CONCISE REPORT

Elimination of Acute Myelogenous Leukemic Cells From Marrow and Tumor Suspensions in the Rat With 4-Hydroperoxycyclophosphamide

By Saul J. Sharkis, George W. Santos, and Michael Colvin

Cell suspensions of normal rat marrow mixed with rat acute myelogenous leukemic cells were prepared and incubated in vitro with graded doses of 4-hydroperoxycyclophosphamide (4HC). The cell suspensions were injected into rats prepared with a lethal dose of total body irradiation. Animals injected with these cells survived fatal irradiation induced aplasia. In a dose related manner 4HC was able to "purge" tumor cells from the cell mixtures. Thus, animals given cell suspensions incubated with the lower doses of 4HC showed prolonged survival before death from leukemia and animals given cell suspensions incubated with higher doses of 4HC survived lethal irradiation without the subsequent appearance of leukemia. These studies clearly establish that tumor cells may be eliminated from normal marrow suspensions without completely destroying the pluripotent stem cells.

ALLOGENEIC bone marrow transplantation has become the therapy of choice in severe aplastic anemia and in certain cases of severe combined immunodeficiency. The role of this procedure in the treatment of malignancy in general and in acute leukemia in particular remains to be firmly established.[1] Recent data, however, suggest that the therapeutic results in acute leukemia may be markedly improved if patients are entered into a transplantation protocol when they are in remission.[2-4] Allogeneic marrow transplantation nevertheless remains limited in its therapeutic potential because of graft-versus-host disease, profound posttransplantation immunodeficiency, and interstitial pneumonitis.[1]

Bone marrow transplantation in hematologic malignancies has shown considerable promise when identical twin donors were employed.[5] These transplants are not complicated by graft-versus-host disease and, in addition, have a marked reduction of interstitital pneumonitis and duration of posttransplantation immunodeficiency.

Unfortunately, identical twin donors are rare, but the therapeutic results obtained with these patients have encouraged a number of transplant centers to consider more imaginative uses of autologous remission marrow for transplantation. Remission marrow suspensions undoubtedly contain undetectable tumor cells. Clearly, if the use of such marrow is to be optimized, remaining tumor cells must be eliminated. Evidence that rodent marrow may be "purged" of tumor cells by antibody without destroying the pluripotent hematopoietic stem cells has been presented.[6-8]

Previously, we reported our experience with syngenic marrow transplantation in a rat model of acute myelogenous leukemia (AML).[9] In that report we indicated that this tumor was remarkedly sensitive to cyclophosphamide (Cy).

The purpose of this article is to report our initial trials in "purging" AML from rat marrow without destroying hematopoietic stem cells by short incubation with the Cy analogue, 4-hydroperoxycyclophosphamide (4HC).

MATERIALS AND METHODS

Animals

Female (Lewis × BN)F_1 (LBNF_1) (Ag-B1,3) rats, 10–12 wk of age, were obtained from Microbiological Associates (Bethesda, Md.). The animals were housed in polycarbonate cages, two to a cage. They were provided with tap water and Purina chow ad libitum.

Drugs and Total Body Irradiation (TBI)

4-Hydroperoxycyclophosphmide (4HC)[10] is an analogue of cyclophosphamide that spontaneously decomposes in aqueous solution to generate either the primary metabolites of cyclophosphamide or compounds with similar chemical and biologic properties to these metabolites.[11] Studies examining the cytotoxic and crosslinking activity of this compound demonstrate that it shows essentially equal activity when compared with the metabolites generated by the microsomal metabolism of Cy.[12] Therefore, the compound was utilized for in vitro studies to test the cytoreductive activity of Cy. The 4HC used in the present experiments was synthesized by one of the authors (M. Colvin). The 4HC was dissolved in RPMI 1640 in the appropriate concentrations.

Cyclophosphamide as compared to several other agents, appears to have less stem cell toxicity.[13] Furthermore, the rat AML tumor that was used in these studies was previously shown to be sensitive to Cy.[9] For these reasons, 4HC seemed to be an appropriate agent to employ in experiments designed to "purge" AML tumor cells from normal marrow.

From the Oncology Center, Johns Hopkins University, Baltimore, Md.

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Address reprint requests to G.W. Santos, Bone Marrow Transplant Unit, Oncology Center, The Johns Hopkins University School of Medicine, Baltimore, Md. 21205.

