period of at least 6 months when kept at 4°C. Anti-J5 was an IgG2a anti-CD10 MoAb (Coulter Immunology, Hialeah, FL).

Treatment of Cells

Immunoconjugates. Suspensions of leukemic cells with a 20-fold excess of irradiated BMMC were treated at 1×10^7 cells/mL with IT. Treatments were performed in RPMI 1640 supplemented with 2.5% FBS for high concentrations of IT (as compatible with in vitro marrow purging) and with 2.5% human AB serum (HAB) for low concentrations of IT (as compatible with in vivo administration). IT concentrations varied from 2.5×10^{-8} mol/L to 5×10^{-13} mol/L and the incubation period at 37°C ranged from 15 minutes to 72 hours.

Complements. Suspensions of leukemic cells with a 20-fold excess of irradiated BMMC were incubated at 1×10^7 cells/mL with MoAb at saturating concentrations for 15 minutes at 4°C followed by incubation with C′ (3- to 4-week-old rabbit serum; Pel-Freeze Inc, Brown Deer, WI) at 37°C for 30 minutes. Treatment with antibody and C′ was repeated twice.

Limiting Dilution Assay

After treatment with either IT or antibody and C′, cells were washed three times and plated in a limiting dilution assay (LDA) as described previously.[12] Briefly, each treatment sample was serially diluted from 5×10^5 to 0.5 cells per 100 μL in RPMI 1640 supplemented with 10% FBS. From 24 to 48 aliquots of each dilution were plated in flat bottom microculture plates (Nunclon, Nunc, Denmark). Cells were fed every 4 days and incubated at 37°C for 14 to 18 days. Growth at each serial dilution was assessed in an "all-or-nothing" (positive or negative) fashion under an inverted phase microscope. Frequency of clonogenic cells within the test population was estimated using χ^2 minimization, which was shown to provide maximum accuracy and precision.[13] This LDA allowed detection of a maximum of 4.4 ± 0.3 logs depletion of HL-60 cells in a 20-fold excess of normal BMMC.

CFU-GM and Leukemic Blast Colony Assay

BMMC or primary AML cells were treated with either IT or MoAb and C′. CFU-GM and leukemic blast colonies (CFU-L) were assayed in semi-solid agar (Agar Noble; Difco Laboratories, Detroit, MI) by a modification of the method of Pike and Robinson.[14] Underlayers (0.5 mL) were composed of 0.5% agar in Iscoves modified Dulbecco's minimum essential medium (IMDM) with 20% FBS. As a source of colony-stimulating factor, 20% conditioned medium from the bladder carcinoma 5637 cell line was added to the underlayer. The overlayer (0.5 mL of 0.3% agar) contained 0.5 to 3.0×10^5 normal BMMC or AML cells. The cultures were set up in quadruplicate in 24-well plastic culture plates (Linbro; Flow Laboratories Inc, McLean, VA) and incubated at 37°C in 5% CO_2 and humidified air. After 7 and 14 days of culture, overlayers were removed, dried onto glass slides, and stained with Gill's hematoxylin (Fisher Scientific, Orangeburg, NY). CFU-GM colonies were considered as aggregates of greater than 40 cells, clusters as aggregates of 8 to 40 cells. All primary AML colonies stained positively for either specific or nonspecific esterase, or both. CFU-L colonies were considered as aggregates of greater than 20 blast cells.

BFU-E and CFU-GEMM Assay

Erythroid colonies were grown in IMDM containing 0.9% methylcellulose, 30% FBS, 0.9% bovine serum albumin (BSA), 2×10^{-4} mol/L 2-mercaptoethanol, 2 U/mL erythropoietin (Amgen Biologicals, Thousand Oaks, CA), and 10% Mo cell-conditioned medium.[15] Cells were plated at 10^5/mL and the large, multicentric, and late hemoglobinizing bursts scored as BFU-E at 14 days. In other replicate cultures, the addition of erythropoietin was delayed for 3 to 5 days, and CFU-GEMM were enumerated in these cultures after 14 days.[16]

RESULTS

Effect of Concentration and Length of Incubation With Anti-MY9-bR on Leukemic Cell Depletion Using a Limiting Dilution Assay

High Anti-MY9-bR concentrations. To determine the effectiveness of Anti-MY9-bR for depletion of leukemic

cells from a marrow graft, we used an in vitro model in which HL-60 cells (CD33+) were mixed with a 20-fold excess of normal irradiated BMMC. This mixture was treated with various concentrations of Anti-MY9-bR for 2 hours and plated in a limiting dilution assay. As controls, cells were treated with either anti-MY9 (CD33) antibody alone or Anti-B4-bR, an IT recognizing CD19, an antigen present on B cells but absent from HL-60 cells. Anti-MY9-bR depleted HL-60 cells in a concentration-dependent manner (Fig 1A). Treatment with 5×10^{-10} mol/L Anti-MY9-bR depleted less than 1 log of leukemic cells, while treatment with 10^{-8} mol/L Anti-MY9-bR eliminated 4.4 logs of HL-60 cells. Treatment with Anti-B4-bR was not significantly more toxic than media control, even at the highest concentration (10^{-8} mol/L). When Anti-MY9-bR 10^{-8} mol/L was incubated for 2 hours with Nalm-6 cells, a cell line not expressing CD33, no cytotoxicity was detected (data not shown).

