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In Vitro Bone Marrow Purging of Multidrug-resistant Cells with a Mouse Monoclonal Antibody Directed against M_r 170,000 Glycoprotein and a Saporin-conjugated Anti-Mouse Antibody[1]

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ABSTRACT

Selective elimination of multidrug resistance-positive cells (LoVo/Dx) was obtained by using the monoclonal antibody MRK 16, which recognizes a surface epitope of the M_r 170,000 glycoprotein, and a sheep anti-mouse immunoglobulin antibody, conjugated to the ribosome-inactivating protein saporin 6. The killing was greatly decreased or even abolished by adding the monoclonal antibody at a 100-fold concentration. Both the MRK 16 and anti-mouse saporin 6 conjugate did not show any killing activity when they were used separately. In cell suspensions composed of 90% normal bone marrow cells and 10% multidrug resistance-positive cells, the monoclonal antibody MRK 16 followed by the anti-mouse immunotoxin caused the elimination of 99% multidrug resistance-positive cells, as revealed by immunofluorescence and immunocytochemistry as well as by a clonal assay. Human normal hematopoietic precursors (granulomonocytic colony-forming units, erythroid burst-forming units, and multipotent granulomonocytic, erythroid, and megakaryocytic-forming units) were not affected by the MRK 16 plus immunotoxin treatment. This technique might be suitable for *ex vivo* bone purging in an appropriate clinical setting, such as autologous bone marrow transplantation.

INTRODUCTION

Repeated exposure of tumor cell lines to cytotoxic agents can lead to the development of sublines that are highly resistant to a number of drugs, including anthracyclines, *Vinca* alkaloids, epipodophyllotoxin derivatives, colchicine, and actinomycin D (1–3). This phenomenon of pleiotropic MDR[4] is based on increased transcription and, sometimes, on amplification of one or more normal genes that code for a membrane gp170 that acts as an energy-dependent drug efflux pump (1–6). The expression of the MDR phenotype has been looked for by measuring cytoplasmic MDR mRNA levels and, more recently, by detecting gp170 by immunoblotting and/or MoAbs against gp170 (1–3, 7). By these techniques it has been shown that MDR is expressed in normal adrenal gland, liver, renal, and colon cells, as well as in the tumors that are derived from these organs (3, 7–10). MDR has also been detected occasionally in several other tumors, including leukemia and lymphoma, especially when they are primarily resistant to chemotherapy or when they relapse after chemotherapy (7, 11–16). Although the clinical and therapeutic importance of MDR has not yet been assessed, available evidence suggests that it can play an important role in determining the outcome of treatment (7).

The MoAb MRK 16 that recognizes a gp170 surface epitope has already been used as a target of immunotoxin or complement-mediated cell lysis (17, 18). In this paper we describe the use of the MRK 16 MoAb as a target of an AM-IT composed of sheep anti-mouse immunoglobulin polyclonal antibodies conjugated to the ribosome-inactivating protein saporin 6. The tests have been performed on MDR+ and MDR− cell lines, normal human bone marrow-derived hematopoietic precursors, and MDR+ cells mixed with normal bone marrow, in order to simulate a therapeutic bone marrow purging. The results indicate that it is possible to eliminate MDR+ cells without affecting the number and the *in vitro* growth of hematopoietic precursors.

MATERIALS AND METHODS

Cell Lines. The colon carcinoma-derived cell line (LoVo) and its doxorubicin-resistant subline (LoVo/Dx) were used throughout all the experiments (19). The K562 cell line that is derived from Ph+ chronic granulocytic leukemia was used as an irrelevant cell line. All cells were maintained in 75-cm^2 flasks (Falcon) in exponential growth until harvesting in complete RPMI 1640 medium (Seromed) with 10% fetal calf serum (Seromed) and 1% glutamine and antibiotics (Flow).

Antibodies and Immunotoxins. The mouse MoAb MRK 16, which recognizes a surface epitope of gp170, was produced and characterized as described (20). It was used at a final concentration of 2.5 μg/ml throughout all the experiments, with the exception of one series in which the AM-IT was incubated in the presence of 250 μg/ml of MRK 16 in order to inhibit the binding of the conjugate. A second mouse MoAb (C219), which recognizes cytoplasmic epitopes of gp170 (a gift of Dr. V. Ling, Ontario Cancer Research Institute, Toronto, ON, Canada), was also used in this study. F(ab')$_2$ sheep anti-mouse IgG antibodies were purchased from Sigma and conjugated to saporin 6, a ribosome-inactivating protein obtained from the seeds of *Saponaria officinalis* (21), using SPDP as a linking agent (22).

The molar ratio between saporin 6 and the antibodies was 4.14. A mouse MoAb (8A) that was produced in our laboratory against a plasma cell membrane-associated antigen (23) was used for the synthesis of an irrelevant saporin immunotoxin.

