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In Vitro and *in Vivo* Effects of a Monoclonal Antibody-Toxin Conjugate for Use in Autologous Bone Marrow Transplantation for Patients with Breast Cancer

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ABSTRACT

We have devised a method utilizing a monoclonal antibody-toxin conjugate (LICR-LON-Fib75/abrin A-chain) for ridding bone marrow of infiltrating breast cancer cells to rescue patients with autologous bone marrow following high dose therapy.

Initially we examined the activity of this conjugate *in vitro*. Five of seven human breast cancer cell lines were killed following exposure at 10^{-8} M for 2 h; this concentration only reduced bone marrow colony formation to 83% (range, 50–100%) of control bone marrow.

We then examined the pattern of bone marrow recovery after high dose melphalan (200 mg/m^2) in patients with advanced breast cancer who were in remission following combination chemotherapy. To do this we compared the time of recovery of the blood count in three patients who received treated marrow and seven who received untreated marrow. Mean time to recovery of the peripheral white count (>1.5 × 10^9/liter) was 16.7 days (treated) and 18.3 days (untreated), respectively. Mean time to recovery of peripheral platelet count (>50 × 10^9/liter) was 23.7 days (treated) and 18.9 days (untreated), respectively.

Patients continued in remission for 1–>14 mo after high dose melphalan, and remission duration was similar in patients who received treated (6.2 mo) and untreated (7.3 mo) bone marrow.

These findings indicate that treatment of bone marrow with LICR-LON-Fib75/abrin A-chain conjugate does not significantly impair bone marrow recovery, and it is, therefore, possible to rescue breast cancer patients with bone marrow that has been cleansed of infiltrating cancer cells. This may have an application in patients with poor-risk primary breast cancer who have micrometastases and who may benefit from intensive therapy, but it has minimal application in patients with more advanced disease.

INTRODUCTION

Bone marrow toxicity is the limiting factor in the use of many cytotoxic drugs, and much larger and possibly more effective doses can be given safely if a portion of the patient's bone marrow is withdrawn before chemotherapy and reinfused when the drug has been metabolized and/or excreted (1). A major objection to this approach has been the possibility of infusing cancer cells along with the bone marrow used to rescue the patient. In an attempt to avoid this problem we have examined a number of MABs[2] recognizing determinants on human cell surfaces for their ability to selectively kill cancer cells while leaving the capacity of the bone marrow to reconstitute hematopoietic function unimpaired.

One such MAB, LICR-LON-Fib75 (2) (a murine IgG2a), was selected on the basis of homogeneous binding to breast cancer cells in all cell lines tested and its ability to kill cancer cells in the presence of added rabbit complement (3). Our earlier studies showed that this MAB was effective in killing cancer cells but that the addition of complement from several sources had a variably deleterious effect on bone marrow survival as assessed by an *in vitro* colony-forming assay. An alternative means of rendering antibodies cytotoxic is to link them to toxins of plant or bacterial origin (see Ref. 4 for a review). We have, therefore, explored the use of a conjugate between MAB LICR-LON-Fib75 and the A-chain of the plant toxin abrin to produce selective cancer cell killing in bone marrow *in vitro* and *in vivo*.

We have already determined that patients with poor-risk primary breast cancer have a high increase of bone marrow micrometastases (5). It is our ultimate aim to treat these patients with high dose therapy and bone marrow rescue. However, we initially wished to determine whether it was possible to use monoclonal-treated marrow to rescue patients with advanced disease, who had received high dose melphalan as part of a program to determine whether intensive therapy of this sort could extend their remission induced by conventional chemotherapy.

MATERIALS AND METHODS

Conjugate Preparation and Properties

The preparation of the conjugate in which MAB LICR-LON-Fib75 was linked by means of a disulfide bridge to the A-chain of abrin has been described elsewhere (6). The cytotoxic potential of the conjugate was characterized using the highly clonogenic cell line EJ, derived from a human bladder carcinoma and which also expresses the antigen recognized by LICR-LON-Fib75 in homogeneous fashion. In a protein synthesis inhibition assay, the conjugate LICR-LON-Fib75/abrin A-chain produced half-maximal killing following exposure for 120 min at a concentration of about 1.5 × 10^{-10} M. The abrin A-chain conjugate was 5 times more cytotoxic than the ricin A-chain analogue and was therefore preferred for clinical evaluation.

