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**GRAFT VERSUS HOST DISEASE
IN RATS AFTER DONOR TREATMENT
WITH CYCLOPHOSPHAMIDE AND
SPLEEN CELLS OF HOST ORIGIN**

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FOREWORD
(Nontechnical summary)

Bone marrow transplantation is a procedure having potential therapeutic value for the treatment of various blood and immune disorders. However, this potential has not been realized due to a frequently occurring complication of marrow transplantation that is known as graft versus host disease (GVHD). This disease manifests itself as body weight loss, skin lesions, infiltration of the liver by donor cells, loss of the cellular lining of the intestinal tract and microbial infections. Death from GVHD nearly always occurs after marrow transplantation in man. These manifestations of GVHD are produced by the lymphoid cells of the graft in response to antigens, known as transplantation antigens, expressed on the cells of the recipient. In our study, a rat model for GVHD was devised to investigate that problem when human marrow is grafted into an individual whose transplantation antigens are different from those of the donor. The model consists of exposing the offspring of two genetically defined strains of rats to a lethal dose of radiation and, shortly thereafter, injecting them with known quantities of parental marrow and spleen cells.

It is well established that animals injected with an antigen such as sheep red blood cells and treated either shortly before or after with an immunosuppressant drug such as cyclophosphamide (CY) develop an inability to respond to that antigen. That is, the animals treated as stated fail to make antibodies when injected again with the same antigen. This phenomenon is termed drug-induced tolerance.

In our experiments, we attempted to apply the principles of drug-induced tolerance to that of GVHD. Thus, rats to be used as donors were injected with viable spleen

cells bearing the transplantation antigens of the recipient and also with CY. We found that the severity of the GVHD could be significantly diminished by such donor treatment. We also observed that the degree of suppression of immune competence was dependent on the interval between the injection of the cellular antigens and the treatment with CY. Additional experiments verified that (1) the altered immune state depended on the interactions of the drug and the antigenic challenge and (2) the immune competence of the grafted animal was suppressed only for the specific antigen given to the donor.

ABSTRACT

Lethally irradiated Lewis-Brown Norway F_1 hybrid (LBN) rats were grafted with either marrow or marrow and spleen cells from normal Lewis (L) donors. All recipients of inocula containing both marrow and spleen cells died of graft versus host (GVH) disease. In other irradiated and grafted rats, the severity of the GVH disease was reduced by challenge of the donor with LBN spleen cells prior to or after treatment with cyclophosphamide (CY). The magnitude of this suppressed immunity depended on the interval between the administration of CY and the allogeneic spleen cells. Maximum suppression was obtained when donors received CY on day 0 and LBN cells on day +1. In all experiments, the treated donors were sacrificed on day +7. The observed immunosuppression was shown to result from the synergistic action of CY and cells bearing the alloantigens of the recipient. Furthermore, the phenomenon was demonstrated to be antigen specific.

I. INTRODUCTION

Drug-induced tolerance is a state of specific immunologic unresponsiveness produced by the treatment of animals with antigen and immunosuppressive drugs.¹¹ Most investigations of this phenomenon have focused on the impaired antibody response of treated animals^{11, 13} or cells from treated animals^{1, 2, 8} following rechallenge with specific antigen. Recently, however, experiments were reported that support the view that drug-induced tolerance can also be demonstrated by assays which reflect antigen-specific suppression of cell-mediated immunity. Nirmul et al.^{6, 7} showed that mouse skin allograft survival can be prolonged by pretreatment of recipients with a combination of cyclophosphamide (CY) and spleen cells of donor strain origin. Van Winkle¹⁴ reported that the intensity of the graft versus host (GVH) reaction as measured by the Simonsen assay¹² was diminished by treatment of prospective spleen cell donors with CY and spleen cells from mice of the recipient strain.

We were prompted by these results to test the possibility that GVH mortality might be prevented by treating prospective donors with CY and viable cells bearing the histocompatibility antigens of the recipient. For these studies, a rat model was developed for the lethal GVH disease encountered in human bone marrow transplantation. This model was used to investigate the degree of specific immunosuppression achieved as a function of the interval between injection of a single dose of CY and treatment with viable cells.

