Immunological Mechanism of Action of an Experimental Nucleoside Drug, Gr1784: Effects on Hematopoiesis and Lymphocyte Mitogenesis

Robert Clive Landis
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IMMUNOLOGICAL MECHANISM OF ACTION OF AN EXPERIMENTAL NUCLEOSIDE DRUG, GR1784: EFFECTS ON HEMATOPOIESIS AND LYMPHOCYTE MITOGENESIS

By

Robert Clive Landis

A Thesis submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science

May

1987
I would like to acknowledge the guidance, advice and perseverance of my committee members, Drs. Paul Gordon, Terry Wepsic, Herbert Mathews, Charles Lange and Allen Frankfater.

I would also like to acknowledge the unfailing friendship of David & Diane Beno, Edward Fitzsimons and Alexander Stern, who have made the journey along the path of postgraduate education an enjoyable, as well as worthwhile, one.
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INTRODUCTION

This thesis presents experiments designed to further our understanding of the immunological mechanism of action of GR1784, a novel synthetic nucleoside drug. Previous observations have indicated that GR1784 may have potential pharmaceutical applications in a number of immunological conditions, in particular the recovery from immunosuppression induced by exposure to X-irradiation or administration of cytotoxic drugs.

The important questions answered in this thesis are:

1) Can GR1784 exert immunomodulation upon a normal, healthy immune system or only upon a damaged immune system (as previously observed)?

2) What are the effects of GR1784, if any, upon certain hematopoietic and cellular immune parameters subsequent to immunosuppression?

In order to address these questions, the drug was administered to groups of normal and immunosuppressed mice in vivo, while temporally assessing its hematological and immunological effects upon a number of cell populations in vitro.

The agent chosen to induce immunosuppression in these studies was cyclophosphamide (Cy). The previously observed immunopotentiating effects of GR1784 were made in animal
models of immunosuppression induced by exposure to X-irradiation or administration of Cy. The parameters assessed in these studies have been shown to be affected in very similar ways by either of these two hematological insults. Cy was preferred because of its ease of administration and more reproducible immunosuppression.

The hematological parameters followed were: 1) Peripheral blood leukocyte count (PBLC), 2) spleen weight and spleen weight index (S.W.I.), 3) bone marrow and spleen cellularity and 4) bone marrow and spleen colony forming units (CFU's). The cellular immune parameters followed were: 1) Splenic T lymphocyte mitogenesis, as assessed by response to Concanavalin A (Con A) and 2) splenic B lymphocyte mitogenesis, as assessed by response to Lipopolysaccharide (LPS).

These parameters provided an indication of both the quantity and functional responsiveness of cell populations at various stages of differentiation, ranging from pluripotent progenitor stem cells of the bone marrow to fully differentiated and mitogen responsive lymphocytes of the spleen. In addition, these parameters have all been shown to be affected by the administration of Cy at the doses employed in these studies.
GR1784 is a Xanthosine analog that belongs to a family of nucleoside drugs, one of which, Isoprinosine, is in clinical use throughout much of the world as an antiviral agent (for review see 1). GR1784 is the ion pair formed between the Choline cation and the Xanthosinate anion. It is the product of a neutralization reaction that occurs in aqueous solution by the mixing of equimolar (0.4 M) quantities of Choline Hydroxide (in Methanol) and Xanthosine.2H₂O. The white Choline Xanthosinate precipitate is formed at a temperature of 80°C and is lyophilised to dryness.

This unusual ion pair association was strongly suggested by Infra Red (I.R.) spectroscopy (Gordon, P. and Ferraro, J., 1985, unpublished data), pH and conductivity data (Gordon, P., 1984, unpublished data). I.R. spectroscopy on solid Choline Xanthosinate in KBr showed no spectral peak shifts compatible with the formation of new covalent bonds. Furthermore, the pH in 0.1 M solution of this salt of a strong base and weak acid was 7.10. This contrasted sharply with the pH of a salt formed between the Sodium cation and Xanthosinate anion, which was 10.48 in 0.1 M solution. In the absence of any covalent interaction, this argues strongly for the formation of an
ion pair between the Choline cation and Xanthosinate anion. Lastly, the conductivity of Choline Xanthosinate was also anomalous: Whereas the equivalent conductivity of Sodium Xanthosinate paralleled that of an ionic salt (NaCl), that of Choline Xanthosinate was dramatically reduced over the same range of concentrations. This again argues for the formation of an ion pair.

