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Bone Marrow Transplantation in Cancer Therapy: Inactivation by Antibody and Complement of Tumor Cells in Mouse Syngeneic Marrow Transplants[1] (40223)

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The main limitation of current systemic cancer therapy is its lack of specificity; life-threatening toxicity, usually in the form of marrow aplasia, is frequently reached before all tumor cells in the patient have been inactivated. The application of bone marrow transplantation to clinical cancer therapy permits the use of marrow ablative chemotherapy and radiation therapy in an attempt to inactivate a greater amount of tumor cells. The otherwise lethal consequences are then averted with a marrow graft (1–3).

Even though clinical bone marrow transplantation is still in its early stages of development, significant numbers of patients with hematopoietic malignancies have been successfully grafted with allogeneic or syngeneic (identical twin) bone marrow (4–6). These are patients who have failed conventional therapy and whose chances of survival with any other treatment(s) are extremely poor. Graft-versus-host disease, the immunosuppression needed to control it, the attendant infectious complications and graft rejection account for a very significant morbidity and mortality in patients receiving allogeneic grafts (7). The absence of many of these complications very likely accounts for the higher survival rate among recipients of syngeneic grafts. Unfortunately, few potential candidates have an identical twin and not all even have a suitable allogeneic donor. One solution to these problems of marrow availability and histocompatibility would be to use the patients own marrow. However, before such an autologous graft could be used, any contaminating tumor cells would have to be inactivated. Thierfelder *et al.* have reported the selective inactivation of AKR lymphoma cells in an intentionally contaminated syngeneic bone marrow graft by treatment with an anti-T cell antiserum and complement (8). In this communication, we report similar findings in the C3H mouse–6C3HED lymphoma model using two antisera. A portion of this work has been previously reported (9).

Materials and methods. Used throughout these experiments were 8- to 12-week old C3H/HeN MTV-mice (abbreviated C3H) of either sex which were obtained from The National Cancer Institute through the courtesy of the Frederick Cancer Research Center, Frederick, Maryland. The 6C3HED lymphosarcoma, which originated in the thymus of a C3H mouse, was obtained from The Jackson Laboratory, Bar Harbor, Maine and maintained in solid form by passage in C3H mice.

A rabbit xenoantiserum against 6C3HED (Ra-a-6C3HED), lot 877-2, was prepared by immunizing a New Zealand rabbit with weekly intramuscular injections of 40 mg of lyophilized, homogenized tumor emulsified in 0.5 ml complete Freund's adjuvant. The rabbit was bled at 4 and 6 weeks; the antiserum obtained from the latter collection was heat-inactivated at 56° for 30 min and is designated as "unabsorbed antiserum." An aliquot was absorbed with 1/10 vol/vol packed C3H/HeN spleen cells for 1 hr at 5° and clarified by centrifugation and sterile filtration. The latter serum was designated "absorbed antiserum." Normal rabbit serum (NRS) was obtained from an unimmunized rabbit. The lytic complement source was freshly frozen guinea pig serum that was absorbed with agar (10). Aliquots of fresh guinea pig serum were rendered nonlytic by heat-inactivation.

The medium used for all manipulations was RPMI 1640 supplemented wth 2% fetal

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bovine serum, penicillin, 100 U/ml, and streptomycin, 100 μgm/ml. The 6C3HED lymphoma was harvested into a single cell suspension by pressing minced tumor through a stainless steel mesh. Bone marrow was obtained by flushing the femurs of normal C3H mice with medium. The bone marrow was disrupted with a pasteur pipette to obtain a single cell suspension. Cell counts and viabilities were assessed using a light microscope, hemacytometer, and dye (0.3% Buffalo Black in saline) exclusion.

Mice received 950 R, which exceeds LD_{100}, from a Cesium-137 dual source, small animal irradiator (Atomic Energy of Canada, Ltd.) at a dose rate of 136 R/min. No more than two mice were housed per cage and were provided with food pellets and water, *ad libitum,* the latter supplemented with streptomycin sulfate (Vet Strep, Merck and Co., Rahway, NJ) at 2.5 mg/ml.

Cytotoxicity tests were performed in the following manner. One volume of nucleated target cells, at a concentration of 10^6/ml, was mixed with one volume of a dilution of rabbit serum (Ra-a-6C3HED or NRS). This suspension was incubated at 37° for 45 min with occasional agitation. Two volumes of agar-absorbed guinea pig serum (lytic or heat-inactivated) were added and the suspension similarly incubated for 90 min. Cell viability was then evaluated.

Bone marrow or marrow-tumor suspensions were administered intravenously to irradiated mice via the lateral tail vein. Cells pretreated with various antiserum-complement combinations using the above-mentioned cytotoxic protocol were washed twice in medium and adjusted to contain the appropriate number of viable cells for infusion.