II. MATERIALS AND METHODS

Animals. Female Lewis (L; Ag-B¹), Lewis-Brown Norway F₁ hybrid (LBN; Ag-B¹/Ag-B³), and Lewis-Buffalo F₁ hybrid (LBuf; Ag-B¹/Ag-B⁶) rats, weighing

150-225 g, were used throughout this study. Inbred rats were obtained from Microbiological Associates, Bethesda, Maryland. Animals were housed three or fewer per cage except for irradiated grafted rats which were always caged individually. Rats were provided with acidified tap water and a standard laboratory diet ad libitum.

Cell preparations. Animals were killed by cervical dislocation. For the preparation of a single-cell suspension of splenocytes, spleens were dissected, minced with scissors, and gently forced through nylon gauze. The cells were washed three times in iced Hanks' balanced salt solution (HBSS) and the final pellet resuspended in HBSS. For the preparation of marrow cell suspensions, femur and tibiae were removed and the marrow forced out of the bone with HBSS delivered from a syringe through an 18-gauge needle. A suspension of separated cells was prepared by gently pressing the marrow pulp through nylon gauze and thereafter washing the cells three times with HBSS. Viable cells were enumerated in a hemocytometer with the aid of trypan blue dye. Spleen and marrow cells prepared in this manner typically had a viability of 90 percent.

Tolerance-induction protocol. CY was obtained from Mead Johnson Laboratories, Evansville, Indiana. The drug was reconstituted with sterile water in the manner described by the manufacturer and administered within 10 min. L rats were injected intraperitoneally on day 0 with 100 mg CY per kg body weight. This dose of CY is approximately 60 percent of the $LD_{50/30}$ for L rats.⁹ At various times relative to the drug treatment, the rats were injected intravenously with 2×10^8 viable LBN or LBuf spleen cells suspended in 1.0 ml of HBSS. On day +7, the treated rats were

sacrificed to provide spleen and marrow cells for the GVH assay. Control treated rats received either CY on day 0 or LBN cells on day +1.

GVH assay. LBN rats were used as recipients and L rats as marrow and spleen cell donors. Bilateral irradiations were performed on animals restrained in individual Lucite cages. A midline dose of 1000 rads was delivered at a dose rate of 40 rads/min by a 10,000 Ci ^{60}Co source. Within 6 hours, the irradiated rats received an intravenous injection via the saphenous vein of 5×10^7 marrow cells and various numbers of spleen cells suspended in 1.0 ml of HBSS.

In all the experiments performed, a relatively constant fraction of animals died within 5-10 days after grafting of infections apparently unrelated to GVH disease. Thus, mortality was expressed as the percentage of animals surviving past day 60 from the number of animals that were alive on day 12. Similarly, mean survival time (MST) was defined as the arithmetic mean of the individual survival times of rats living greater than 12 but less than 60 days.

In all instances, animals dying of GVH disease manifested characteristic symptoms of the syndrome as previously reported.³ Briefly, these symptoms included weight loss, skin lesions (most often around the eyes), depilation, ulcerations on the skin and kyphotic posture.

Statistical analyses. The significance of differences in mortality was determined with the χ^2 test. The significance of differences in MST was determined with the Student's "t" test for unpaired data.

III. RESULTS

GVH mortality produced by marrow and spleen cells from normal donors. Preliminary experiments were conducted to develop a model for lethal GVH disease in the adult rat. In the first experiment, 15 irradiated LBN rats were grafted with 5×10^7 marrow cells from normal L donors. Mortality for this group of animals was 47 percent and the MST (\pm SE) was 36.57 ± 3.64 days. In the next experiment, irradiated LBN rats were grafted with 5×10^7 marrow and various numbers of spleen cells from normal L donors. Mortality was 100 percent for each spleen cell dose administered. In addition, there was an inverse linear relationship over the dose range studied between MST and the logarithm of the number of spleen cells injected (Figure 1).