Its neutral pH, together with its good solubility in aqueous solvents, make GR1784 ideal for safe pharmaceutical application. A caveat to its administration in liquid form, is that it is unstable in aqueous solution. It has been shown to be 67% hydrolyzed into one of its constituents, Xanthine, after 7 days in distilled water (Landis, R.C., 1984, unpublished data).

While no pharmacologic properties have been previously reported for Xanthosine or its analogs, unpublished data from this laboratory, employing both animal models and in vitro culture systems, suggest that GR1784 may possess immunoregulatory properties. For example, GR1784 has been shown to provide significant protection towards lethal and sublethal X-irradiation in Fisher F344 rats (Gordon, P. and Wepsic, T., 1985, unpublished data). In a rat model of chronic Cy induced immunosuppression, GR1784 was shown to exhibit immunorestorative properties similar to those previously reported for Isoprinosine (2). Using Isoprinosine as an
internal control, a significant reversal of Cy cytotoxicity, with respect to body weight and spleen weight, was demonstrated. In addition, a partial (not significant) restoration of depressed Con A mitogenesis was observed, which was at least as effective as that achieved by Isoprinosine (Gordon, P., 1985, unpublished data). In contrast to these immunopotentiating properties, GR1784 was also shown to significantly enhance the formation of suppressor cells (Gordon, P., 1985, unpublished data), in an in vitro model of Con A induced suppressor cell generation (3).

These findings suggest that GR1784 may possess immunomodulating properties very similar to those ascribed to Isoprinosine (4, 5, 6).

The C57Bl/6 mouse model was used because it has been widely employed as a standard model in which to perform X-irradiation and Cy cytotoxicity studies (9, 10, 11, 12, 13).

Cy was chosen as the agent with which to induce hematosuppression in an attempt to combine the previously observed radiation protection and Cy cytotoxicity reversal data of GR1784 into a unified mechanism of action.

The four hematopoietic parameters assessed (see Introduction) have all been shown to be affected in basically similar ways by both X-irradiation and Cy administration in both rats and mice (13, 14, 15, 16). The
chronological order of events following recovery from either of these two hematological insults has been shown to be the same: Survival and recovery of the pluripotent hematopoietic stem cells in the bone marrow and subsequent repopulation of depleted mature cell compartments in the peripheral blood, spleen and bone marrow (17, 18, 19, 20).

The CFU parameter is a measure of the colony forming capacity of an early stem cell population that represents the first link in the chain of events leading to recovery following hematological insult. The possibility that GR1784 could be acting as a radioprotector (Gordon, P. and Wepsic, T., 1985, unpublished data) by stimulating hematopoiesis is therefore also investigated. This possibility is also consistent with its significant restoration of the spleen weight index during Cy immunosuppression in rats (Gordon, P., 1985, unpublished data).

The parameters that measure differentiated cell numbers are important not only because they reflect the recovery of pluripotent stem cells but also because they can provide information on any potential direct protection of mature cells by GR1784.

Another means by which GR1784 might be exerting its radioprotective effects is by increasing the immunological responsiveness of the surviving lymphocyte compartment following X-irradiation. Such a mechanism of action was
examined by assessing the responsiveness of splenic lymphocytes to both T & B cell mitogens at several time points following Cy administration.

The various hematopoietic and mitogenic parameters outlined above were assessed on days 2, 5, 9 & 16 following Cy administration on day 0. This was necessary due to the dynamic nature of both the circulating lymphocyte (13, 21, 23) and hematopoietic stem cell compartments (8, 12, 15, 16, 17, 18, 19, 20, 21 and for review 22) following Cy treatment. The assay days were chosen to reflect the three phases of; depression, rebound, and normalisation, characteristic of Cy immunosuppression. In normal mice these time points represented days following the beginning of the GR1784 treatment regimen. This selection of time points therefore permitted the detection of both early and late potential drug effects of GR1784 in normal mice.
MATERIALS AND METHODS