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Table 1. "Purging" of AML Cells From $LBNF_1$ Rat Marrow With 4-Hydroperoxycyclophosphamide

Treatment of Recipient	Cells Injected	Treatment of Cells	Individual Survival in Days
TBI*	None	—	7, 8, 8, 10, 10, 11, 11, 12
TBI	Marrow†	None	100^+, 100^+, 100^+, 100^+, 100^+, 100^+, 100^+, 100^+
TBI	Marrow	80 nmole/ml 4HC§	100^+, 100^+, 100^+, 100^+, 100^+, 100^+, 100^+, 100^+
None	AML‡	None	16, 16, 16, 18, 18, 19, 19, 20, 21, 21, 21, 21, 21
None	Marrow + AML	None	10, 18, 22, 22, 23, 23, 23, 23
TBI	Marrow + AML	20 nmole/ml 4HC	24, 27, 27, 27, 28, 28, 28, 29
TBI	Marrow + AML	40 nmole/ml 4HC	10¶, 10¶, 28, 29, 29, 30, 30, 31, 32, 32, 100^+, 100^+, 100^+, 100^+, 100^+, 100^+
TBI	Marrow + AML	60 nmole/ml 4HC	10¶, 100^+, 100^+, 100^+, 100^+, 100^+, 100^+, 100^+
TBI	Marrow + AML	80 nmole/ml 4HC	20, 100^+, 100^+, 100^+, 100^+, 100^+, 100^+, 100^+

*Total body irradiation (1000 rad).

†Normal $LBNF_1$ marrow (64×10^6 cells i.v.).

‡Acute myelogeneous leukemia (10^6 cells i.v.).

§4-Hydroperoxycyclophosphamide.

¶These animals died in aplasia without histologic evidence (spleen and marrow) of AML. With the exception of the first control group, all others died with leukemia.

A duel-source ^{137}Cs small-animal irradiator delivering 136 rad/min was used for TBI.

Acute Myelogenous Tumor

The transplantable BN AML tumor was induced in female BN rats with 7, 12 dimethylbenzanthracene, and was kindly supplied to us by Dr. A. Hagenbeek and Dr. D. W. van Bekkum. After the tumor was passaged several times in the $LBNF_1$ rat, the growth characteristics appeared similar to the original model in the BN rat. Limiting dilution assay indicated that one tumor cell would produce death from florid leukemia in 40 days.[14]

Preparation of Cell Inocula

Normal $LBNF_1$ donor animals were killed by cervical dislocation. Marrow from the femur, tibia, and humerus was collected in cold RPMI 1640 solution, cell counts were made, and viability (90%–95%) was estimated with trypan blue, as described previously.[15] Marrow was given intravenously (i.v.) in a constant 1-ml volume at a concentration of 64×10^6 cells/ml. In our experience (unpublished), 32×10^6 $LBNF_1$ marrow cells is required to consistently protect 100% of rats from the lethal effects of 1000 rad of TBI. Doses of marrow lower than this are considerably less effective.

Spleens were taken from leukemic $LBNF_1$ rats (who had received 10^6 leukemic cells 3–4 wk previously), minced, and gently teased in Petri dishes containing cold RPMI 1640. Clumps of cells were gently dispersed with the aid of a small syringe. Cell suspensions were counted and adjusted to the proper concentration as noted above. Viability as judged by trypan blue exclusion was 85%–95%. Cells were injected i.v. into rats in a constant 1-ml volume containing 10^6 cells.

Incubation Procedure

Marrow, tumor, or mixtures of marrrow and tumor were incubated with appropriate concentrations of 4HC in RPMI 1640 at 37°C for 30 min and then directly (without centrifugation and washing) injected i.v. into recipients. Viability was not changed by the incubation procedure.

RESULTS

In preliminary studies, AML cells and normal $LBNF_1$ marrow cells were separately incubated with 40, 80, or 100 nmole/ml of 4HC for 30 min at 37°C. The tumor cells were transferred to normal and the marrow cells to lethally irradiated (1000 rad) recipients. All animals (5/group) survived for greater than 100 days.

In a final series of experiments (Table 1), normal marrow (64×10^6 cells) was mixed with tumor cells (10^6 cells) and incubated with 0, 20, 40, 60, and 80 nmole/ml of 4HC immediately before transfer to groups of lethally irradiated (1000 rad) rats. Animals were observed for survival and presence of leukemia for 100 days. The data clearly indicate the ability of 4HC to clear a mixture of normal marrow and tumor cells of AML cells in a dose-dependent manner that did not affect the ability of the cell suspensions to prevent the otherwise lethal effects of 1000 rad of TBI. The three animals that died of aplasia and the one that died of leukemia in the last three experimental groups in Table 1 appear to be exceptions that do not invalidate the above conclusions.

DISCUSSION

The use of autologous bone marrow transplantation is attractive, since it allows very intensive cytoreductive therapy to be applied to a greater number of patients with malignancy than would ordinarily be permitted. The use of autologous marrow also has the same potential advantages of syngeneic marrow in that many of the problems associated with allogeneic transplantation (e.g., GVHD) can be avoided. The major disadvantage in using autologous marrow is the possible contamination of the marrow by tumor cells.