In Vitro Studies of Breast Cancer Cell Lines

Because of the difficulty of demonstrating toxin-mediated cell kill of breast cancer cells which are found to infiltrate the bone marrow (7) of a majority of patients with advanced breast cancer such as these, a series of model experiments was carried out using a panel of 7 well-characterized epithelial human breast tumor cell lines. Preliminary experiments using the highly clonogenic human bladder tumor cell line EJ demonstrated that LICR-LON-Fib75/abrin A-chain conjugate at a concentration of 10^{-10} M resulted in a 50% diminution in viability, as measured by enumeration of clones, while at 10^{-8} M the surviving fraction represented <0.1% of the treated cells. Addition of human nucleated bone marrow cells at a 100:1 ratio with respect to the tumor cells did not significantly shift this dose-response curve for inhibition of clonal growth.

None of the human breast tumor cell lines tested in this study possessed as high an intrinsic cloning efficiency as the EJ line. The effect of the toxin conjugate on them was, therefore, assessed by monitoring the growth of bulk cultures treated as a monolayer with the toxin-conjugate at concentrations ranging from 10^{-11}–10^{-8} M and maintained for a period of 10–14 days to determine at what concentration

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[2] The abbreviations used are: MABs, monoclonal antibodies; CFU-C, colony-forming units; ER, estrogen receptor.

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the majority (>90%) of the cells were killed. All cultures were treated with LICR-LON-Fib75/abrin A-chain for 2 h in serum-containing medium.

Cells to be treated were plated out in replicate aliquots in 24-well Costar tissue culture plates in complete tissue culture medium (Dulbecco's modified Eagle's medium) with 10% FCr. When the wells were approximately 25% confluent as assessed by phase contrast microscopy they were treated, in quadruplicate, with doses of 10^{-11}, 10^{-10}, 10^{-9}, and 10^{-8} conjugate; control wells were untreated.

Plates were examined daily by phase contrast to determine the dose at which cell proliferation was inhibited and at which cell death ensued as evidenced by detachment from the monolayer and cytolysis. When control wells had reached confluence, or at 10–14 days posttreatment, whichever was the sooner, the plates were fixed in formol saline, and the residual monolayers were stained with Giemsa so that the final cell density achieved after conjugate treatment could be assessed. If the residual monolayer was 10% or less of the control wells and no proliferation had been detected during the posttreatment phase, the corresponding conjugate concentration was decreed effectively cytotoxic.

Treatment of Bone Marrow

Initial Evaluation. The CFU-C survival of bone marrow samples from 17 patients was assessed after treatment *in vitro* with the LICR-LON-Fib75/abrin A-chain conjugate. Seven of these samples were from normal donors, and 7 were from patients scheduled for high-dose melphalan who had received induction chemotherapy and who received untreated autologous bone marrow (Table 1). The remaining 3 patients were those whose bone marrows were subject to treatment with the Fib/abrin-A conjugate before reinfusion.

Mononuclear cells were separated from the bone marrow samples using Lymphoprep (Nyegaard). Untreated aliquots were assayed for colony-forming cells of the granulocyte-monocyte series by the standard CFU-C method (8). Further aliquots were incubated for 2 h at 37°C with 10^{-8} M LICR-LON-Fib75/abrin A-chain conjugate and then washed twice and plated out for CFU-C. Controls included LICR-LON-Fib75 monoclonal antibody alone, abrin A-chain alone, and untreated incubated bone marrow.

Preparation of Marrow for Patient Rescue. Five hundred to 1500 ml of bone marrow were removed into plastic bags using heparinized syringes. There were approximately $2–5 \times 10^8$ nucleated cells/kg of patient body weight. All the bone marrow was placed in an International Business Machines cell separator, and the buffy coat was removed under sterile conditions. This was further separated to give a mononuclear cell suspension which contained approximately 4×10^7 cells/kg in a volume of 100–200 ml and at a cell concentration of not more than 2×10^7 cells/ml. Between one-third and one-half of the marrow was cryopreserved. LICR-LON-Fib75/abrin A-chain conjugate was added to the remaining half of the marrow (final concentration, 10^{-8} M) and incubated for 2 h at 37°C. The marrow was then washed twice with saline and 10% autologous plasma and resuspended in 100 ml of the same medium and maintained at 4°C until reinfusion 12 h after administration of melphalan (200 mg/m^2).

Table 1 *Details of patients treated*

This table compares the clinical characteristics of patients whose marrow was treated and untreated. Most patients had visceral metastases and had received endocrine therapy in the past.