Materials  Concanavalin A (Con A) and Pokeweed Mitogen (PWM) were purchased from Sigma Chemical Corporation (St. Louis, MO). Hanks balanced salts solution (HBSS), fetal calf serum (FCS), Eagles MEM essential amino acids, Eagles MEM non-essential amino acids and sodium pyruvate were purchased from Grand Island Biological Company (Grand Island, NY). RPMI 1640, L-glutamine, Penicillin/Streptomycin (10000 U/ml & 10000 μg/ml) and HEPES buffer were purchased from Irvine Scientific (Santa Ana, CA). E. coli 0127:B8 LPS and bacto DIFCO agar was purchased from DIFCO Laboratories (Detroit, MI). Flat bottomed 96 well microtiter plates and 35 mm² plastic petri dishes were purchased from Falcon laboratories (Oxnard, CA). 3H-Thymidine (6.7 mCi/mol) was purchased from ICN Chemicals & Radioisotope Division (Irvine, CA). Pseudocumene scintillation liquid was purchased from Beckman instruments Inc. (Fullerton, CA).

Mice  Male C57Bl/6NCR mice, 22 weeks old, were purchased from Charles River Laboratories (Wilmington, MA) and were used in the Cy dose titration and colony stimulating factor (CSF) titration experiments. The age of these mice, well...
past the optimum for mitogenic studies, was deemed acceptable for the purposes of titration of a CSF source. Unknown CSF activity was determined by comparison with a known CSF standard. Female C57Bl/6 mice, 8-10 weeks old, were purchased from Cumberland View Farms (Clinton, TN) and were used in the experiments examining the immunomodulating effects of GR1784. Male CBA/2J mice, 22 weeks old, were purchased from Jackson Laboratories (Bar Harbour, Maine) and were used to prepare spleen conditioned growth medium for the CFU experiments.

Drugs Cyclophosphamide [Neosar] was purchased from Adria Laboratories (Columbus, Ohio) and was injected intraperitoneally (i.p.) at either 150 or 200 mg/kg body weight on day 0. GR1784 (Dr. P. Gordon, Loyola University of Chicago, Maywood, IL) was prepared in physiological saline and was injected subcutaneously (s.q.) at 0.1 mg/kg/day, the dose shown to most effective in previous radioprotection studies (Gordon, P. and Wepsic, T., 1985, unpublished data). It was prepared freshly each day immediately prior to use, because of its labile nature in aqueous solution (see Literature Review).

Peripheral blood leukocyte count (PBLC) Peripheral blood was obtained by retroorbital puncture, diluted in physiological saline to 1 % and counted, after erythrocyte
lysis, with a Hycel automated cell counter (Hycel Co., Houston, TX).

**Spleen weight and spleen weight index (S.W.I.)** Spleens were removed aseptically, all excess fat trimmed away and each weighed individually on sterile gauze pads. The spleen weight index is the spleen weight as a percent of body weight and is defined by the equation: \( \frac{\text{spleen weight}}{\text{body weight}} \times 100 \).

**Bone marrow and spleen cell preparations** Spleens and hind leg femurs were removed observing sterile technique and single cell suspensions made by homogenising individual spleens with ground glass homogenisers and washing out femurs with HBSS using a 22 gauge syringe. Nucleated cells from individual animals were counted in Turk's stain. Cell viabilities were also determined by Trypan Blue dye exclusion of pooled cell populations. Cell counts represent the average ± S.E. of total spleen and total bone marrow/femur cell numbers for each treatment group.

**Splenic lymphocyte mitogenesis** The mice used in the Cytitration experiment were male C57Bl/6NCR (Charles River Laboratories, Wilmington, MA) and in the GR1784 immunomodulation experiment female C57Bl/6 (Cumberland View Animal Farms, Clinton, TN). Individual spleen cell
suspensions were pooled for each treatment group and washed 3 x with HBSS. The final resuspension was in culture medium (RPMI 1640 supplemented with 10 % FCS, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2 mM L-glutamine, 20 mM HEPES buffer and 50 μM 2-Mercaptoethanol). Nucleated spleen cells were counted in Turk's stain and viability determined in 0.04 % Trypan Blue. Cells were placed at 2 x 10^5 cells/well in flat bottomed 96 well microtiter plates and incubated in the presence of mitogen for 72 h at 37°C and 5 % CO₂. Con A was included at 0.5, 1.0, 3.0 & 5.0 μg/ml and LPS at 0.5, 1.0 & 2.0 μg/ml. Cells were pulsed with ³H-Thymidine over the last 18 h of incubation. They were harvested and then counted for radioactive decay in the ³H channel using a Beckman LS 5801 scintillation counter. Counts represent average ± S.E. DPM's of triplicate wells.