The cytotoxic activity of Anti-MY9-bR was also found to be dependent on time of exposure (Fig 1B). Anti-MY9-bR (5×10^{-9} mol/L) destroyed 1 log of leukemia cells after 15 minutes, and up to 3.6 logs after 6 hours. Under these conditions, Anti-B4-bR was not toxic to HL-60 cells.

Low Anti-MY9-bR concentrations. To simulate conditions compatible with in vivo administration of Anti-MY9-bR, HL-60 cells were treated with lower concentrations of

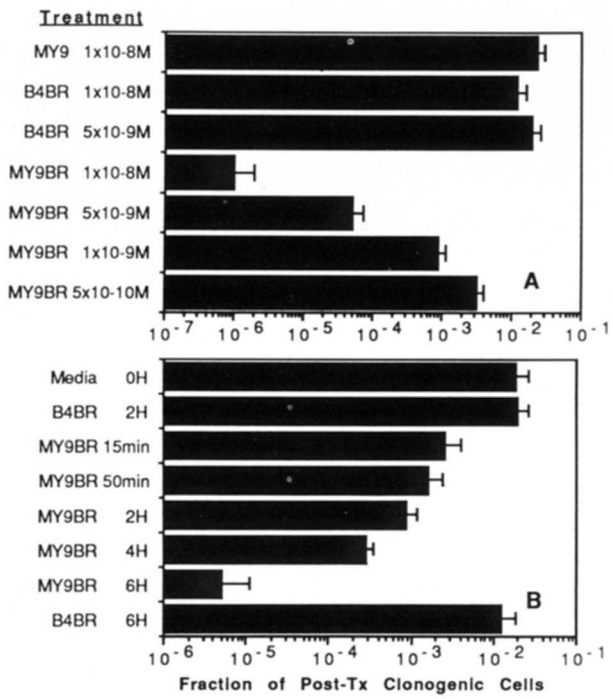

Fig 1. Effectiveness of Anti-MY9-bR for depletion of leukemia cells using limiting-dilution analysis. (A) HL-60 cells in a 20-fold excess of irradiated normal BMMC were incubated for 2 hours with unconjugated anti-MY9 (MY9), Anti-B4-bR (B4BR), and Anti-MY9-bR (MY9BR) at concentrations varying from 10^{-8} mol/L to 5×10^{-10} mol/L. (B) HL-60 + BMMC were incubated with Anti-MY9-bR 5×10^{-9} mol/L for up to 6 hours. The number of post-Tx clonogenic cells is expressed as a fraction (mean ± SE of two experiments) of the total number of cells plated (HL-60 + BMMC).

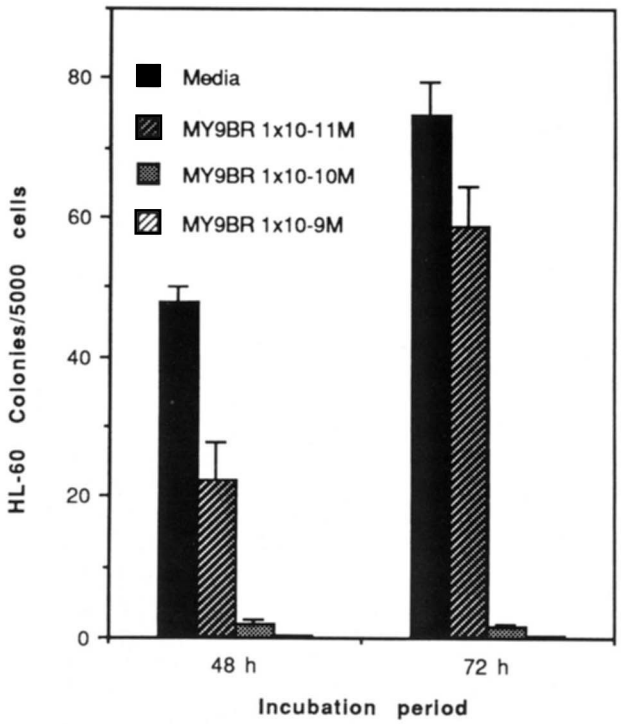

Fig 2. Effect of Anti-MY9-bR used at low concentrations for prolonged incubation periods. HL-60 cells were incubated for 48 and 72 hours with Anti-MY9-bR at concentrations of 10^{-9} mol/L to 10^{-11} mol/L and clonogenic growth (mean ± SE) evaluated in semisolid agar.