	Marrow treated	Marrow untreated
No. of patients	3	7
Age (yr)		
Mean	49	48
Range	37–60	43–51
No. of ER positive	1/2	1/5
Prior endocrine therapy	2	2
No. of courses of induction chemotherapy (mean)	5	5
Dominant sites of metastases		
Soft tissue	1	2
Liver	2	2
Lung	0	3

Patients

Ten patients have been studied (Table 1). All had histologically proved advanced breast cancer, and their ages ranged from 37–60. All had received between 4 and 6 courses of induction combination chemotherapy consisting of vincristine (1.5 mg/m^2), Adriamycin (40 mg/m^2), and cyclophosphamide (750 mg/m^2) prior to high dose therapy with melphalan (200 mg/m^2) and autologous bone marrow transplantation (1). Patients were only considered for this procedure if they had achieved a partial response, assessed using standard UICC criteria (9). Four patients had received endocrine therapy before induction, but all had failed to respond. Two of 7 patients had tumors containing ER.

RESULTS

Effect of LICR-LON-Fib75/Abrin A-Chain Conjugate on Colony Formation of Breast Cancer Cells *in Vitro*. Table 2 summarizes the results of the monoclonal conjugate on various human breast cancer cell lines. Five of the 7 cell lines tested were killed at concentrations of 10^{-8} M or less, as assessed by their capacity for continued survival and growth *in vitro* after treatment. The well-differentiated ER-positive lines seemed particularly susceptible, but only a small number of cell lines was tested. Two of these were clearly killed by the conjugate, as judged by complete absence of colony formation, although somewhat higher concentrations were required in 2 others (BT474 and MDA-MB-415), while 2 (MDA-MB-134 and ZR-75-30) were clearly refractory. Treatment of the latter with 10^{-8} M toxin-conjugate resulted in no significant cell death even after 14 days. Examination of the expression of LICR-LON-Fib75 antigen on both of these cell lines with the use of a fluorescent second antibody staining procedure and quantifying levels as fluorescence per cell with fluorescence-activated cell sorting analysis using the T-47D line as a control showed, interestingly, that both lines expressed the antigen at levels comparable to susceptible lines.

Effect of LICR-LON-Fib75/Abrin A-Chain Conjugate on Bone Marrow Colony Formation *in Vitro*. Incubation of the conjugate with bone marrow in 9 normal donor patients showed a residual CFU-C of 60–100% (mean, 88%). Residual CFU-C from 10 patients with breast cancer was 67–100% (mean, 81.4%), and these results are shown in Table 3. Although a minor degree of

Table 2 *Effect of LICR-LON-Fib75/abrin A-chain conjugate on viability and growth of breast cancer cell lines*

This table demonstrates that 5 of 7 breast cancer cell lines are sensitive to 10^{-8} M–10^{-9} M toxin-conjugate.

Cell line[a]	Estrogen receptor status	Toxin-conjugate concentration[b] (M)
MCF-7	Positive	10^{-9}
ZR-75-1	Positive	10^{-9}
T-47D	Positive	10^{-9}
BT474	Negative	10^{-8}
MDA-MB-415	Negative	10^{-8}
MDA-MB-134	Negative	$>10^{-8}$
ZR-75-30[c]	Negative	$>10^{-8}$

[a] Ref. 14 details all original references, except for T-47D which is described in Ref. 15.

[b] Concentrations indicated are the minimum for obtaining at least a 90% reduction in cell number within 10–14 days after treatment.

[c] This cell line was derived from a different tumor and patient than that from which ZR-75-1 was obtained (16).

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Table 3 *Effect of LICR-LON-Fib75/abrin A-chain conjugate on CFU-C in vitro*

The effect of LICR-LON-Fib75/abrin A-chain conjugate on bone marrow colony formation *in vitro* is shown in patients who had not received chemotherapy prior to sampling and patients who had received chemotherapy before sampling.