Protein Synthesis Inhibition Assay. LoVo/Dx cells, adjusted at a concentration of 10^5/ml, were incubated in Falcon tubes for 30 min at 0°C (wet ice) with 2.5 μg/ml of MRK 16 MoAb, washed twice in phosphate-buffered saline, pH 7.4, and resuspended in 900 μl of complete RPMI 1640 medium. The cells were then incubated for 2 h at 37°C in a water-saturated, 5% CO_2 atmosphere with AM-IT (final dilution ranging from 0.1 to 100 nmol) and washed again with phosphate-buffered saline. Additional samples were also incubated (*a*) with MRK 16 alone, (*b*) with saporin 6 alone, (*c*) with AM-IT alone without any previous incubation with MRK 16, and (*d*) with an excess of MRK

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[4] The abbreviations used are: MDR, multidrug resistance; gp170, M_r 170,000 glycoprotein; MoAb, monoclonal antibodies; AM-IT, anti-mouse immunotoxin; MDR+, multidrug resistance-positive; MDR−, multidrug resistance-negative; CFU-GM, granulomonocytic colony-forming units; BFU-e, erythroid burst-forming units; CFU-GEMM, monocytic, erythroid, and megakaryocytic-forming units.

16 MoAb (250 μg/ml), without any additional wash before incubation with the AM-IT. A series of control experiments was performed using an irrelevant immunotoxin (8A-saporin 6) or an irrelevant cell line (K562). After 48 h at 37°C, the cells were centrifuged, and the medium was substituted with leucine-free RPMI 1640 (Eurobio) containing 0.5 μCi of L-[^{3}H]leucine. After 24-h incubation at 37°C, the samples were transferred onto glass filter diskettes by means of a Skatron cell harvester, and the radioactivity was counted with a scintillation β-counter with Instagel scintillation liquid (Packard). Each experiment was carried out in quadruplicate.

Tumor Cell Lines' Cloning Inhibition Assay. The cells were treated in the same way as above and, after the washes following incubation with the AM-IT, were seeded in Petri dishes (1000 cells/dish). The medium contained 850 μl of RPMI 1640 and 150 μl of human citrated plasma. The clotting of the medium was obtained by addition of 40 μl of a solution of calcium chloride (55 mg/ml). Colonies were counted with an inverted microscope after 6 to 8 days, according to cell cycle kinetics (22).

Human Normal Hematopoietic Precursors Inhibition Assay. Bone marrow cells were obtained by iliac puncture of fully informed human healthy volunteers. The mononuclear fraction, obtained by Ficoll-Hypaque gradient, was T-cell depleted by the erythrocyte rosette technique. The final percentage of T-cells was <1% in all samples. The cells were then adjusted at a concentration of 1×10^6 cells/ml in RPMI 1640 medium and incubated with MRK 16 MoAb and anti-mouse saporin 6 immunotoxin (10 nmol), as described above for cell lines. After incubation, the cells were washed and assayed for the growth of CFU-GM, BFU-e, and CFU-GEMM.

(*a*) To assay for granulomonocytic precursors (CFU-GM), 5×10^5 cells were resuspended in 2.5 ml of Dulbecco's minimal essential medium (Gibco) supplemented with 0.9% methylcellulose, 20% fetal calf serum (Flow), and 10% phytohemagglutinin-conditioned medium (a supernatant obtained from a 7-day human mononuclear fraction culture stimulated with 10 μg/ml of phytohemagglutinin) used as source of colony-stimulating activity (24). Cells were plated in Petri dishes (35 x 10 mm), and colonies (>50 cells) and clusters (20 to 50 cells) were scored after Days 7 and 14 of culture at 37°C. Experiments were performed in triplicate.

(*b*) To assay for erythroid precursors (BFU-e), 0.25×10^5 marrow cells were plated in 0.25 ml of methylcellulose containing Iscove's modified Dulbecco's medium supplemented with bovine serum albumin, transferrin, lecithin, natrium selenite, β-mercaptoethanol, 30% fetal calf serum, and human erythropoietin (25). Cells were plated in a cluster dish (24-well plates; Falcon) and kept at 37°C and 5% CO_2 in fully humidified air. Cultures were performed in quadruplicate. Colonies (more than 50 cells) were counted at Day 14.

(*c*) To assay for multipotent progenitors (CFU-GEMM), multipotent progenitors giving rise to mixed colonies were grown as described by Fauser and Messner (26). Colonies were scored at Day 15. Mixed colonies defined as containing erythroid cells plus at least 5% myeloid cells were always verified cytologically.

