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ATTEMPTS TO MODIFY GRAFT-VERSUS-HOST DISEASE WITH CHLORPHENESIN

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ABSTRACT

Because graft versus host (GVH) disease remains a clinical problem, investigation of potentially useful drugs continues to be of interest. Chlorphenesin (CH) a compound found to have immunosuppressive effects *in vitro* and an immunophylactic result *in vivo*, was tested here in a parent-to-F_1 hybrid mouse transplantation model designed to reveal any ameliorative action on GVH. Cumulative mortality data demonstrated that early (4-5 week) survival in groups given spleen and CH was slightly better than that in those given spleen but no drug. However, the ameliorative effect did not persist beyond the second month. Insofar as these data might be predictive for the clinical situation, CH can not be recommended for use to improve long term survival.

Key Words: Graft versus host, secondary disease, chlorphenesin

INTRODUCTION

Graft-versus host disease (GVHD) was recognized early in the history of tissue transplantation (1, 2), and despite the development and use of palliative treatments (3-7), remains a serious clinical problem. Attempts have been made in laboratory models to eliminate the precursors of immunocompetent cells responsible for GVHD by sublethal irradiation (8) of donor marrow or by separation of stem cells (9, 10). Marrow-irradiation, on adequate testing, was found not to be an effective treatment, inasmuch as the surviving stem cells not only repopulated myelopoietic tissues but also supported regeneration of a donor-type immune system: GVHD in the form of secondary disease persisted (11). Recent experiments have shown that pretreating cells with anti Thy 1 ($\alpha\Theta$) serum plus complement eliminated early or acute GVHD in mice injected with allogenic bone marrow and spleen (12-14). The relative success of this approach and of those experiments using separated stem cells (9, 10) raises the interesting question of how selected populations, depleted presumably only of mature immunocompetent cells, are amenable to tolerance induction.

Chlorphenesin (3-ρ-chlorophenoxly-1-2-propanediol) (CH) was examined by Stites _et al_. (15) in a wide variety of _in vitro_ assays for cellular immunity. They suggested that the predominant inhibition of proliferation measured in both B and T cell responses might reduce expansion of immunocompetent clones _in vivo_. Because chlorphenesin (CH) has been reported to reduce primary responses to bacterial and erythrocyte antigens when injected intravenously in rabbits, guinea pigs, or mice (16, 17), and because of its potential clinical usefulness in tissue transplantation, it seemed important to test the drug's ability to modify GVHD. The present paper reports data from such tests.

MATERIALS AND METHODS

Animals

Donors were female or male C57BL/6 (B6) mice, and recipients were (B6+XDBA/2♂)F_1 hybrids (or B6D2F_1). All mice were house conventionally, 4-5 per cage; were allowed free access to Purina mouse chow and acid water; and were checked daily for three months to determine mortality values.

Irradiation

Recipients in a lucite chamber were given a single whole-body exposure to 1200-1250r from a ^{60}Co source at a rate of 28-20 r/minute 20 to 24 hours before being injected intravenously (IV) with cells.

Preparation, drug treatment, and injection of cell suspensions

Spleens were removed from decapitated mice and made into single cell suspensions in medium RPMI 1640 by disruption with a teflon pestle in a loosely fitted test tube. Suspensions were passed through nylon filters to remove debris. Marrow was removed from tibias and femurs by expelling plugs from bones into a small amount of RPMI 1640 using a short 2 ml glass syringe fitted with a 22 or 23g needle. Suspensions were prepared by further passage of marrow through 25g and then 27g needles. Cell suspensions were counted in a hemocytometer after dilution with Eosin Y (0.2%, w/v in physiological saline). A Coulter count was made to assure accuracy. After appropriate dilution of cell suspensions followed by lysing of erythrocytes with Zapoglobin (Coulter Diagnostics), viable cell content was established, and final dilutions for intravenous injection were made in medium RPMI 1640 in the case of spleen (0.5 ml/mouse) or phosphate buffered saline (PBS) for marrow (1.0 ml/mouse). In all cases in which mice received suspensions from both spleen and marrow, spleen

cells were administered first and marrow 20 minutes to 2 hours later.

CH was dissolved in PBS, pH 7.2-7.3, at 37°C in a concentration twice that required. An equal volume of spleen cells at 3.2-.8 x 10^7/ml was then added, and suspensions were incubated in a 37°C water bath for 5-10 minutes and then in a CO_2 incubator for an additional 20-25 minutes after which cells were appropriately diluted for injection. Control suspensions not exposed to drug were similarly handled, the initial 2-fold dilution before incubation being made with PBS. Fetal calf serum (5% v/v) was added to the suspending medium for all spleen cells that were to be incubated at 37°C. For the final experiment CH was mixed with spleen cells immediately (1/2-4 minutes) before they were administered (method suggested by Dr. F. M. Berger, private communication); in this particular experiment spleen and drug were in 1.0 ml volume, BM 0.5 ml.