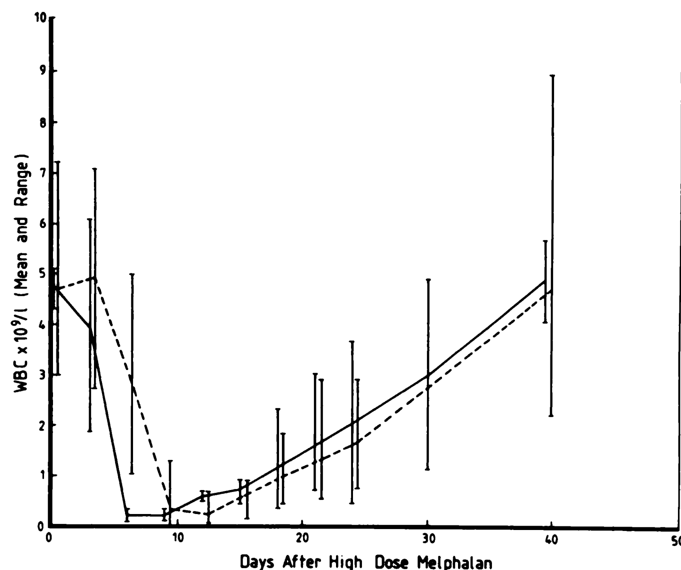
No.	Colonies/10^5 cells plated		% of recovery
	Pretreatment	Posttreatment	
No prior chemotherapy			
1	20.3	21.8	100
2	366.8	307.6	84
3	58.6	29.4	50
4	38.6	33.6	87
5	165.1	162.5	98
6	202.5	198.1	98
7	148.1	151.8	100
8	70.1	76.4	100
9	44.3	33.3	75
Mean			88
Prior chemotherapy			
1	47.8	38.5	81
2	84.7	56.9	67
3	80.2	91.9	100
4	7.0	4.2	60
5	98.4	90.8	92
6	47.3	37.1	78
7	92.3	66.1	72
8	129.4	92.5	71
9	49.6	50.5	100
10	125.0	116.6	93
Mean			81.4

suppression when compared to control marrow is seen, the degree of suppression is slight, and in no case was this greater than 50% of the control results.

Effect of Exposure of Bone Marrow to LICR-LON-Fib75/Abrin A-Chain Conjugate on Recovery of Bone Marrow *in Vivo*. Fig. 1 and Table 4 compare the pattern and time of marrow recovery of the 3 patients whose bone marrows were pretreated with the 7 patients whose bone marrows were returned without being treated. The pattern of recovery is similar in the 2 groups. Thus, the average total number of days in which the white cell count was $<1.5 \times 10^9$/liter was 16.7 (treated) compared to 18.3 (untreated). The figures for the duration of thrombocytopenia ($<50 \times 10^9$/liter) were 23.7 days (treated) and 18.9 days (untreated), respectively.

No micrometastases were observed in treated bone marrow prior to reinfusion after high dose therapy. No other difference was seen in terms of number of bleeding episodes or infective episodes. All patients' blood counts returned to normal within 8 wk. The majority of patients left the hospital within 35 days of the administration of high dose melphalan.

Patients remained in remission for 1->14 mo after high dose melphalan. Mean remission duration was 7.3 mo in patients receiving uncleansed bone marrow and 6.2 mo in patients receiving cleansed bone marrow. Only 2 patients are still in remission, one at 14 mo and one at 7 mo after high dose therapy.



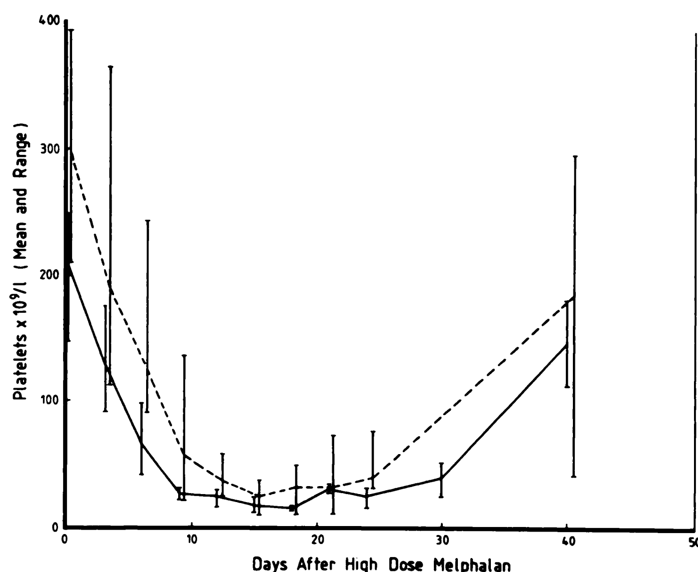


Fig. 1. The peripheral white cell count (*WBC*) (*upper*) and peripheral platelet count (*lower*) after high dose melphalan. *Bars*, range of the count. - - - -, count of patients who received untreated bone marrow; ——, mean count of patients receiving bone marrow treated with LICR-LON-Fib75/abrin A-chain conjugate.