Animals were autopsied within 12 hr of death and samples of lung, liver, kidney and spleen fixed in a solution of 10% formalin. These samples were embedded in paraffin from which 6 μm sections were obtained and stained with hematoxylin and eosin for microscopic examination.

Results. The unabsorbed antiserum to 6C3HED contained considerable reactivity to both the lymphoma and normal C3H bone marrow as assessed by *in vitro* complement-dependent lysis (Fig. 1A). Reactivity to normal marrow was removed by a single absorption with C3H spleen with a modest lowering of the antiserum titre against tumor (Fig. 1B). As expected, combinations including nonimmune serum or heat-inactivated complement were without *in vitro* cytotoxic activity (not shown). The goal of subsequent experiments was to compare the ability of both antisera to selectively inactivate 6C3HED lymphoma cells in intentionally contaminated C3H bone marrow. A 1/50 dilution of the unabsorbed antiserum and a 1/30 dilution of absorbed antiserum were used.

Both antisera were first compared for their ability to suppress C3H bone marrow hematopoietic stem cells. Bone marrow cells were treated with either unabsorbed or absorbed antiserum and lytic complement, or left untreated. Varying numbers of recovered viable cells were then infused into lethally irradiated mice (Fig. 2). Treatment by both antisera inactivates hematopoietic stem cells since untreated bone marrow was quantitatively more efficient in protecting irradiated mice. It is clear, however, that the absorbed antiserum was considerably less suppressive to normal marrow.

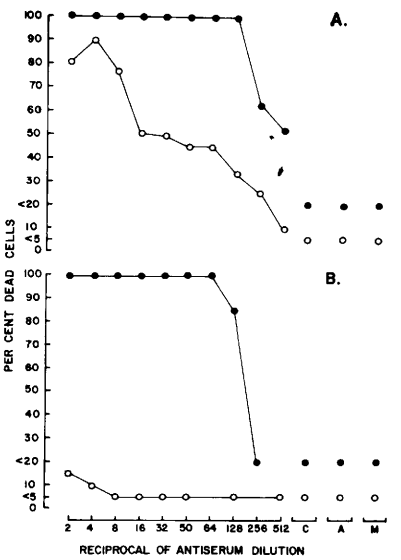

FIG. 1. Cytotoxicity of Ra-a-6C3HED and lytic complement against 6C3HED (closed circles) and normal C3H bone marrow (open circles) target cells. Controls include cells exposed only to complement (C), antiserum (A) or medium (M). Panel A, unabsorbed antiserum. Panel B, antiserum absorbed with C3H spleen cells.

Our experience in tumor suppression experiments with these antisera is summarized in Table I. All C3H mice received 950 R whole body irradiation. The invariable radiation death (group A) could be prevented by an infusion of 5×10^6 viable nucleated C3H bone marrow cells (group B). Mice receiving bone marrow and 10^4, (group C), 10^3 (group D) and 10^2 (group E) lymphoma cells all died of disseminated tumor by days 12, 13, and 16, respectively. Most mice receiving marrow and 10^1 lymphoma cells died of tumor (group F). Mice receiving a standard marrow-tumor mixture (5×10^6 marrow + 10^4 6C3HED) treated with unabsorbed Ra-a-6C3HED (1/50 dilution) and lytic complement (group G) all survived longer than 45 days except one mouse that died of infection with evidence of engraftment but no tumor. Treatment with absorbed antiserum (1/30 dilution) and lytic complement (group J), likewise, permitted survival of all mice. Antiserum treatment alone (group K) or in combination with heat-inactivated complement (group H) produced greater than 50% long-term survival.

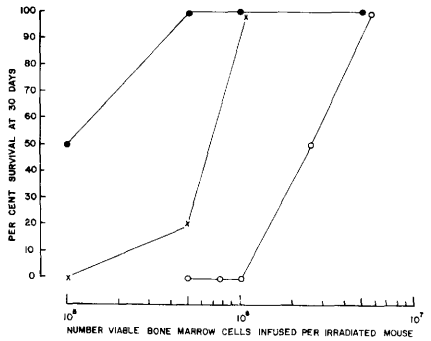

FIG. 2. Survival at 30 days of irradiated C3H mice receiving varying numbers of untreated (closed circles), absorbed antiserum and complement treated (crosses) or unabsorbed antiserum (open circles) and complement-treated C3H bone marrow. Each point represents four to ten mice.