Anti-MY9-bR for more prolonged periods of time. As shown in Fig 2, incubation for 48 hours with Anti-MY9-bR at 10^{-11} mol/L, 10^{-10} mol/L, and 10^{-9} mol/L eliminated 0.33, 1.4, and 2.4 logs of clonogenic cells, respectively, as assayed in semisolid agar. Leukemic cells incubated in media for 72 hours grew more than cells incubated in media for 48 hours, but the extent of depletion nevertheless depended on the period of incubation with Anti-MY9-bR (Fig 2). Anti-MY9-bR at 10^{-10} mol/L for 72 hours destroyed 1.7 logs of these cells and at 10^{-9} mol/L induced depletion of at least 2.6 logs of clonogenic cells (the lower limit of detection of this assay).

Mechanism of Anti-MY9-bR Toxicity

To evaluate if the B-chain binding sites of blocked-ricin in Anti-MY9-bR were completely blocked, we used our LDA assay to measure cytotoxicity towards CD33+ target cells in the presence and absence of galactose (Fig 3A). Incubation of Anti-MY9-bR (5×10^{-9} mol/L) with galactose (0.1 mol/L) for 90 minutes depleted the same number (1.5 ± 0.1 log) of HL-60 cells in BMMC as incubation with Anti-MY9-bR alone. In contrast, after addition of a 100-fold excess of unconjugated anti-MY9 MoAb, depletion of HL-60 cells by Anti-MY9-bR decreased dramatically. Addition of both galactose and anti-MY9 to Anti-MY9-bR did not inhibit further the activity of Anti-MY9-bR, confirming specific targeting of leukemia cells through the MoAb moiety.

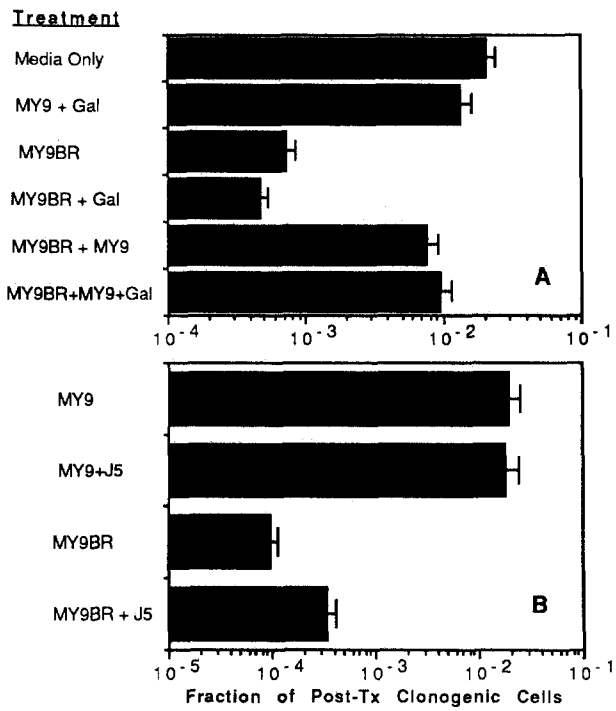

Fig 3. Specificity of the anti-leukemia activity of Anti-MY9-bR. HL-60 + BMMC were incubated with (A) Anti-MY9-bR 5 × 10^{-9} mol/L for 90 minutes in the presence of galactose 0.1 mol/L (Gal) and/or a 100-fold excess of anti-MY9 MoAb (CD33). The possibility of Fc binding was evaluated by incubating HL-60 + BMMC with (B) Anti-MY9-bR 5 × 10^{-9} mol/L for 2 hours with or without adding an MoAb of the same IgG2a isotype (J5).

Anti-MY9-bR could still have bound to targets through the Fc portion instead of the Fab portion of its anti-MY9 MoAb moiety. To examine this possibility, a 100-fold excess of an irrelevant MoAb of the same IgG2a isotype, anti-J5 (CD10), was added to Anti-MY9-bR. Anti-J5 does not react specifically with target cells but Fc binding does occur and competes with Fc binding of anti-MY9. Incubation of HL-60 cells alone with Anti-MY9-bR (5 × 10^{-9} mol/L for 2 hours) inhibited growth of 98.6% ± 0.1% (mean ± SE) of HL-60 cells as measured with the same LDA, while the addition of anti-J5 to Anti-MY9-bR caused 91.9% ± 1.1% inhibition of growth. This 6.7% difference in the elimination of HL-60 cells may be attributable to Fc binding. When HL-60 cells were treated in the presence of BM (Fig 3B), Anti-MY9-bR eliminated 99.5% ± 0.1% of leukemic cells, and after addition of anti-J5, 98.3% ± 0.2% of HL-60 cells was inhibited. Thus, the activity of Anti-MY9-bR decreased only slightly in the presence of anti-J5, especially in the presence of BM, suggesting that little Fc binding was occurring and that binding through the antigen-binding sites of anti-MY9 mediated killing by Anti-MY9-bR.

Effect of Cell Concentration on Leukemic Cell Depletion With Anti-MY9-bR

To determine the optimal conditions for in vitro treatment of BM with Anti-MY9-bR, we evaluated the effect of cell density on depletion of HL-60 cells in a 20-fold excess of BMMC. Five different concentrations of HL-60 + BMMC (ranging from 1 × 10^7 to 1 × 10^8 total cells/mL) were treated with either unconjugated anti-MY9 or Anti-MY9-bR at 5 × 10^{-9} mol/L for 2 hours at 37°C. For each cell concentration evaluated, the ratio of HL-60 cells to BM cells was constant. Over the range tested, concentration did not affect Anti-MY9-bR toxicity, which was 2.1 ± 0.2 logs.