DISCUSSION

We have shown here for the first time that treatment of bone marrow with a monoclonal-toxin A-chain conjugate capable of killing contaminating cancer cells does not impair the ability of bone marrow to rescue patients following intensive therapy for breast cancer. A previous study has outlined the use of monoclonal antibody/toxin conjugates for ridding marrow of normal T-cells thought to be responsible for graft-*versus*-host disease and has shown that bone marrow recovers satisfactorily using this approach (10). Other workers have examined the use of monoclonal antibodies with complement in lymphoid neoplasms (11), and we initially investigated this approach. However, we found this procedure to be unreliable and variably toxic to bone marrow function (Ref. 3; Footnote 3).

Unlike the situation in neuroblastoma where a heavy cancer cell infiltration is common, and where the effects of a cytotoxic monoclonal on cell numbers are readily appreciated (12), infiltration of marrow by breast carcinoma cells is less obvious. With such small numbers of cells it is often difficult, because of heterogeneity, to detect their presence and also to establish that they have been killed by the procedure. It was for this reason that we used several cell lines to establish the effect of conjugate on their viability. These showed that the LICR-LON-Fib75/abrin A-chain conjugate was highly toxic to most of the cell lines examined.

Although the advantage of using the monoclonal LICR-LON-Fib75 is clear in that this antibody recognizes the vast majority of individual cells in human cancer cell lines, some patients' tumor cells may not be killed by this monoclonal

[3]Unpublished observations.

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Table 4 *Time of major marrow suppression in both groups of patients*

The number of days of thrombocytopenia and leukopenia is shown in both groups of patients. Similar times to recovery are seen in patients whose bone marrow was treated and untreated.

	No. of days					
	WBC (× 10⁹/liter)			Platelet count (× 10⁹/liter)		
	<0.5	0.5–1.5	Total	<30	30–50	Total
Marrow treated						
Patient 1	5	11	16	14	7	21
Patient 2	16	6	22	24	8	32
Patient 3	3	7	10	10	8	18
Mean	8	8	16	16	7.7	23.7
Marrow untreated						
Patient 4	7	7	14	13	6	19
Patient 5	8	9	17	7	5	12
Patient 6	9	16	25	31	7	38
Patient 7	6	15	21	15	4	19
Patient 8	6	8	14	3	6	9
Patient 9	9	12	21	5	8	13
Patient 10	7	9	16	13	9	22
Mean	7.4	10.9	18.3	12.4	6.4	18.9

antibody-toxin conjugate as demonstrated by our *in vitro* results (Table 2). Since LICR-LON-Fib75 antigen, described by McIlhinney *et al.* (13), is expressed equally in susceptible and resistant cell lines, the origin of the toxin insensitivity must be sought elsewhere. It may be of significance that ZR-75-30 and MDA-MB-134 were the slowest growing of all the lines tested, with doubling times in our hands of approximately 4.5 and 6 days, respectively, compared with 28 h for MCF7, indicating that more slowly growing tumors may be less readily killed by this method, but further studies utilizing more cell lines are needed. Despite these results with cell lines it remains to be demonstrated that LICR-LON-Fib75 is expressed on all breast cancers. We may achieve a wider range of killing of tumor cells by the addition of other monoclonal antibodies such as LICR-LON-M8, or anti-EMA, currently used in detecting cancer cells in bone marrow (5). The limitation of these antibodies is, however, that their expression is heterogeneous within the cells of an individual tumor.

The procedure outlined here is fairly straightforward and compatible with treatment of marrow in bulk, since only 1–3 mg of toxin-conjugate is needed making the procedure operationally feasible.

Following an induction regimen for metastatic breast cancer, high dose melphalan and marrow rescue can be used to achieve further response. It is unlikely, however, that killing tumor cells in the bone marrow used for rescue after high dose therapy is necessary in patients with advanced breast cancer since these patterns of relapse indicate that disease recurred in the original metastatic site. It seems likely that the practical utility of this approach ultimately depends on the efficacy of high dose therapy in early disease. We believe that our recent observation of the detection of micrometastases in patients with primary breast cancer (5) will permit us to select patients with poor prognosis at presentation, for whom we can first reduce microscopic tumor burden by conventional chemotherapy and subsequently, by monitoring the number of micrometastases in the bone marrow, treat with high-dose therapy while rescuing them with bone marrow that has been cleansed of tumor cells. It is in this situation that ridding the marrow of tumor cells is likely to be crucial to prevent reseeding.

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