**Generation of colony stimulating factor (CSF-C.L.)** Pooled spleens from two male CBA/2J mice were homogenised and washed 3 x with HBSS. The final resuspension was in culture medium (RPMI 1640 supplemented with 10 % FCS, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2 mM L-glutamine, 20 mM HEPES buffer and 50 μM 2-Mercaptoethanol). Nucleated cells were counted in Turk's stain. CSF was generated by incubating 50 ml spleen cells, at 2x 10^6 cells/ml, with 0.2 ml 1 % PWM for 7 days at 37°C and 5 % CO₂. The supernate
was removed, vacuum filtered (pore size 45 μm) and stored at -20°C until required.

**Colony forming unit (CFU) assay** Bone marrow and spleen CFU's were measured by employing a soft agar assay (12). Specifically, spleen and bone marrow cell suspensions from individual animals were pooled for each treatment group and were washed 3 x with HBSS. The final resuspension was in RPMI 1640 (supplemented with 15 % FCS, 0.045 % sodium bicarbonate, 1 mM sodium pyruvate, 0.8 % Eagles MEM essential amino acids (100X), 0.4 % Eagles MEM non-essential amino acids (100X), 0.4 % Eagles MEM vitamins (100X), 0.8 mM L-glutamine, 50 U/ml Penicillin and 50 μg/ml Streptomycin). Nucleated spleen and bone marrow cells were counted in Turk's stain and viability determined in 0.04 % Trypan Blue. 0.7 ml bone marrow or spleen cell suspensions, at a final concentration of 7.5 x 10^4 bone marrow and 7.5 x 10^5 spleen cells/ml, were mixed at 37°C with 0.1 ml 3 % molten bacto DIFCO agar and plated in 35 mm^2 petri dishes containing 0.2 ml of the CSF source. PWM spleen conditioned CSF source was included at 0 & 25 μl volumes for each treatment group. CSF-C.L. was prepared as described above and CSF-R.Y. was the gift of Rita Young (Department of Pathology, Loyola University, Maywood IL). The number of colonies (> 50 cells) per 35 mm^2 petri dish was determined microscopically at 40 x magnification after
5 days incubation at 37°C and 5 % CO₂. CFU counts represent the average ± S.E. number of colonies/petri dish. Background colony counts, occurring in the absence of an exogenously added CSF source, were subtracted for each determination.

Statistics The students t test was used to evaluate differences between treatment groups, significance being attributed at P values < 0.05.
The experiments presented in this thesis are concerned with studying the effects of GR1784 upon a number of immunological parameters in normal and Cy immunosuppressed mice. Two important steps that had to be taken before this study could be initiated, however, were a determination of the Cy dose used to induce immunosuppression and a standardisation of the two *in vitro* culture assays employed, the mitogenesis and CFU assays. The remainder of the parameters pertained to determination of spleen weight and quantitation of several hematological cell populations. These did not require standardisation before evaluation of their response to GR1784 treatment.

A Cy dose titration was therefore first performed, following the suppression of lymphocyte mitogenesis as a measure of immunosuppression. A dose of Cy was selected that resulted in a significant suppression of mitogenesis but not so large as to overshadow any potential immunorestorative effects of GR1784. This experiment also served the dual purpose of standardising the B and T lymphocyte mitogenesis assays for subsequent use.

The other *in vitro* assay that required standardisation was the soft agar CFU assay of bone marrow
and spleen. This measures the colony forming response of progenitor cells to an exogenously added colony stimulating factor (CSF) source. Such a CSF source was obtained from Pokeweed mitogen (PWM) conditioned spleen cells, as described in Materials and Methods, and was assayed for colony stimulating activity against a positive control in a standard soft agar CFU assay. The dose of CSF source used in subsequent assays was therefore established at the same time as standardising the CFU assay.