Anti-MY9-bR Is Toxic to Primary Clonogeneic AML Cells

The cytotoxic activity of Anti-MY9-bR was subsequently measured against clonogenic AML cells obtained from 12 different patients. At high concentrations of Anti-MY9-bR (10^{-9} mol/L to 10^{-8} mol/L), elimination of AML cells was dose- (Fig 4A) and time-dependent (Fig 4B). Depletion of 94.7% of AML cells occurred after 2 hours of incubation with Anti-MY9-bR 10^{-8} mol/L. With this short incubation period, very low concentrations (2 × 10^{-10} mol/L) of Anti-MY9-bR were not toxic. Anti-B4-bR was nontoxic under identical conditions. Elimination of primary AML cells with lower concentrations of Anti-MY9-bR for longer incubation periods was also evaluated. At these lower concentrations (5 × 10^{-13} mol/L to 10^{-9} mol/L), increasing the incubation period with Anti-MY9-bR again showed elimination of AML cells in a dose- and time-dependent manner. Thus, 46% of AML cells were depleted after 24 hours of incubation with Anti-MY9-bR (5 × 10^{-11} mol/L) (data not shown) and prolonging the incubation to 48 hours increased depletion of primary AML cells to 78% (Fig 4C). Even concentrations of Anti-MY9-bR as low as 5 × 10^{-13} mol/L eliminated 28% of leukemic cells when the incubation period was extended to 48 hours.

Comparison of Anti-MY9-bR and Anti-MY9 Plus Rabbit Complement

The efficacy of in vitro treatment with Anti-MY9-bR IT used in conditions simulating autologous tumor cell purging was compared with that of treatment with anti-MY9 MoAb and baby rabbit complement. Three sequential treatments with anti-MY9 + C′ depleted 3.6 ± 0.3 logs of clonogenic HL-60 cells in a 20-fold excess of normal irradiated BMMC while anti-MY9 alone or complement alone did not cause significant killing. Treatment with Anti-MY9-bR 10^{-8} mol/L for 5 hours eliminated all detectable leukemic cells (LDA detection threshold = 4.4 ± 0.3 logs of HL-60 cells).

Effect of Anti-MY9-bR on Normal Hematopoietic Progenitor Cells

Because some normal hematopoietic progenitors express CD33 (MY9) antigen, we evaluated the toxicity of Anti-MY9-bR against CFU-GM, BFU-E, and CFU-GEMM. With a 2-hour incubation period, elimination of CFU-GM by Anti-MY9-bR (10^{-8} mol/L) was concentration-dependent (Fig 5A). Longer periods of exposure to Anti-MY9-bR (2.5 × 10^{-10} to 10^{-8} mol/L) resulted in increased toxicity (Fig 5B). Incubation of BMMC with 2.5 × 10^{-9} mol/L Anti-MY9-bR for 14 and 24 hours destroyed 76.3% and 89.3% of CFU-GM, respectively. Normal BM was also incubated with anti-MY9 or Anti-MY9-bR at the highest

