

Selective resistance of bone marrow-derived hemopoietic progenitor cells to gliotoxin

(epipolythiodioxopiperazines/bone marrow transplantation/graft-versus-host disease)

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ABSTRACT The fungal metabolite gliotoxin at low concentrations prevents mitogen stimulation of mature lymphocytes as a result of gliotoxin-induced genomic DNA degradation. Bone marrow, on the other hand, contains a subpopulation of cells resistant to gliotoxin at similar concentrations. This population includes the hemopoietic progenitor cells that grow *in vitro* in response to appropriate colony-stimulating factors and cells that form colonies in the spleens of lethally irradiated recipients. Gliotoxin treatment of lymph node cell-enriched bone marrow significantly delayed the onset of graft-versus-host disease in fully allogeneic bone marrow chimeras.

Graft-versus-host disease (GvHD) is still a major complication associated with bone marrow transplantation (1, 2). Present evidence suggests that mature T cells in the bone marrow inoculum are responsible for the induction of GvHD (1, 3), and elimination of T cells is therefore an important step prior to transplantation. However, T-cell depletion may lead to other complications such as lack of chimerism—i.e., failure of engraftment (4, 5). T-cell depletion is mainly accomplished by treatment of bone marrow with monoclonal antibodies specific for T-cell characteristic cell surface antigens and with complement (6, 7). The major disadvantages of such a depletion method lie in the variability of the biological reagents—thus requiring constant standardization, successive treatments, and the generally low cell concentration necessary for efficient cell depletion (8).

We have reported previously on the immunomodulating activity of naturally occurring secondary fungal metabolites possessing an epipolythiodioxopiperazine ring structure such as gliotoxin (GT) (9-11). Recently Braithwaite *et al.* (12) have shown that epipolythiodioxopiperazine compounds selectively cause DNA degradation of leukocytes. Here we describe how GT selectively inhibits proliferation of mature B and T cells in the bone marrow without inhibiting the ability of bone marrow cells to recolonize lethally irradiated recipients.

MATERIALS AND METHODS

Animals. CBA/H (H-2^k) and BALB/c (H-2^d) mice were obtained from the breeding unit of the John Curtin School and were used when >6 weeks old.

Gliotoxin. GT was prepared from fungal cultures of *Penicillium terlikowskii* as described in detail (11). GT was dissolved in absolute ethanol at 1.0 mg/ml, stored at -20°C, and diluted in the appropriate medium before use. All cells were treated at a cell concentration of 10⁶ cells/ml. Cell viability assessed by trypan blue exclusion was not affected after exposure to GT for 1 hr.

DNA Extraction and Size Fractionation. The method has been described in detail (12). In brief, CBA/H spleen cells and bone marrow cells were first erythrocyte depleted, pulsed with GT in Eagle's minimal essential medium F-15 medium (GIBCO) for 1 hr at 37°C at 10⁶ cells/ml, washed, and then left incubating for 16-20 hr in F-15 containing 5% fetal calf serum. Cells were then lysed and treated with Pronase; the DNA was extracted with phenol and chloroform and finally precipitated with ethanol. After 4- to 8-hr RNase treatment (bovine pancreatic ribonuclease A, Sigma R-4875) at pH 8.0 in 10 mM EDTA, the DNA was electrophoresed overnight in Tris acetate on a 0.4% agarose gel (Bio-Rad) at 25 mA. DNA size markers included uncut bacteriophage λ [48.3 kilobase pairs (kbp)] and *Hind*III restriction enzyme digest of λ (23.0, 9.4 and 6.5 kbp).

Measurement of DNA Synthesis. Bone marrow and spleen cells were erythrocyte depleted and pulsed with various concentrations of GT in F-15 for 1 hr at a cell concentration of 10⁶ cells/ml. Cells were washed; the spleen cells were resuspended in F-15 medium containing 5% fetal calf serum, and the bone marrow cells were resuspended in RPMI 1640 containing 10% fetal calf serum. The spleen and bone marrow cells were distributed at 2 × 10⁵ and 1 × 10⁵ cells per well, respectively, into 96-well microculture plates. The medium for splenocytes contained either 4 μ g of Con A per ml or 10 μ g of lipopolysaccharide per ml. Bone marrow cells were cultured in the presence of 500 units of recombinant interleukin 3 per ml (a gift of A. J. Hapel, Australian National University), 500 units of recombinant granulocyte/macrophage-colony-stimulating factor per ml (GM-CSF) (a gift of S. Bartelmez, Peter McCallum Institute, Melbourne) or 500 units of CSF-1 per ml (derived from tumor cell line L929 culture medium). The cells were pulsed with 0.5 μ Ci (1 Ci = 37 GBq) of [³H]thymidine after 48- and 72-hr culture for the spleen and bone marrow cells, respectively. The water-insoluble radioactivity was measured, and the results are expressed as a percentage of untreated control cultures.