With these preliminary experiments completed, the main study assessing the potential immunomodulating effects of GR1784 in C57Bl/6 mice, was undertaken.
RESULTS

**Cy dose titration: Suppression of splenic lymphocyte mitogenesis**

Cy was observed to exert a dose dependent suppression of both B and T lymphocyte mitogenesis with respect to untreated control animals (Fig 1.). This suppression became more pronounced with time, reaching maximum levels on the last assay point, 9 days following Cy treatment. The suppression of B lymphocyte mitogenesis, as assessed by response to LPS, was more profound than the suppression of T lymphocyte mitogenesis, as assessed by response to Con A.

Suppression of T lymphocyte mitogenesis by 150 mg/kg and 200 mg/kg Cy varied from 13 % (N.S.) and 16 % (P < .02) respectively on day 3, to 40 % and 70 % (P < .001 in each case) respectively on day 9. In comparison, B lymphocyte mitogenesis was already significantly suppressed by both doses on day 3 (P < .001 in each case). Suppression increased steadily with time and reached 79 % and 92 % for the 150 mg/kg and 200 mg/kg doses respectively on day 9.

The Cy dose of 150 mg/kg was chosen to induce immunosuppression in subsequent studies investigating the potential immunomodulating effects of GR1784.
Figure 1  Cy dose titration: Suppression of splenic B & T cell mitogenesis. Male C57Bl/6NCR mice (n=5) were injected i.p. on day 0 with either saline or Cy at 150 mg/kg (open symbols) or 200 mg/kg (closed symbols). Spleens were removed on days 3, 6 & 9, homogenised and seeded into 96 well microtiter plates at a concentration of $2 \times 10^5$ cells/well. Cells were incubated for 72 h at 37°C and 5% CO$_2$ with either media alone, Con A at 3 μg/ml (triangles) or LPS at 1 μg/ml (squares). $^3$H-Thymidine uptake over the last 18 h of incubation was assessed by liquid scintillation spectroscopy. Counts represent the average ± S.E. DPM's of triplicate wells, expressed as a % of untreated controls.
## CSF TITRATION

<table>
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<tr>
<th>μl CSF</th>
<th>CSF source</th>
<th>A) Bone Marrow</th>
<th>B) Spleen</th>
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<tr>
<td>0</td>
<td></td>
<td>1 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>C.L.</td>
<td>188 ± 18.6</td>
<td>21.0 ± 2.0</td>
</tr>
<tr>
<td>25</td>
<td>R.Y.</td>
<td>142 ± 3.2</td>
<td>15.0 ± 1.5</td>
</tr>
<tr>
<td>50</td>
<td>C.L.</td>
<td>250 ± 8.5</td>
<td>25.3 ± 2.9</td>
</tr>
<tr>
<td>50</td>
<td>R.Y.</td>
<td>170 ± 10.5</td>
<td>18.3 ± 1.8</td>
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Table 1  Titration of the CSF preparation for activity in a CFU assay  The PWM induced CSF preparation, CSF C.L. (see Materials and Methods) was compared for colony stimulating activity with a positive control, CSF R.Y., in a soft agar Colony Forming assay. Final 1 ml reaction mixtures contained A) 7.5 x 10⁴ Bone Marrow cells or B) 7.5 x 10⁵ spleen cells, 0.3 % bacto DIFCO agar and CSF at the volumes indicated. Colonies ( > 50 cells) were counted microscopically at 40 X magnification after 5 days incubation at 37°C and 5 % CO₂. Numbers represent the average colonies ± S.E. per 35 mm² petri dish of triplicate reaction mixtures.
CSF production and dose titration

The CSF source to be used in subsequent CFU assays, CSF C.L., was prepared from PWM conditioned CBA/2J splenocytes as described in Materials and Methods. It was then titrated for colony stimulating activity with a positive control, CSF R.Y., in a standard soft agar colony forming assay (Table 1).

Both CSF sources were shown to possess significant colony stimulating activity, when included in reaction mixtures at volumes of 25 \( \mu l \) and 50 \( \mu l \). The colony stimulating activity of CSF C.L. was greater than that of the positive control, CSF R.Y. In fact, CSF C.L. possessed a colony stimulating activity at 25 \( \mu l \) approximately equal to that of CSF R.Y. at double that volume.

Effect of GR1784 upon hematopoietic parameters in normal and Cy immunosuppressed mice

1) **Peripheral Blood Leukocyte Count (PBLC)**

In normal mice, GR1784 was observed to cause a significant depression of PBLC's on days 2 (P < .05) and 9 (P < .02), but not on days 5 and 16 (Fig. 2.). A steady increase in the PBLC level was also observed in the untreated control group as the study progressed, indicating a certain amount of variability in the assay.