Tumor might be eliminated from marrow suspensions by physical methods, such as cell fractionation,[16] immunologic means,[6–8] or by pharmacologic means. Depending on the type of tumor to be cleared, the most

efficient procedure might prove to be a combination of these techniques.

The data reported here indicate that the incubation of marrow–tumor mixtures with 4HC may effectively eliminate tumor cells contaminating marrow suspensions without destroying the ability of the cell suspensions to reverse the lethal effects of high doses of TBI. Previously, Shand[17] had shown that the incubation of parental spleen cells with 100 μg/ml of microsomal-activated Cy was able to abolish GVHD in mice without destroying hematopoietic stem cells. Thus, the data of Shand[17] as well as the present report indicate that the incubation of marrow with chemotherapeutic agents may show considerable promise in eliminating not only tumor cells for autologous marrow transplantation but also cells capable of initiating GVHD in allogeneic marrow transplantation.

REFERENCES

1. Santos GW: Bone marrow transplantation, in Stollerman GH (ed): Advances in Internal Medicine, vol 24. Chicago, Year Book Medical 1979, p 157

2. Thomas ED, Sanders JE, Flournoy N, Johnson FL, Buchner CD, Clift RA, Fefer A, Goodell BW, Storb R, Weiden PL: Marrow transplantation for patients with acute lymphoblastic leukemia in remission. Blood 54:468, 1979

3. Beutler E, Blume KG, Bross KJ, Chillar RK, Ellington OB, Fahay JL, Farbstein MJ, Schmidt GM, Spruce WE, Turner MA: Bone marrow transplantation as the treatment of choice for "good risk" adult patients with acute leukemia. Clin Res 27:508A, 1979 (abstr)

4. Thomas ED, Buckner CD, Fefer A, Johnson FL, Neiman P, Sanders JE, Storb R: Marrow transplantation for acute myelogenous leukemia (AML) in remission. Proc Am Assoc Cancer Res Am Soc Clin Oncol 20:30, 1979 (abstr)

5. Fefer A, Einstein AB, Thomas ED, Buckner CD, Clift RA, Glucksberg H, Neiman PE, Storb R: Bone marrow transplantation for hematologic neoplasia in 16 patients with identical twins. N Engl J Med 290:1389, 1974

6. Thierfelder S, Rodt H, Netzel B: Transplantation of syngeneic bone marrow incubated with leucocyte antibodies. I. Suppression of lymphatic leukemia of syngeneic donor mice. Transplantation 23:459, 1977

7. Economou JS, Shin HS, Kaizer H, Santos GW, Schron DS: Bone marrow transplantation in cancer therapy: Inactivation by antibody and complement of tumor cells in mouse syngeneic marrow transplants. Proc Soc Exp Biol Med 158:449, 1978

8. Bast RC Jr, Feeney M, Greenberger JS, Knapp RC: Eliminatino of leukemic cells from rat bone marrow using antibody and complement (C'). Proc Am Assoc Cancer Res Am Soc Clin Oncol 20:239, 1979 (abstr)

9. Santos GW, Sharkis SJ: Experience with syngeneic marrow transplantation in BN and WF rat models of acute myelogeneous leukemia, in Baum SJ, Ledney GD (ed): Experimental Hematology Today. New York, Springer-Verlag, 1978, p 187

10. Takamizawa A, Matsumoto S, Iwata T, Katagiri K, Tochino Y, Yamaguchi K: Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985, 1973

11. Fenselau C, Kan M-NN, Rao SS, Myles A, Friedman OM, Colvin M: Identification of aldophosphamide as a metabolite of cyclophosphamide in vitro and in vivo in humans. Cancer Res 37:2538, 1977

12. Hilton J, Colvin M: Unpublished data

13. Sensenbrenner LL, Owens AH Jr, Heiby JR, Jeejeebhoy HF: Comparative effects of cytotoxic agents on transplanted hematopoietic and antibody-producing cells. J Natl Cancer Inst 50:1027, 1973

14. Vaughan WP, Burke PJ, Jung J: BN rat myeloid leukemia transferred to the (LEW × BN)F_1 rat. J Natl Cancer Inst 61:927, 1978

15. Santos GW, Owens AH Jr: Syngeneic and allogeneic marrow transplants in the cyclophosphamide pretreated rat, in Dausset J, Hamberger J, Mathé J (ed): Advances in Transplantation, Proceedings of the First International Congress of the Transplantation Society. Copenhagen, Munksgaard, 1968, p 431

16. Dicke KA, Zander AR, Spitzer G, Verma DS, Peters LJ, Vellekoop L, Thomson S, Stewart D, Hester JP, McCredie KB: Autologous bone marrow transplantation in relapsed adult acute leukemia. Exp Hematol 7:170, 1979

17. Shand FL: The capacity of microsomally-activated cyclophosphamide to induce immunosuppression in vitro. Immunology 35:1017, 1978