Treatment with anti Thy-1 (αΘ) serum

Bone marrow pellets of known cell number were suspended at 10^7 cells/ml in αΘ serum at a dilution of 1/40 in RPMI 1640 medium at room temperature for 20 minutes. Cells were then gently pelleted by centrifugation and resuspended to the same volume in guinea pig complement (absorbed on mouse thymocytes, cells from one thymus per one ml serum) at 1/6 in medium for 30' in a 37°C water bath. They were pelleted once again, resuspended in fresh medium, counted, and diluted appropriately for injection. αΘ serum was prepared in a rabbit against C57BL/6 mouse brain in Freund's complete adjuvant and then absorbed according to the methods described by Mishell and Shiigi (18). Serum from each bleeding was labeled, absorbed, titrated against ^{51}Cr-labeled thymocytes, and kept separate. All bleedings from this rabbit released label from more than 90% of the cells at dilutions up to 1/512.

Test for Chimerism

A few drops of retroorbital-sinus blood were removed for red cell typing from each of 151 chimeras that survived beyond day 60 in six of the experiments and from 29 appropriate, untreated control mice. Hemolysis, in the presence of rabbit complement, resulting from specific isoimmune sera prepared to each of the two parental strains (B6 and D2) was compared to that in nonimmune serum plus complement and to that in distilled water (19). Full (100% donor) erythrocyte chimerism in the B6→B6D2F_1 combination is easily recognized by complete lack of hemolysis in the presence of B6 anti D2 serum.

RESULTS

Eight experiments contributed data to this study, five of them (using 1049 mice) establishing baseline values for survival/mortality at several bone marrow and/or spleen cell doses. At least 20 mice (and as many as 30 when available) were assigned to each experimental group. In those experiments in which several doses of bone marrow were administered to different groups in the absence of spleen cells, the greater the dose of B6 marrow given hybrid recipients, the more death seen in the secondary-disease period. When tested for chimerism, more than 90 days after transplantation, all surviving mice in groups that had been given 16, 8, 4 or 2 million bone marrow cells had only donor-type red cells, whereas 13 out of 18 tested that had received only one million cells had at least partially reverted to F_1 hybrid-type erythrocytes. Discounting one early experiment in which, because of a low dose rate from the ^{60}Co source the radiation exposure used was inadequate (20) to kill more than 87% of control (untreated) mice in 30 days, we determined that 8 x 10^6 bone

marrow cells was the best dose to use for testing CH effects on GVH induced by spleen cells. The higher doses tested, 16 and 32 x 10^6 cells, resulted in considerable secondary disease, and the doses lower than 8 x 10^6 promoted much poorer 90-day survival.

No dose of spleen cells, administered without bone marrow, was found to have sufficient hemopoietic stem cells and at the same time few enough cells with GVH potential to promote 30-day survival. Spleen cells added to an adequate dose of marrow, established as described above, accelerated death from GVHD in a dose-dependent manner. All recipients of no marrow or marrow plus spleen at 32, 26, or 8 million cell doses were dead by day 40 in one experiment. Second and third studies, replicating five of the seven groups tested in the first experiment confirmed those findings (data not shown).

From the seven studies just described, involving 90 day survival studies on 1578 recipient mice, a bone marrow dose of 8 million cells and two spleen cell doses, 8 and 4 million, were finally chosen for further investigation of the effect of CH on mortality from GVHD. Because our own data (unpublished) and that of others (12-14) indicate that GVH is curtailed or abolished when donor marrow is treated with $\alpha\Theta$ serum, we decided to eliminate the complication with which mature T lymphocytes might burden our interpretation by removing then from the marrow suspension in the final experiment presented here in Figure 1. As can be seen from both figure parts a) and b), separated by spleen cell dose for clarity of presentation, there were no long term (beyond 60 days) survivors in any group given spleen along with bone marrow. Nonetheless, CH was able to afford some protection in that treatment with 400 μg/ml shifted the mortality curve to the right at both doses of spleen cells (4 and 8 million). At the lower spleen cell dose, all three levels of CH (100, 200, and 400 μg/ml) appeared to ameliorate or delay death from GVH in the first four weeks.