Spleen Colony-Forming Assay. Mice were irradiated with 950 rad (1 rad = 0.01 Gy) from a ⁶⁰Co source 24 hr before reconstitution with GT-treated or untreated bone marrow cells. Spleens were removed 10 days postreconstitution and fixed for 3 days in Bouin's fixative; the surface colonies were then counted.

RESULTS

Size Fractionation of Genomic DNA from GT-Treated Bone Marrow and Spleen Cells. Our previous observations of selective inhibition by GT of cell proliferation and DNA degradation of mature cells of hemopoietic lineage (12) led us to investigate whether GT can act selectively on cells at

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Abbreviations: GT, gliotoxin; GvHD, graft-versus-host disease; GM-CSF, granulocyte/macrophage colony-stimulating factor.

different stages of maturation. Bone marrow and spleen cells of strain CBA/H mice were treated with various concentrations of GT for 1 hr at 1×10^6 cells per ml, washed, and incubated for 16–20 hr before cell lysis and DNA extraction.

Fig. 1 shows a comparison of genomic DNA from spleen and bone marrow cells. Spleen cell DNA is completely degraded after a pulse of ≥ 30 ng of GT per ml. A portion of DNA in bone marrow was also affected at 30 ng of GT per ml, but a residual band of DNA migrated to a gel position identical to untreated control DNA at GT concentrations of up to 1000 ng per ml. This suggests that a subpopulation of bone marrow cells is resistant to GT-induced DNA breakage.

Effect of GT on Proliferative Response of Bone Marrow and Spleen Cells. Erythrocyte-depleted bone marrow and spleen cells both pulsed with various concentrations of GT for 1 hr were tested for their proliferative activity in response to mitogens for splenocytes and colony-stimulating factors for bone marrow cells. Fig. 2 shows a representative example of such a proliferative assay. Splenocytes, pulsed with low concentrations of GT, showed a reproducible enhancement of thymidine incorporation in response to the mitogens Con A and lipopolysaccharide. Thymidine incorporation is completely abrogated after a pulse of ≥ 30 ng of GT per ml. Bone marrow cells, on the other hand, required a 10-fold higher concentration of GT to prevent subsequent proliferation induced by three different growth factors: interleukin-3, GM-CSF, and colony-stimulating factor I. Proliferation induced by GM-CSF was dramatically enhanced at GT concentrations of 1–30 ng per ml. Total inhibition of proliferation was reached at ≥ 1000 ng per ml.

Spleen Colony-Forming Assay with GT-Treated Bone Marrow Cells. The efficacy of GT-treated bone marrow cells in reconstituting lethally irradiated animals was tested using the Till and McCulloch spleen colony-forming assay (13). CBA/H mice, lethally irradiated (950 rad) 6 hr previously, were reconstituted with either 10^5 or 10^6 syngeneic bone marrow cells pulsed with GT for 1 hr. Spleens were removed and fixed 10 days later, and surface colonies were counted (Table 1). Experiment 1 was performed with aliquots of bone marrow cells also used in the proliferative experiment (Fig. 2). In both experiments the number of colonies on spleen reconstituted with 30 or 100 ng of GT-treated bone marrow per ml was not significantly different from those colonies on spleen reconstituted with untreated bone marrow cells. However, a reduction by 0.5–0.8 logarithmic units did occur in repeat experiments using 300 ng of GT per ml.