Discussion. In this study, we were able to selectively and completely inactivate tumor cells in contaminated mouse bone marrow using antiserum and complement. This observation has been previously reported by Thierfelder and colleagues using the AKR leukemia and an anti-T cell antiserum. They report that this extensively absorbed antiserum spares hematopoietic stem cells and, that

TABLE I.[a]

Group	Total number lethally irradiated mice	Number of separate experiments	Intravenous inoculum: Number of C3H bone marrow cells	Intravenous inoculum: ± Number 6C3H-ED	Inoculum pretreatment: Antiserum ± complement	Survival of mice: Fraction of survivors	Survival of mice: Mean day ± SE of death	Survival of mice: Cause of death
A	53	6				0/53	11.02 ± 0.43	Marrow failure
B	35	6	5×10^6			34/35	5	Infection
C	26	4	5×10^6	+ 10^4		0/26	11.54 ± 0.37	Tumor
D	5	1	5×10^6	+ 10^3		0/5	11.75 ± 1.60	Tumor
E	5	1	5×10^6	+ 10^2		0/5	16.00 ± 0.00	Tumor
F	4	1	5×10^6	+ 10^1		1/4	18.33 ± 0.33	Tumor
G	16	3	5×10^6	+ 10^4	Unabsorbed Ra-a-6C3HED + Lytic (1/50)	15/16	10	Infection No tumor
H	6	1	5×10^6	+ 10^4	Unabsorbed Ra-a-6C3HED + Heat inactivated (1/30)	3/6	22.00 ± 1.53	Tumor
I	6	1	5×10^6	+ 10^4	NRS + Lytic	0/6	11.20 ± 0.20	Tumor
J	5	1	5×10^6	+ 10^4	Absorbed (1/30) Ra-a-6C3HED + Lytic	5/5	—	—
K	5	1	5×10^6	+ 10^4	Absorbed (1/30) Ra-a-6C3HED	3/5	22.00 ± 1.00	Tumor

[a] Survival of irradiated mice receiving indicated treatments. All mice were observed for at least 60 days.

pretreatment of an inoculum of 2×10^7 marrow cells – 10^5 leukemia cells with antiserum and complement produced 40% long-term survival in lethally irradiated (800R) recipient AKR mice (8). Treatment with antiserum alone was not reported.

A rabbit antiserum raised against the 6C3HED lymphoma was absorbed once with normal C3H spleen cells and shown to mediate markedly preferential complement-dependent lysis of tumor cells (Fig. 1B). By comparison, the unabsorbed antiserum demonstrated considerable marrow toxicity in the complement-lysis assay (Fig. 1A). Both sera suppressed hemopoietic stem cells as assessed by the survival of irradiated mice receiving antiserum-complement treated bone marrow cells. The absorbed antiserum, however, was clearly less suppressive and additional absorptions would likely have removed stem cell reactivities not detected by complement-mediated lysis.

Treatment of a tumor-marrow mixture with either the unabsorbed or absorbed antiserum and complement permitted 100% of irradiated recipient mice in four separate experiments to sustain long-term survival. This observation indicates that: (a) all clonogenic tumor cells were inactivated, and (b) sufficient numbers of bone marrow hemopoietic stem cells survived the treatment of adequately repopulate lethally irradiated mice.

Treatment with antiserum alone produced considerable tumor suppression with a 50% cure rate. This phenomenon of host suppression of antibody-coated cells is well recognized and may be mediated by such host effectors as macrophages, lymphocytes, platelets and/or complement (11). Our experience with this and another preparation of antiserum (data not presented) is that the magnitude of suppression by antibody alone is unpredictable. Nevertheless, this capacity for *in vivo* suppression, however variable, would provide an opportunity for the suppression of any tumor cells that escape *in vitro* cytolysis. Indeed, our consistent protection of all mice receiving marrow-tumor mixtures treated with antiserum and lytic complement may be due to these dual opportunities for tumor inactivation. Additional studies are in progress to quantitate their relative contributions.

Clinical trials utilizing autologous bone marrow transplantation following intensive cytotoxic therapy in the treatment of responsive malignancies is receiving renewed attention (12–14). A major problem with this approach is the possibility that the patient's bone marrow is already contaminated with small numbers of tumor cells. The efficiency of the *in vitro* treatment of tumor contaminated marrow with heterologous antiserum and complement in eliminating clonogenic tumor has now been demonstrated in two animal models. While much remains to be done before such methodology can be applied in a clinical setting, these results are sufficiently encouraging to suggest that the development of appropriate cytotoxic antisera for human tumors be pursued.

Summary. Mouse bone marrow, contaminated with lymphoma cells, was treated *in vitro* with anti-lymphoma antiserum and complement in an effort to selectively inactivate the tumor cells. The effectiveness of this treatment was demonstrated by the successful use of such treated marrow to repopulate lethally irradiated mice without the development of tumor. This approach, if it can be applied to man, may facilitate the use of bone marrow transplantation in human cancer therapy by providing a source of tumor-free autologous marrow.

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