Unfortunately the early improvement in survival did not persist. Long term survival was found to be no better in two analogous experiments in which similar and even higher CH doses were tested, although, again there was suggestive evidence that CH at 400 μg/ml delayed early GVH death. These studies (data not shown) used 4×10^6 marrow and 16×10^6 spleen cells incubated with the drug, and they were done earlier than that shown in Figure 1. As in the early experiments establishing appropiate cell doses, chimerism tests were done for all mice living beyond 60 days in this final experiment. In confirmation of those other findings, all were full chimeras (i.e., had circulating red cells of donor, B6, type).

DISCUSSION

The preliminary experiments carried out here as a basis for testing the anti GVH capacity of chlorphenesin confirm rather old, but sometimes forgotten, transplantation findings. First, there is an inverse relationship between the quantity of donor marrow transplanted and the incidence of graft reversion to host type within a given nonisogenic donor-host combination, all other conditions being the same. This finding, apparent in Popp's data twenty years ago (21) and confirmed here by tests of red cell chimerism, has probably been fully appreciated clinically, availability of donor marrow being a severe limitation on the maximum dose administered. However, despite (or perhaps because of) the better survival of marrow grafts in our experiments when higher doses were injected initially, recipients survived less well. Chimeric mice died in that period when secondary or GVH disease is usually seen. Second, radiation dose rate is of critical importance in determining total dose in those situations that require an exposure that is lethal to the hemopoietic system (20). A

whole-body ^{60}Co exposure that is adequate for a given mouse strain on one occasion will not obtain when the experiment is repeated six months or two years later.

The selection of CH doses to be tested in this study was based on earlier work of Stites et al. (15) and Berger et al. (17). The greatest exposure used here, 400 μg/ml, either injected immediately after mixing with cells or first incubated with them for 30 minutes, was well below the level at which significant lysis of mouse splenocytes was recorded in the former study and presumably therefore was not toxic. The other doses we tested, 200 and 100 μg/ml, were within the range at which were recorded significant in vitro effects on human and mouse cells in mixed lymphocyte and mitogenic responses (15). It is impossible to compare these doses accurately to effective in vivo levels achieved by Berger et al. (17), because no measurements of drug concentration at the critical site(s) were made, nor probably was such measurement feasible. At 50 mg/kg body weight, one would expect 50 μg/ml at any location if CH were distributed evenly and quickly throughout the body. Initially, assuming blood volume to be 6.5% body weight (22), we could expect an intravenous injection of such a dose to produce a concentration of 769 μg/ml whole blood in a 20 gram mouse. Clearly the actual CH concentration necessary for a decrease in the primary immune responses reported by those investigators was between 50 and 769 μg/ml, within which limits we chose our test doses.

It should be noted that CH-induced reduction of a primary immune response could be shown only if drug was introduced concomitantly with (but not before or after) the antigen (16, 17). Moreover, despite the near ablation of a primary, challenge with the same antigen at a later time evoked a secondary response. It is interesting that Berger et al. (17), in view of this and other in vivo findings, such as the lack of effect of CH on circulating lymphocyte

numbers, chose to describe its effects as "immunophylactic rather than immunosuppressive."

After our early somewhat disappointing results, we consulted with Dr. Berger again and decided to inject CH along with the spleen immediately after the two were mixed. Another possibility would have been to inject the drug separately before the cells. However, the cells to be "immunized" in this case are the parental spleen cells being administered, not the hybrid host's. The simultaneous injection of CH and spleen cells, immediately after their mixing to avoid possible in vitro metabolic alteration of the drug, would therefore afford the best exposure of target cells to the drug. This protocol, chosen for the final studies, closely resembles that used in studies discussed above of primary and secondary responses to nonproliferating antigens. It produced mortality data suggesting that early GVH had been ablated but that the capacity of donor spleen cells to recover this potential remained. This interpretation is compatible with the immune response findings insofar as the capacity for a secondary response was demonstrated in those studies, and the spleen cells we injected in our own study were fully able to persist and proliferate, providing the basis for an analogous secondary response.

From the experiments presented here, carefully designed to test the effectiveness of CH in combating the GVH seen when marrow and/or spleen cells are transplanted into nonisogenic recipients, we can conclude that the drug ameliorates early mortality but that long-term survival is unaffected. Use of CH cannot, therefore, be strongly recommended as a replacement for or as an adjunct to $\alpha\Theta$ treatment of donor material or to other therapies calculated to eliminate GVH disease.