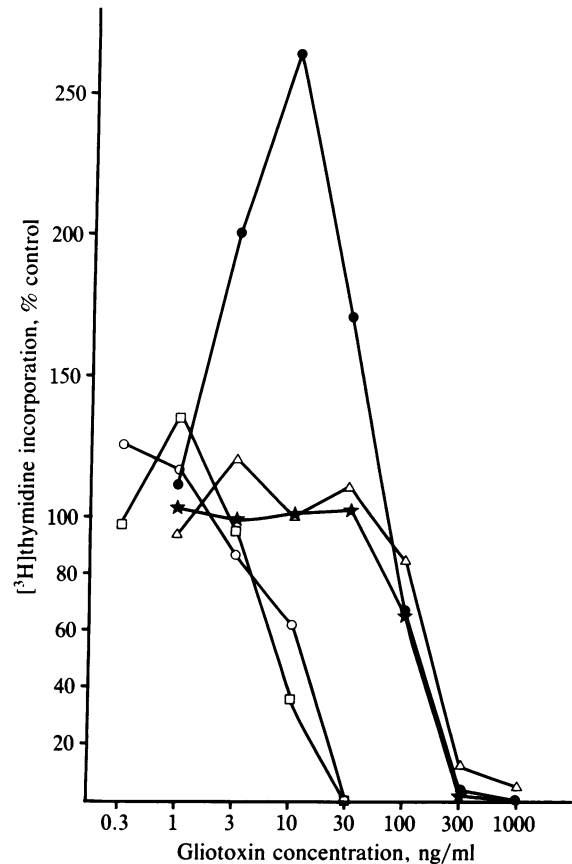


FIG. 2. Effect of GT on proliferative response of bone marrow and spleen cells. Proliferation response of spleen cells to $4 \mu\text{g}$ of Con A per ml (\circ) or $10 \mu\text{g}$ of lipopolysaccharide per ml (\square) and of bone marrow cells in the presence of 500 units of interleukin-3 per ml (Δ), 500 units of GM-CSF per ml (\bullet) or 500 units of colony-stimulating factor per ml (\star). The values for the control cultures were: spleen, Con A, 15.8×10^3 cpm; spleen, lipopolysaccharide, 4.0×10^3 cpm; bone marrow, interleukin-3, 7.2×10^3 cpm; bone marrow, GM-CSF, 31.5×10^3 cpm; and bone marrow colony-stimulating factor 1, 21.1×10^3 cpm.

Histological examination showed that colonies from recipients of untreated bone marrow were well differentiated, whereas those colonies derived from animals receiving bone

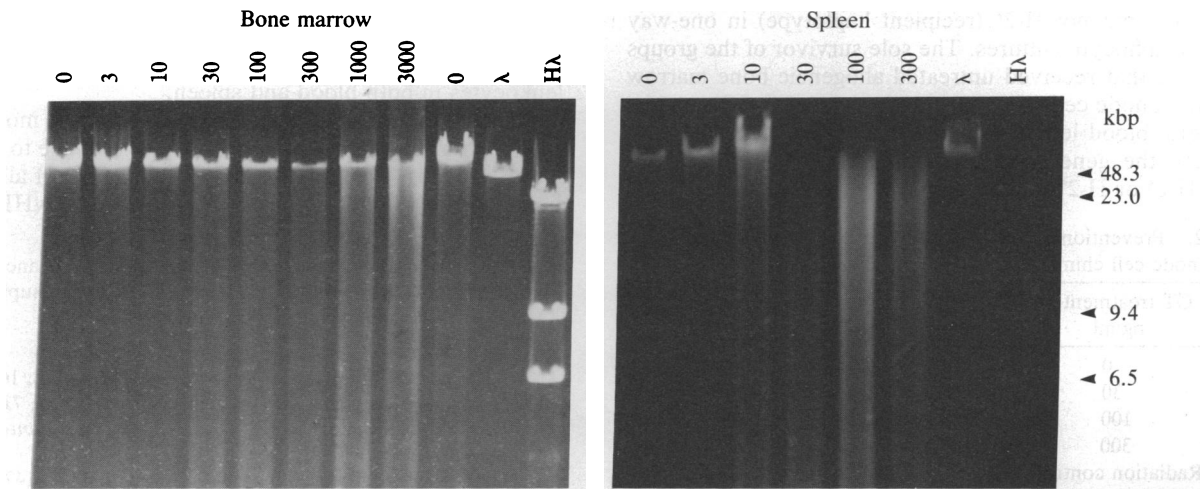


FIG. 1. Size fractionation of genomic DNA. Extracted DNA from cells pulsed with GT for 1 hr were size-fractionated in 0.4% agarose for 21 hr, and the gel was photographed immediately. DNA size markers of uncut bacteriophage lambda (λ) and *Hind*III restriction of lambda ($H\lambda$) are included. Numbers along the gel lanes represent concentrations of GT in ng/ml.

Table 1. Spleen colony-forming assay with GT-treated syngeneic bone marrow cells

GT treatment, ng/ml	Spleen surface colonies,* mean \pm SEM	
	Viable cells transferred, 10^5	Viable cells transferred, 10^6
	Experiment 1	
0	15.5 \pm 1.5 (2)	TM
30	17.7 \pm 2.3 [†] (3)	TM
100	16.0 \pm 2.0 [†] (4)	TM
300	1.0 \pm 0.4 (4)	24.7 \pm 0.8 (3)
	Experiment 2	
0	13.0 \pm 1.7 (4)	TM
30	15.0 \pm 1.9 [†] (4)	TM
100	11.5 \pm 1.0 [†] (4)	TM
300	2.8 \pm 0.9 (4)	20.3 \pm 5.6 (4)

Numbers in parentheses represent *n*.

*Count taken at 10 days postreconstitution. TM, too many to count.

[†]Not statistically different from control, as tested by Student's *t* test (*P* > 0.20).

marrow treated with 30, 100, or 300 ng of GT per ml, although similar in size, showed a marked lack of differentiation.