The administration of 150 mg/kg Cy caused a significant depression (P < .001) of PBLC's to 30 % of
Fig 2  Effect of GR1784 upon PBLC's in normal and Cy immunosuppressed mice. Female C57Bl/6 mice were injected i.p. on day 0 with either saline (circles) or 150 mg/kg CY (triangles). Open symbols represent control groups and closed symbols GR1784 treated groups (0.1 mg/kg/day, s.q.). Blood was obtained on days 2, 5, 9 & 16 by retroorbital puncture, diluted in physiological saline to 1% and counted, after erythrocyte lysis, on a Hycel automated cell counter. Points represent the average ± S.E. PBLC's from 5 animals.
Peripheral Blood Leukocyte Count (PBLC)

Days post Cyclophosphamide treatment

PBLC x 10^6/ml
untreated control levels. While a gradual return to normal was observed on days 9 and 16, PBLC levels still remained significantly depressed on these days ($P < .002$ and $P < .01$ respectively). GR1784 had no significant effect upon PBLC's in Cy immunosuppressed mice.

2) Spleen weight and S.W.I.

Both spleen weight and S.W.I. expressed very clearly the three phases characteristic of CY cytotoxicity: Significant spleen weight depression on days 2 and 5 ($P < .001$), significant rebound and splenomegaly on day 9 ($P < .001$) and complete normalisation by day 16 (Fig. 3a. & b.).

There was clearly no GR1784 treatment effect upon either of these parameters in either normal or Cy immunosuppressed mice.

3) Spleen and bone marrow cellularity

The response of spleen cellularity was observed to follow that of spleen weight very closely (Fig. 4a.), exhibiting the three phases of Cy cytotoxicity described above. There was no GR1784 treatment effect upon spleen cellularity in either normal or Cy immunosuppressed.

Bone marrow cellularity was significantly depressed by Cy on day 2 ($P < .001$), levels returning to normal by days 9 and 16 (Fig. 4b.). No rebound phase similar to that described for spleen cellularity was observed. No GR1784
Fig. 3 a. & b. Effect of GR1784 upon spleen weight and S.W.I. in normal and Cy immunosuppressed mice. Female C57Bl/6 mice were injected i.p. on day 0 with either saline (circles) or 150 mg/kg CY (triangles). Open symbols represent control groups and closed symbols GR1784 treated groups (0.1 mg/kg/day, s.q.). Spleens were removed and weighed on days 2, 5, 9 & 16. Spleen Weight Index is defined by the equation: \((\text{spleen weight/body weight}) \times 100\). Points represent A) the average spleen weight \(\pm\) S.E. (in mg) or B) the average S.W.I. \(\pm\) S.E. of 5 animals.
Fig. 4a. & b. Effect of GR1784 upon bone marrow & spleen cellularity in normal and Cy immunosuppressed mice.

Female C57Bl/6 mice were injected i.p. on day 0 with either saline (circles) or 150 mg/kg CY (triangles). Open symbols represent control groups and closed symbols GR1784 treated groups (0.1 mg/kg/day, s.q.). Spleens and hind leg femurs were removed on days 2, 5, 9 & 16. A) Spleens were homogenised using a ground glass homogeniser and B) femurs were flushed using a 22 gauge needle. Nucleated cells were counted in Turk's stain. Points represent the average ± S.E. nucleated cells/spleen or hind leg femur of 5 animals.
A) Spleen Cellularity

B) Bone Marrow Cellularity
related drug effects were observed on this parameter in either normal or Cy immunosuppressed mice.

4) **Spleen and bone marrow CFU's**

Bone marrow CFU's were rapidly elevated by Cy on day 2 to 350 % of untreated control levels. They passed through normal levels on day 5 and rebounded to 15 % of untreated control levels on day 9 (Fig. 5a.). There was no GR1784 treatment effect in either normal or immunosuppressed mice.

Spleen CFU's were unchanged by Cy on day 2 but became dramatically elevated on days 5 and 9 to 800 % and 1700 % of untreated control levels respectively (Fig. 5b.). GR1784 exerted no effect upon this parameter in immunosuppressed mice. In normal mice, however, it significantly depressed spleen CFU's to 40 % and 37 % of untreated control levels on days 5 and 9 respectively (P < .01 in each case), but not on day 2.