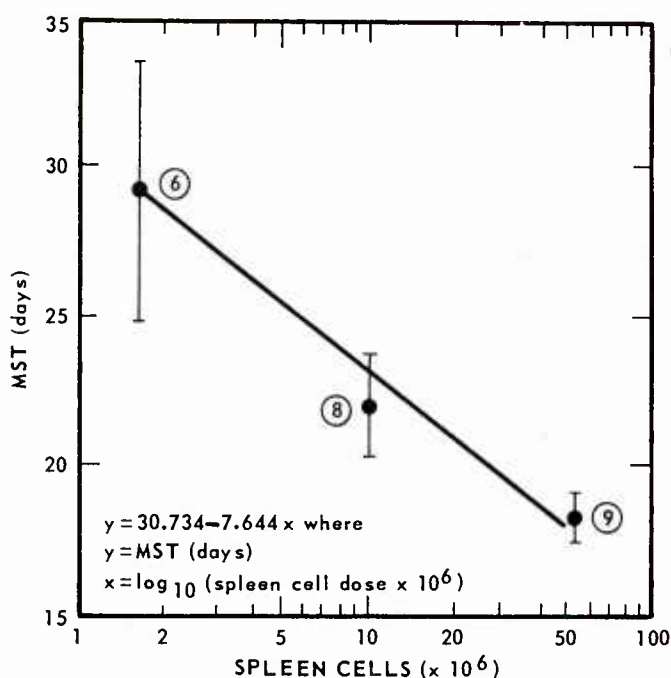


Figure 1.
Survival of LBN rats given 1000 rads and 5×10^7 L marrow and various quantities of L spleen cells. The ordinate represents the MST in days while the abscissa represents the log of the number of transplanted spleen cells. Each point represents the geometric mean (\pm 1 SE) of the number of animals indicated within the circle adjacent to each point.

On the basis of these results, we concluded that mortality and MST could be used as measures of the competence of marrow plus spleen cell inocula to initiate the GVH reaction in the rat. A standard dose of 5×10^7 marrow and 5×10^7 spleen cells was used in all subsequent experiments.

GVH assay of cells from donors treated with CY and allogeneic spleen cells.

Presented in Table I are the mortality and MST data obtained by grafting irradiated recipients with marrow and spleen cells from donors treated with CY on day 0 and LBN spleen cells on day -5, -2, -1, 0, +1, or +5. Also shown are the data obtained with normal donors and donors treated only with CY. Recipients of inocula derived from donors treated only with CY lived significantly longer than recipients of cells obtained from normal donors. The immunosuppression resulting from donor treatment with CY alone, however, was not sufficient to affect GVH-associated mortality. Treatment of donors with LBN cells on days -5 or +5, in addition to CY on day 0, was no more immunosuppressive than the CY treatment alone. On the other hand, CY in combination with cells administered on day -2, -1, 0, or +1 was significantly more immunosuppressive than CY treatment alone. Furthermore, mortality was significantly reduced for those recipients grafted with inocula from donors treated with LBN spleen cells either the day before or the day after administration of CY.

Based on both the mortality and MST data, the best protocol for diminishing the severity of GVH disease in irradiated hosts consisted of donor treatment with CY on day 0 and allogeneic cells on day +1. Comparison of the results obtained with this protocol to those obtained by grafting irradiated LBN recipients with normal L marrow cells shows that there are no substantial differences in either mortality or MST.

Table I. Survival of LBN Rats Given 1000 Rads and 5×10^7 Spleen and 5×10^7 Marrow Cells Obtained from L Donors Previously Injected with CY and Sensitized with 2×10^8 LBN Spleen Cells.

Group number	Donor treatment*	Day of antigen treatment†	Number of animals in group	MST data			Mortality data	
				MST \pm SE (days)	P value cf. group 1	P value cf. group 2	Percent	P value cf. groups 1 and 2
1	None	--	9	18.22 \pm 0.80	--	--	100	NS
2	CY	--	8	24.50 \pm 1.28	<.001	--	100	--
3	LBN + CY	-5	9	27.00 \pm 2.53	<.01	NS	100	NS
4	LBN + CY	-2	7	38.00 \pm 3.61	<.001	<.001	100	NS
5	LBN + CY	-1	10	38.57 \pm 4.77	<.001	<.01	70	<.05
6	CY + LBN	0	8	36.88 \pm 3.83	<.001	<.01	100	NS
7	CY + LBN	+1	12	42.83 \pm 3.33	<.001	<.001	50	<.025
8	CY + LBN	+5	17	26.59 \pm 1.69	<.005	NS	100	NS