Bone Marrow Purging Simulation. Bone marrow mononuclear cells and LoVo/Dx cells were mixed in a 9:1 proportion, incubated for 30 min with MRK 16 MoAb, washed, incubated for 2 h with 10 nM AM-IT, washed again, and divided in two aliquots. The first aliquots were used for a clonal inhibition assay, as described above. After 8 days of culture, the clones were counted, picked up, and checked for their phenotype. The second aliquot was allowed to stay for 48 h at 37°C. Cells were then incubated with the same antibody and with fluorescein isothiocyanate-conjugated goat anti-mouse and analyzed with Facstar equipment (Becton-Dickinson) to evaluate the elimination of MRK 16-positive cells. A fraction of the same cells was centrifuged onto slides, fixed for 10 min in cold acetone, and stained with C219 antibody followed by biotin-conjugated rabbit anti-mouse immunoglobulin (Dako) and by Texas Red-conjugated streptavidin (Amersham) in order to evaluate residual MDR+ cells by means of a different epitope of the gp170 (27).

RESULTS

LoVo/Dx Cell Line. The MRK 16 MoAb and AM-IT brought about a strong inhibition of protein synthesis in the Adriamycin-resistant LoVo/Dx cell line. In fact, at 10 and 100 nM concentration, expressed as saporin content, the inhibition was 73% and 87% of control values, respectively. The AM-IT alone and the irrelevant immunotoxin caused an inhibition similar to that of free saporin 6. The inhibition was abolished or greatly decreased (at an immunotoxin concentration of 10 and 100 nmol, respectively) in the presence of 250 μg/ml of free MRK 16 MoAb (Table 1).

The effective killing of LoVo/Dx cells was confirmed by the high inhibition of clonogenicity in the samples that were treated with both MRK 16 and AM-IT, with a 50% inhibitory dose of 0.1 nmol (as saporin 6). The specificity of killing was proved by the absence of cloning inhibition when the same treatment was performed on LoVo and K562 cell lines, which do not express the gp170 epitope recognized by the MRK 16 MoAb (Fig. 1).

Hematopoietic Precursors. Either MRK 16 MoAb (2.5 μg/ml) or MRK 16 MoAb (2.5 μg/ml) with AM-IT (10 nmol as saporin 6) did not damage or inhibit normal human myeloid progenitors (CFU-GM, BFU-e, and CFU-GEMM) in three different experiments. The same growth rate was observed in both control and treated samples (data not shown).

Purging Simulation. In a series of 5 experiments, a 2-log elimination of MRK 16-positive cells was obtained by treatment with MRK 16 and AM-IT. The results were also confirmed by the evaluation of cytospins, using the C219 MoAb as a marker of MDR+ residual cells. In the untreated samples the percentage of MDR+ cells ranged from 11% to 13%. In the samples incubated with the MRK 16 (2.5 μg/ml) and AM-IT (10 nmol as saporin 6), MDR+ residual cells, as determined by fluores-

Table 1 *Protein synthesis inhibition of the LoVo/Dx cell line after incubation with 2.5 μg/ml of MRK 16 plus AM-IT*

Controls were performed with MRK 16 alone, saporin 6 alone, AM-IT alone, and AM-IT in the presence of free MRK 16 (250 μg/ml), with irrelevant immunotoxin. A significant inhibition of protein synthesis was shown only upon addition of MRK 16 (2.5 μg/ml) plus AM-IT (10 and 100 nmol; as saporin 6 content).

Addition	Protein synthesis	
	Mean ± SD (cpm)	% of controls
None	26,826 ± 718	100
MRK 16	24,118 ± 688	89
Saporin 6		
100	19,272 ± 612	71
10	25,965 ± 815	97
1	27,587 ± 799	100
0.1	27,186 ± 824	100
AM-IT		
100	17,952 ± 568	67
10	25,761 ± 724	97
1	25,913 ± 777	97
0.1	26,455 ± 782	99
MRK 16 (2.5 μg/ml) + AM-IT		
100	3,515 ± 212	13
10	7,424 ± 234	27
1	16,314 ± 414	61
0.1	22,518 ± 522	84
Excess of free MRK 16 (250 μg/ml, not washed) + AM-IT		
100	23,658 ± 664	88
10	24,986 ± 722	93
1	25,615 ± 726	97
0.1	27,112 ± 790	100
Irrelevant immunotoxin (8A-saporin 6)		
100	21,279 ± 650	79
10	24,414 ± 688	93
1	24,866 ± 706	97
0.1	26,119 ± 754	99