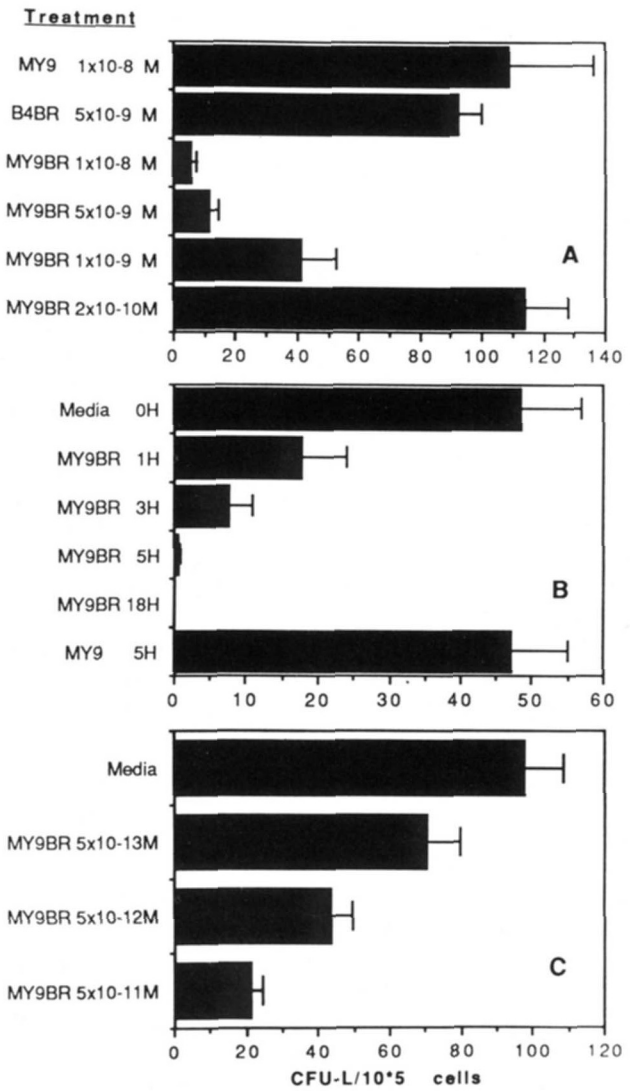

Fig 4. Activity of Anti-MY9-bR on primary AML cells. Clonogenic growth of primary AML cells after incubation with (A) high concentrations (10^{-9} mol/L to 2×10^{-10} mol/L) of unconjugated anti-MY9, Anti-B4-bR, or Anti-MY9-bR for 2 hours (mean ± SEM of AML cells from three patients), (B) unconjugated anti-MY9 or Anti-MY9-bR (10^{-8} mol/L) for incubation periods varying from 0 to 18 hours (mean ± SEM of AML cells from three patients), and (C) low concentrations of Anti-MY9-bR (5×10^{-11} to 5×10^{-13} mol/L) for 48 hours (mean ± SEM of AML cells from six patients).

concentration (10^{-8} mol/L) for periods up to 18 hours. Recovery of CFU-GM, BFU-E, and CFU-GEMM was identical after treatment with anti-MY9 MoAb or media alone. As shown in Table 1, increasing the incubation period with Anti-MY9-bR gradually decreased recovery of day 7 and 14 CFU-GM. After 5 hours of incubation with Anti-MY9-bR 10^{-8} mol/L, 70.7% and 77.3% of day 7 and 14 CFU-GM were depleted, respectively. After 18 hours, the same concentration of IT inhibited growth of more than 99% of CFU-GM.

The effect of different incubation periods with Anti-MY9-bR 10^{-8} mol/L on BFU-E and CFU-GEMM is also detailed in Table 1. Inhibition of BFU-E and CFU-GEMM clonogenic cells increased progressively with the length of the incubation period with Anti-MY9-bR. After 18 hours of incubation, Anti-MY9-bR inhibited growth of BFU-E by 46.1% and CFU-GEMM by 76.9%.

Comparison of Anti-MY9-bR Versus Anti-MY9 + C' Toxicity Against Normal Hematopoietic Progenitors

The number of normal hematopoietic progenitors remaining after treatment with unconjugated anti-MY9 and C' for three cycles was compared with that after treatment with

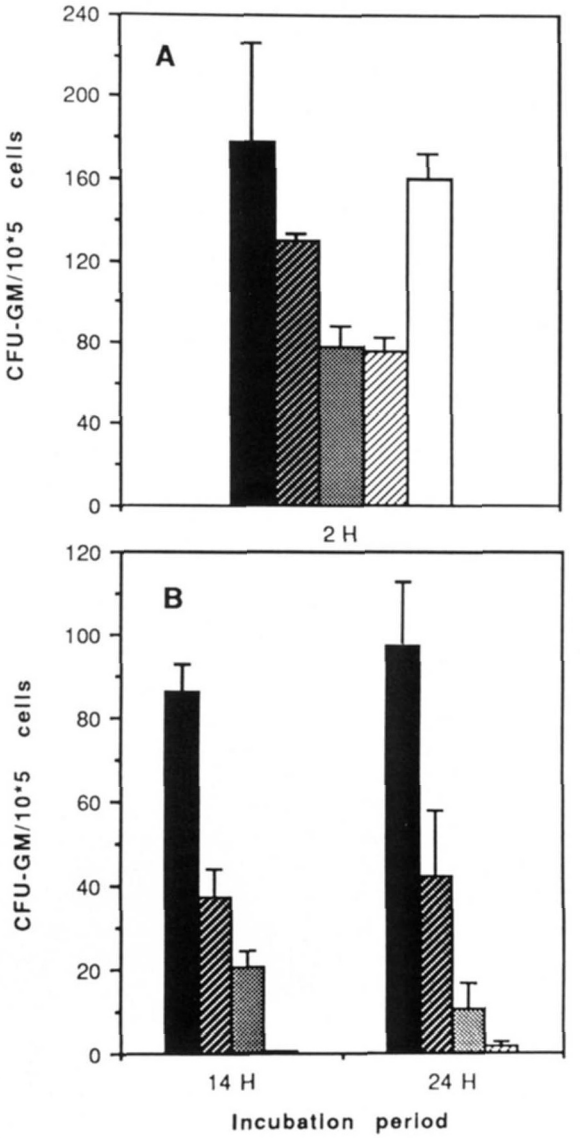

Fig 5. Toxicity of Anti-MY9-bR on CFU-GM progenitors. Normal BMMC were incubated for (A) 2 hours and (B) 14 and 24 hours with media alone, Anti-MY9-bR, or Anti-B4-bR at concentrations ranging from 10^{-8} mol/L to 2.5×10^{-10} mol/L and the number of CFU-GM colonies and clusters (mean ± SEM) scored after 14 days. Concentrations are as follows: (A) (■) Media. MY9BR: (▨) 1×10^{-9}, (▩) 5×10^{-9}, (▨) 1×10^{-8} mol/L. (□) B4BR 1×10^{-8} mol/L. (B) (■) Media. MY9BR: (▨) 2.5×10^{-10}, (▩) 2.5×10^{-9}, (▨) 2.5×10^{-8} mol/L.