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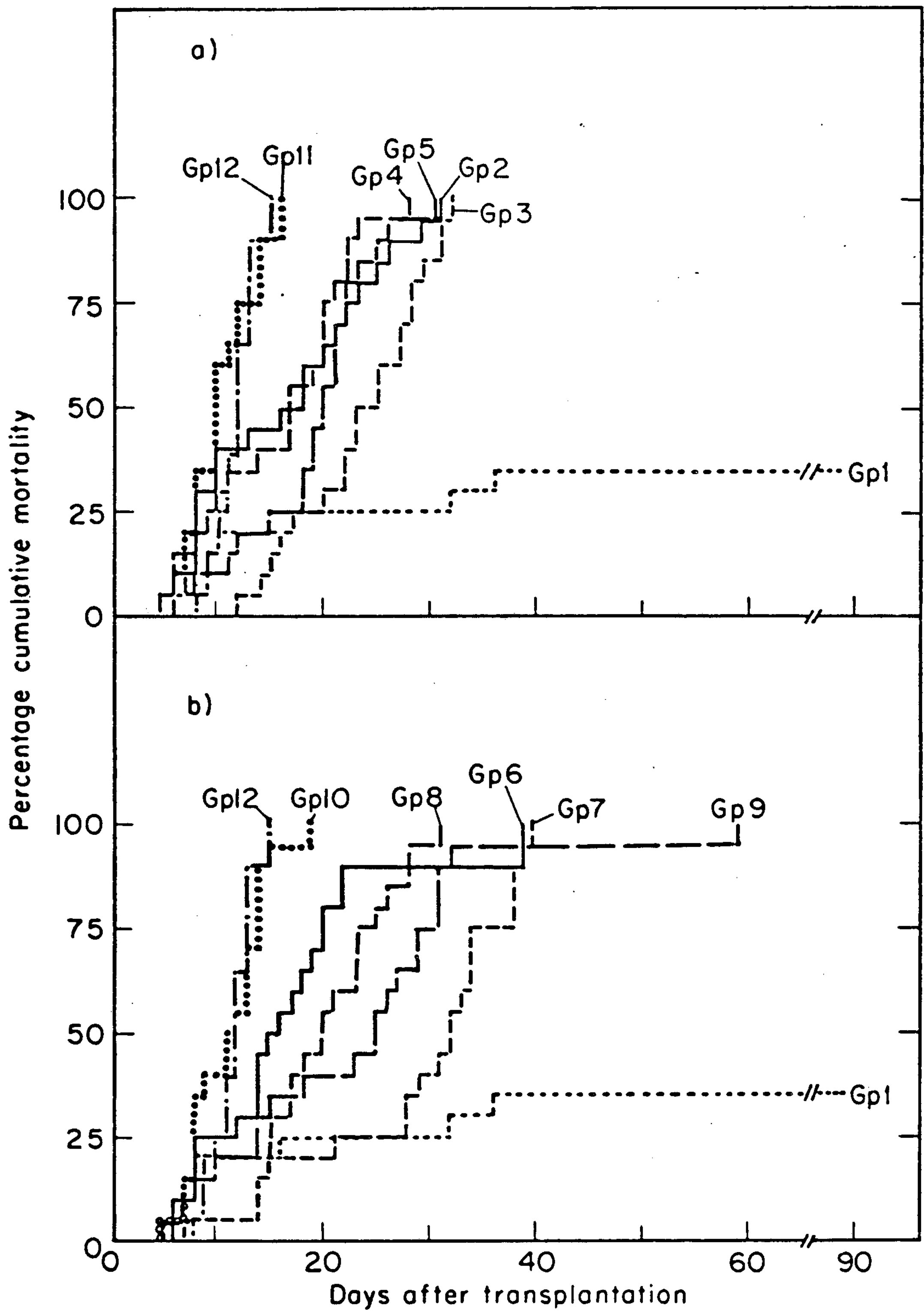
FIGURE LEGEND

Figure 1. Cumulative mortality in 12 groups, each of 20 irradiated B6D2F_1 mice given 8 x 10^6 αΘ treated B6 bone marrow cells: GP1 •••••• marrow only, GP10&11 oooooo spleen only, GP12 -•-•-• untreated controls, GP2&6 ——— marrow plus untreated spleen, GP5&9 —— —— ——marrow plus 100 μg/ml CH spleen, GP4&8 — — — marrow plus 200 μg/ml CH spleen, GP3&7 ------- marrow plus 400 μg/ml spleen. Spleen cell dose 8 x 10^6 in part a), 4 x 10^6 in part b).


a)
Gp12
Gp11
Gp5
Gp4
Gp2
Gp3
Gp1
100
75
50
25
0
b)
Gp12
Gp10
Gp8
Gp6
Gp7
Gp9
Gp1
Percentage cumulative mortality
0
20
40
60
90
Days after transplantation
XBL836-3784

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