* All donor rats were sacrificed on day +7 after CY treatment

† All animals that received CY were injected with the drug on day 0

Specificity of immunosuppression. As stated previously, maximum immunosuppression was obtained by treating prospective donors with CY on day 0 and LBN spleen cells on day +1. This protocol served as a model for experiments designed to determine (1) if the immunosuppression was antigen specific, and (2) if donor treatment with only LBN cells was sufficient to diminish the intensity of the GVH reaction. In the first experiment, L rats were injected on day 0 with CY and on day +1 with LBuf (i.e., third party) spleen cells. The second experiment consisted of treating L rats with LBN spleen cells on day +1 (i.e., 6 days before sacrifice). As is shown in Table II, treatment of donors with CY and third-party spleen cells resulted in survival

Table II. Survival of LBN Rats Given 1000 Rads and 5×10^7 Spleen and 5×10^7 Marrow Cells Obtained from L Donors Injected Either with CY and 2×10^8 LBuf Spleen Cells or Only with 2×10^8 LBN Spleen Cells

Group number	Donor treatment*	Day of antigen treatment ⁺	Number of animals in group	MST data			Mortality data	
				MST \pm SE (days)	P value cf. group 1	P value cf. group 2	Percent	P value cf. groups 1 and 2
9	CY + LBuf	+1	11	23.36 \pm 1.36	< .01	NS	100	NS
10	LBN	+1	8	22.63 \pm 2.04	NS	NS	100	NS

* All donor rats were sacrificed on day +7 after CY treatment

⁺ All animals that received CY were injected with the drug on day 0

times indistinguishable from those obtained by treatment with only the immunosuppressive drug. Donor treatment with LBN spleen cells alone resulted in no significant alteration in immune responsiveness.

IV. DISCUSSION

The data presented here support the view that donor treatment with CY and cells of host origin can diminish the intensity of a systemic GVH reaction. There are two possible explanations for this immunosuppression.

According to the first, CY diminishes the size of the endogenous lymphoid cell compartment of the donor thus permitting the development of a chimera when allogeneic cells are administered. A parallel is drawn here to the experiment of Santos and Owens,¹⁰ in which L rats were treated with CY (225 mg/kg body wt) and then injected with Brown Norway (BN) marrow cells. Seventy days later, 80-90 percent of the cells in the spleens of the L rats were killed by a BN specific antiserum. This experiment differs from the present one in that a much larger dose of CY was used,

the allogeneic cells were of marrow rather than splenic origin, and were not prevented by genetic considerations from initiating a GVH reaction. Although the data presented here cannot rule out this hypothesis, one observation does render it unlikely that it provides the whole answer: LBN spleen cells administered prior to CY treatment were effective in conferring tolerance. Presumably, the lymphocyte depleting properties of CY should be as effective in diminishing the numbers of LBN as well as L cells.

The second explanation attributes the altered immune reactivity to a phenomenon akin to that described by Schwartz and Dameshek¹¹ as drug-induced tolerance. Aisenberg¹ postulated that drug-induced tolerance resulted from immunologic stimulation of drug-damaged cells leading to a destruction of the clone of lymphocytes reactive to the immunizing antigen. That our data are consistent with this hypothesis is supported by the following argument.

In our study, and in previous investigations by others which focused on either humoral^{1,13} or cellular⁷ immunologic unresponsiveness, it was found that antigenic stimulation must occur during a critical span of about 4 days bracketing the time of CY administration. In an elegant study by Aisenberg,¹ it was found that the period of susceptibility after the injection of CY corresponds to a period of depressed DNA synthesis in the spleens of treated animals. The critical timing of antigen treatment before CY administration is probably associated with the persistence time of the immunogen in the animal. The ability of antigen to induce tolerance when administered prior to CY treatment is in contrast to the data obtained when the immunosuppression is provided by antilymphocyte serum (ALS).⁵ When ALS is the immunosuppressive agent, the antigenic challenge must occur after the lymphocyte population is depleted. It is not

surprising then that in ALS-induced tolerance, chimerism is associated with the unresponsive state.

It is entirely plausible that the specific unresponsiveness reported here results from both the establishment of a chimeric condition and the partial depletion of the antigen reaction clone. Experiments are presently underway in our laboratory designed to provide further information on the mechanism involved.

It is interesting that the GVH associated mortality observed for recipients of marrow and spleen cells from donors treated according to the optimal protocol was the same as that observed for recipients of normal marrow cells alone. These data are consistent with the idea that the observed immunosuppression was at the level of mature T cells present in the spleen and not at the level of T cell progenitors present in the bone marrow.⁴

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