Table 1. Effect of Anti-MY9-bR Incubation Times on Hematopoietic Progenitors

Progenitor Cells	Control*	Anti-MY9-bR 1 h†	Anti-MY9-bR 3 h	Anti-MY9-bR 5 h	Anti-MY9-bR 18 h‡
CFU-GM	126.8 ± 33.4	79.8 ± 30.5	66.2 ± 17.7	37.2 ± 16.3	0.8 ± 0.5
Day 7	(0)	(37.1)	(47.8)	(70.7)	(99.1)
CFU-GM	69.7 ± 22.2	32.2 ± 13.9	23.2 ± 9.1	15.8 ± 6.6	0.3 ± 0.3
Day 14	(0)	(53.8)	(66.7)	(77.3)	(99.4)
BFU-E	32.6 ± 5.1	24.6 ± 3.8	19.5 ± 3.0	18.9 ± 2.7	6.9 ± 0.9
	(0)	(24.5)	(40.2)	(42.0)	(46.1)
CFU-GEMM	2.3 ± 0.4	2.2 ± 0.5	1.6 ± 0.4	1.3 ± 0.3	0.3 ± 0.2
	(0)	(4.3)	(30.4)	(43.5)	(76.9)

Mean ± SD (% inhibition of growth calculated as compared with control) of three experiments with each count in quadruplicate.

*Anti-MY9 unconjugated antibody (10^{-8} mol/L) incubated at 37°C for 5 hours.

†Anti-MY9-bR (10^{-8} mol/L) incubated at 37°C for all incubation periods.

‡(% inhibition of growth) for Anti-MY9-bR 18 hours is calculated as compared with BMMC cultured in anti-MY9 unconjugated MoAb for 18 hours.

Anti-MY9-bR 10^{-8} mol/L for 5 hours. As shown in Table 2, CFU-GM were decreased after both types of marrow treatments, but while anti-MY9 + C′ treatment eliminated all day 7 and 14 CFU-GM, Anti-MY9-bR preserved some CFU-GM ($P < .005$). Although there was large variability in the number of BFU-E and CFU-GEMM remaining in different patient samples, we found no difference in the inhibition of BFU-E and CFU-GEMM after treatment with either anti-MY9 + C′ or anti-MY9-bR.

Comparative Activities of Anti-MY9-bR Activity Against Various Targets

The overall effect of Anti-MY9-bR concentration on depletion of different MY9+ cell populations was evaluated in conditions simulating in vitro and in vivo administration. Results following incubation of HL-60, primary AML, and normal BM cells with Anti-MY9-bR at relatively high concentrations (5×10^{-10} mol/L to 10^{-8} mol/L) for short (2-hour) incubation periods are compiled in Fig 6A. HL-60 cells were more sensitive to Anti-MY9-bR than primary AML cells. Interestingly, primary AML cells were more sensitive to Anti-MY9-bR than CFU-GM. When the incubation period was prolonged to 24 hours, lower concentrations of Anti-MY9-bR were found to exhibit significant cytotoxicity. As shown in Fig 6B, HL-60 cells were more sensitive to Anti-MY9-bR than primary AML cells. In contrast to short incubations, primary AML cells and CFU-GM cells were similarly sensitive to long (24-hour) incubations with Anti-MY9-bR. For comparison, the same concentrations of Anti-MY9-bR caused little toxicity to CD33− Namalwa target cells.

DISCUSSION

We report here an IT that is highly toxic to AML cells in vitro. The first feature differentiating Anti-MY9-bR from other IT is its toxin moiety, which consists of both ricin A-chain and a modified B-chain that has its galactose binding sites blocked. Previous experiments with conjugates of anti-MY9 linked to purified ricin A-chain showed little cytotoxicity (J.D. Griffin, unpublished results), suggesting that the functions of the B-chain could not be entirely replaced by a MoAb. When free B-chains were added to single ricin A-chain IT, the cytotoxicity was markedly enhanced,[17] and it was suggested that the B-chain possibly facilitated transport of the IT into target cells.[18] In this study, whole "blocked" ricin conjugate displayed high levels of toxicity against CD33+ cells, providing support to the hypothesis that both chains, and possibly linkage of these chains, play an important role in generating cytotoxicity.