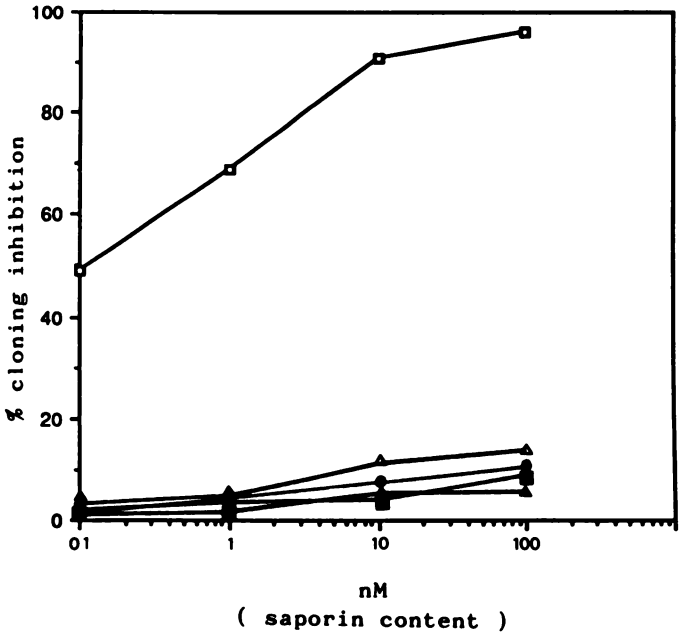

Fig. 1. Inhibition of clonogenic activity of target (LoVo/Dx) and nontarget (LoVo and K562) cell lines after treatment with MRK 16 and AM-IT. ■, LoVo/Dx cells treated with free saporin 6; △, AM-IT alone; □, MRK 16 + AM-IT; ▲, LoVo cells treated with MRK 16 + AM-IT; ●, K562 cells treated with MRK 16 + AM-IT.

cent (MRK 16 MoAb) or immunocytochemical (C219 MoAb) methods, ranged from 0.08% to 0.8%, depending on the method of evaluation. No residual MDR+ colonies could be detected. In fact, the very few tumor colonies that grew after immunotoxin treatment were analyzed for their phenotype and shown to be all MDR−.

DISCUSSION

The rapid development of the knowledge of pleiotropic MDR has provided a number of tools, including several MoAbs recognizing different epitopes of gp170. These reagents could be used to recognize and to evaluate the frequency and the clinical relevance of MDR in human cancer and leukemia (27, 28). Alhough the MDR phenotype might confer to tumor cells a selective advantage over the normal cells, nevertheless it could provide a distinctive difference between tumor and normal cells (29). This difference can be exploited from a therapeutical point of view to verify the possibility of killing tumor cells while sparing the normal ones.

This study showed that LoVo/Dx cells, which are MDR+, were recognized by MRK 16 and killed after the exposure to the MoAb and AM-IT. The specificity of the killing effect was demonstrated by the survival of the LoVo/Dx cells when incubated with an irrelevant immunotoxin as well as with an excess of free MRK 16 MoAb. Moreover, subsets of normal human hemopoietic stem cells (CFU-GM, BFU-e, CFU-GEMM) were not recognized by either the MRK 16 MoAb alone or the AM-IT/MRK 16 MoAb complex.

These results fit with previous experiments in which the MRK 16 MoAb was already used as a carrier of *Pseudomonas* esotoxin (17) or as a target of complement-mediated cell lysis (18) in order to obtain a selective killing of MDR+ cells.

The method described may offer advantages in comparison with other systems on the following bases. (*a*) The killing rate does not rely on complement, so that the variability and the toxicity due to different activities of rabbit serum batches are avoided (22, 30, 31). (*b*) Saporin 6 is one of the most powerful ribosome-inactivating proteins described to date and does not contain B-chain activity, thus avoiding unspecific binding; thus its conjugates have low *in vivo* toxicity in mice and primates (32). (*c*) More than one molecule of AM-IT can bind the same MoAb, so that several molecules of saporin 6 can be delivered to cells via each monoclonal antibody. (*d*) Moreover, the use of an indirect immunotoxin could allow the simultaneous targeting of both MDR-related protein and tumor-associated antigens without any need of preparing immunotoxins with different antibodies. (*e*) Finally it is noteworthy that, in our experiment, only a short (2-h) incubation time was required to produce MDR+ cell killing, either when LoVo/Dx cells were treated alone or mixed with normal bone marrow cells, and that under these experimental conditions the latter were completely spared. Therefore, the system described above can be suitable for the selective *ex vivo* elimination of MDR+ cells in the clinical setting of autologous bone marrow transplantation (33). To prove that it can be of value it has to be tested with naturally occurring MDR+ tumor or leukemia cells. In the latter, membrane gp170 expression can be lower than in laboratory-obtained MDR+ cell lines, and this could be a major problem that could be partly managed by simultaneous or sequential exposure to more than one gp170-directed MoAb, or by MDR-related drugs like *Vinca* alkaloids, to the patients before bone marrow harvesting, to increase MDR expression.

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