Abrogation of Graft-Versus-Host Disease (GvHD) in Fully Allogeneic Bone Marrow and Lymph Node Cell Radiation Chimeras. To assess the efficacy of GT-treated bone marrow cells to prevent GvHD, CBA/H animals lethally irradiated (950 rads) were reconstituted with GT-treated or untreated 10^7 viable nucleated cells of BALB/c bone marrow and lymph node origin. The ratio of bone marrow cells to lymph node cells was 10:1. Lymph node cells were added to enhance the GvHD.

Table 2 gives the result of one such experiment. Groups of eight mice were reconstituted with allogeneic cells. Radiation control animals died on days 12–14. One animal of the untreated group died on day 15, the same day that the remaining animals were weighed. Animals that received GT-treated cells (30, 100, or 300 ng/ml at 10^6 cells/ml) had statistically significant higher body weights than did untreated animals (*P* < 0.05 for 30 ng/ml, and *P* < 0.001 for 100- and 300-ng/ml-treated cells). Animals were sacrificed on day 80 postreconstitution and tissue-typed. All GT-treated cell recipients had peripheral blood leukocytes and splenocytes of donor (BALB/c H-2^d) origin. This was determined by their ability to stimulate C57BL/6 (H-2^b) splenocytes to generate alloreactive cytotoxic T cells specific for H-2^d (donor haplotype), but not H-2^k (recipient haplotype) in one-way mixed lymphocyte cultures. The sole survivor of the groups of animals that received untreated allogeneic bone marrow and lymph node cells showed severe signs of GvHD, and its peripheral blood leukocytes or splenocytes were unable to stimulate the generation of cytotoxic T cells specific for either H-2^k or H-2^d haplotypes.

Table 2. Prevention of GvHD in fully allogeneic bone marrow lymph node cell chimeras

GT treatment,* ng/ml	Body weight, [†] g	Survival [‡]
0	14.4 \pm 0.7 (7)	1/8
30	16.8 \pm 0.8 (8)	4/8
100	19.2 \pm 0.5 (8)	8/8
300	18.1 \pm 0.4 (8)	7/8
Radiation controls	NM	0/8

Numbers in parentheses represent *n*. NM, not measured.

*Cells were treated with GT for 1 hr before transfer.

[†]Mean body weight \pm SEM measured 15 days postreconstitution.

[‡]Survival at day 80 posttransplant.

DISCUSSION

The results presented here clearly demonstrate that progenitor cells are less sensitive to GT than are mature cells of hemopoietic origin. The cell-proliferation assay substantiates the findings on genomic DNA degradation of splenocytes versus bone marrow. There is evidence (14; R.D.E., P.W., A.W.B., and A.M., unpublished results) that this DNA breakage is due to the production of reactive oxygen species generated during oxidation of reduced GT; however, the reason for the tissue selectivity seen here and in an earlier report (12) is not at present understood. We have speculated on a number of possible mechanisms that could account for such specificity (12).

The consistent enhancement (in many replicate experiments seen at low GT concentrations) in the proliferative response of splenocytes to mitogens and, in particular, the dramatic enhancement of bone marrow cells to GM-CSF are interesting observations. This enhancement may result from the inactivation of a particular cell type or cellular process that exerts selective control over (i) maturation and/or (ii) proliferation of immature cells reactive to GM-CSF and (iii) splenocytes to mitogens.

This difference of GT susceptibility on mature-versus-immature hemopoietic cells is further demonstrated by the results of the spleen colony-forming assay. The lack of differentiation seen in the colonies of spleens of animals that received GT-treated bone marrow suggests that cells in the transplant, sensitive to GT, provide additional sources of factors necessary for differentiation and maturation of bone marrow stem cells. The decreased engraftment seen in bone marrow transplant models in which mature T cells have been depleted may be due to a similar lack of differentiation (4, 5). Such delay in the differentiation of spleen colony-forming cells may be overcome by exogenously administered growth and differentiation factors. We have not yet encountered decreased engraftment efficiency in the murine model. Establishment of fully allogeneic bone marrow irradiation chimeras that survived in non-pathogen-free conditions for >80 days is further evidence for the efficacy of GT in eliminating cells that induce lethal GvHD.

In the experiment reported here, allogeneic lymph node cells were used to enrich the bone marrow inoculum of cells able to induce GvHD. This treatment accelerated the disease onset, so that the majority of untreated bone marrow recipients had died by day 26 post-transplant. However, GvHD had already manifested itself on day 15, as signaled by significantly reduced body weights and one death among the control animals compared with the GT-treated animals (Table 2). All surviving animals that received GT-treated bone marrow were fully reconstituted with donor-type (H-2^d) leukocytes in both blood and spleen.

Thus, in the murine bone marrow transplant model, GT treatment offers a fast and convenient alternative to existing T-cell depletion methods for the establishment of allogeneic bone marrow chimeras and the prevention of GvHD.

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