Several IT have been developed, mostly against T- and B-cell surface receptors,[2-4] but Anti-MY9-bR is one of the few reported IT with selective activity against myeloid cells[19] and the first IT against AML cells with potential for in vivo therapy. The specificity of Anti-MY9-bR for CD33+ cells was shown by demonstrating that galactose, the natural ligand of ricin, did not inhibit cytotoxicity, suggesting that unblocked ricin was not mediating the observed cytotoxicity. Further, Anti-MY9-bR was greater than 3 logs more toxic for CD33+ cells than CD33− cells and the cytotoxicity could be blocked by an excess of unconjugated anti-MY9 MoAb. Finally, addition of an isotype-specific MoAb did not inhibit Anti-MY9-bR from killing HL-60

Table 2. Inhibition of Normal Hematopoietic Progenitors by Anti-MY9 + C′ and Anti-MY9-bR

	CFU-GM D7	CFU-GM D14	BFU-E	CFU-GEMM
Anti-MY9 + C′*	99.0 ± 1.2	99.0 ± 1.5	39.6 ± 32.2	52.5 ± 46.2
Anti-MY9-bR†	77.8 ± 13.3‡	87.6 ± 8.0‡	33.2 ± 46.0	34.5 ± 59.2

Mean ± SD of % inhibition of colony formation of at least three separate experiments with each measurement performed in duplicate or quadruplicate.

*Treatment with anti-MY9 + C′ repeated for a total of three cycles.

†Treatment with Anti-MY9-bR (10^{-8} mol/L) incubated at 37°C for 5 hours.

‡Anti-MY9 + C′ versus Anti-MY9-bR: $P < .005$.

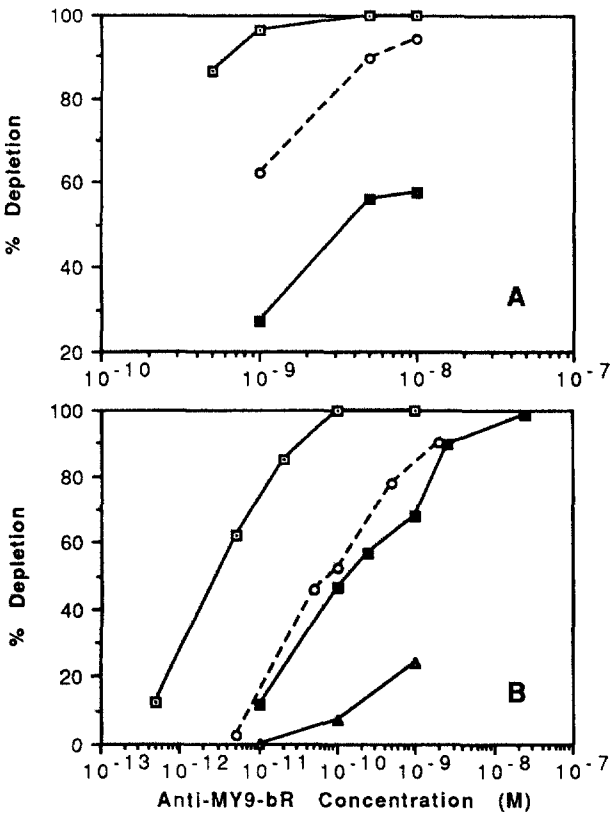

Fig 6. Comparative activities of Anti-MY9-bR on leukemia cells and normal hematopoietic progenitors. Percentage of depletion of HL-60 (—□—), primary AML (--○--), CFU-GM (—■—), and Namalwa cells (—△—) after (A) 2 hours and (B) 24 hours of incubation with Anti-MY9-bR at various concentrations.

cells, excluding the possibility that nonspecific Fc binding mediated the cytotoxicity of Anti-MY9-bR.

There is clinical evidence that patients with leukemia transplanted with autologous marrows in second or subsequent remission without purging of their marrow grafts do relatively poorly.[20,21] Thus, a number of studies have evaluated marrow purging to improve patient outcome. These studies have shown that autologous BMT was not only a feasible alternative for patients with ALL in second or subsequent complete remission who did not have an HLA-compatible donor,[22,23] but that the disease-free survival of patients transplanted with such purged marrows was not significantly different from that of patients treated by allogeneic BMT.[24] Similarly, purging methods, consisting mainly of 4-hydroperoxycyclophosphamide and MoAb + C′, were developed to eliminate residual AML cells before autologous BMT.[25,26] In this study, we showed that an IT, Anti-MY9-bR, was effective for purging of autologous BM, depleting more than 4.4 logs of leukemic cells. Interestingly, when Anti-MY9-bR was compared with anti-MY9 + C′, the IT could be used in conditions that eliminated more leukemia cells than anti-MY9 + C′. Compared with other purging methods, Anti-MY9-bR also provided the added benefit of not necessitating the complicated standardization procedures associated with complement use, or the sophisticated apparatus required for magnetic or laser purging methods. These observations suggest that Anti-MY9-bR is a potentially ideal agent for purging of AML cells.

The cytotoxic activity of Anti-MY9-bR against patient AML cells was evaluated using CFU-L as targets. More than 2 logs of these in vitro clonogenic AML cells, which are thought to represent the in vivo clonogenic AML cells,[6] were eliminated by this IT. In this study, the high levels of depletion of CFU-L exceeded the percentages (60% to 91%) of MY9-positive AML cells. This finding could be explained by the fact that some AML cells express few molecules of MY9 on their surface and escape detection by flow cytometry. In addition, it is also known that AML cells form a heterogeneous population of clonogenic and nonclonogenic cells. Previous studies have shown that these two populations of cells can be differentiated phenotypically.[6,27,28] Ia and MY9 antigens were found to be expressed on a significantly higher percentage of CFU-L than on the total AML cell population. In contrast, few CFU-L expressed Mo1 (CD11b) and Mo2 (CD14) and these antigens were found on a lower percentage of CFU-L than total AML cells.[28] These studies suggest that although AML cells have heteregeneous phenotypes, AML colony-forming cells are less heterogeneous and that the MY9 (CD33) antigen is expressed preferentially on the AML CFU-L.

One concern with using Anti-MY9-bR for purging is that it eliminates the majority of myeloid progenitors and could therefore prevent hematologic engraftment. At our institution, 11 patients have undergone autologous BMT with purging of the marrow graft using anti-MY9 + C′ treatment.[29] All patients engrafted, with a median time to absolute neutrophil count greater than 500/μL of 45 days after BMT (range 16 to 75). Time to white blood cell (WBC) engraftment was longer in these patients than in patients transplanted with autologous MoAb + C′ purged marrows for acute lymphoblastic leukemia[23] or lymphoma.[30] Although patients with AML had delayed engraftment, which correlated with total elimination of CFU-GM progenitors after anti-MY9 + C′ treatment, all these patients engrafted. Hematologic reconstitution thus probably originated from CFU-GEMM progenitors, which were only partially eliminated by such treatment, and from the hematopoietic stem cells that do not express CD33. The fact that all patients engrafted after anti-MY9 + C′ marrow treatment suggests that marrow treatment with Anti-MY9-bR, which targets the same MY9+ cells, will not prevent engraftment. In addition, Anti-MY9-bR, used in conditions for in vitro purging, depleted significantly less CFU-GM than anti-MY9 + C′. This finding suggests that patients receiving a marrow treated with Anti-MY9-bR might engraft more rapidly than after anti-MY9 + C′ treatment of the marrow, decreasing risks for infections and improving patient prognosis.

Surprisingly, when we compared the effect of Anti-MY9-bR on CFU-L and CFU-GM, we found that, for short incubation periods, leukemia cells were more sensitive to Anti-MY9-bR than normal hematopoietic progenitors. This difference in sensitivity between primary AML cells and

CFU-GM is difficult to explain because both types of cells express high levels of CD33 antigen.[5,28] It is possible that normal and leukemic cells have different internalization rates of IT, different sensitivities to intracellular IT, or different intracellular metabolism of IT. Purging of BM grafts provides an opportunity to take advantage of these different sensitivities of normal and leukemic cells to short incubations of Anti-MY9-bR.

An important aspect of IT is their potential for in vivo use. Early trials in which MoAb only were given intravenously to patients with leukemia showed that antibodies were effectively delivered to leukemic cells in both peripheral blood and BM, yet the clearance of circulating cells was transient and BM blasts were not affected.[31] These transient responses were primarily due to the limited host mechanisms available to kill antibody-coated leukemia cells.[32] Linking a toxic molecule to the MoAb could solve many of the problems of low leukemic toxicity, but covalent conjugation of MoAb with toxins has often been complicated by loss of activity.[2,3] In this study, we showed that blockage of galactose binding sites and coupling with anti-MY9 was achieved while preserving the cytotoxic potential of Anti-MY9-bR.

In vivo administration of IT is also very difficult because of the large distribution volume of the patient, IT metabolism, and an environment at 37°C that all contribute to decrease IT serum concentrations. In addition, enhancers like lysosomotropic amines or carboxylic ionophores,[33,34] that can be used in vitro to potentiate the toxicity of IT, are only active at very high concentrations that cannot be achieved in vivo. Thus, IT with potential for in vivo administration must be capable of maximal efficacy at low serum concentrations. A phase I study evaluating Anti-B4-bR, a very similar IT, for in vivo administration is presently ongoing at our institution. In these patients, doses of IT ranging from 10 to 30 μg/kg/d administered by continuous infusion were minimally toxic and achieved stable serum levels of 1 to 4 × 10^{-10} mol/L of Anti-B4-bR.[35] In the present study, Anti-MY9-bR at 10^{-10} mol/L depleted 1.7 logs of HL-60 cells after only 72 hours of incubation and even concentrations as low as 5 × 10^{-13} mol/L were able to eliminate 55% of primary AML cells after 48 hours without addition of enhancers. If we can extrapolate from these observations, Anti-MY9-bR should be a most active agent for in vivo treatment of patients with AML. A potential problem with prolonged infusions of Anti-MY9-bR is the depletion of MY9+ progenitors. In similar in vitro conditions, we found that CFU-GM were as sensitive to Anti-MY9-bR as AML cells. Thus, we would expect that doses of Anti-MY9-bR active against AML cells in vivo will also cause neutropenia. The fact that MY9 is absent from the surface of the hematopoietic stem cell combined with our previous experience with anti-MY9 + C′ purging of marrow grafts suggest that this toxicity will only be transient.

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