**Effect of GR1784 upon T & B cell mitogenesis in normal and immunosuppressed mice**

In confirmation of the results observed in the Cy dose titration experiment (Fig. 1), the suppressive effect of 150 mg/kg Cy was more profound on B lymphocyte mitogenesis than T lymphocyte mitogenesis (Fig. 6a. & b.).
Fig. 5a. & b. Effect of GR1784 upon bone marrow and spleen CFU's in normal and Cy immunosuppressed mice.

Female C57Bl/6 mice were injected i.p. on day 0 with either saline (circles) or 150 mg/kg CY (triangles). Open symbols represent control groups and closed symbols GR1784 treated groups (0.1 mg/kg/day, s.q.). Single cell suspensions were obtained (see Materials and Methods) on days 2, 5 & 9 and tested for A) bone marrow and B) spleen colony forming capacity in a standard soft agar colony forming assay. Final 1 ml reaction mixtures contained 7.5 x 10⁴ bone marrow cells, 7.5 x 10⁵ spleen cells, 0.3 % bacto DIFCO agar and 25 µl CSF. Colonies were counted microscopically at 40 X magnification after 5 days incubation at 37°C and 5 % CO₂. Points represent the average ± S.E. CFU's of 5 animals, expressed as a % of untreated controls.
A) Bone Marrow CFU's

B) Spleen CFU's
Fig. 6 a. & b. Effect of GR1784 upon T and B lymphocyte mitogenesis in normal and Cy immunosuppressed mice. Female C57Bl/6 mice were injected i.p. on day 0 with either saline (circles) or 150 mg/kg CY (triangles). Open symbols represent control groups and closed symbols GR1784 treated groups (0.1 mg/kg/day, s.q.). Spleens were removed on days 2, 9 & 16, homogenised and seeded into flat bottomed 96 well microtiter plates at a concentration of $2 \times 10^5$ cells/well. Cells were incubated for 72 h at 37°C and 5% CO$_2$ with media alone or in the presence of A) LPS at 1 μg/ml or B) Con A at 3 μg/ml. $^3$H-Thymidine uptake over the last 18 h of incubation was assessed by liquid scintillation spectroscopy. Points represent the average ± S.E. DPM's of triplicate wells, expressed as a % of untreated controls.
A) B Lymphocyte Mitogenesis (LPS)

B) T Lymphocyte Mitogenesis (Con A)
B lymphocyte mitogenesis, as assessed by response to LPS, became significantly suppressed through days 2 to 9 (P < .001 in each case), to 10% of untreated control values. T lymphocyte mitogenesis, as assessed by response to Con A, became significantly suppressed through these study days to 25% of untreated control values (P < .001 in each case). However, at the additional 16 day time point, neither T nor B lymphocyte mitogenesis were still significantly depressed.

There was no significant GR1784 treatment effect upon B cell mitogenesis in either normal or immunosuppressed mice. There was, however, a significant decrease in T cell mitogenesis on day 9 (P < .05), but not on days 2 and 16, in normal mice. There was also a significant elevation in T cell mitogenesis on day 2 (P < .02), but not on days 9 and 16, in immunosuppressed mice. The elevation in T cell mitogenic responsiveness in immunosuppressed mice was of particular interest, since it might provide a partial explanation, at least, for the radioprotective properties of GR1784.
DISCUSSION

The experiments described in this thesis were designed to answer two basic questions: 1) Could GR1784 act as a pure immunostimulant in the context of an undamaged immune system? 2) Could previously observed immunorestorative properties of GR1784 be explained by an increase in hematopoiesis or an enhancement of lymphocyte function following hematological insult?

With respect to the first question, PBLC's were significantly depressed on days 2 and 9 but were unchanged on days 5 and 16 (Fig. 2). However, standard error bars were relatively large and the untreated control values could not be reproduced over all study days. Taking these factors and the lack of any effect in immunosuppressed mice into consideration, it was concluded that GR1784 had no reproducible effect on PBLC in normal mice.

A significant depression of spleen CFU's was also observed on days 5 and 9, although not on day 2 (Fig. 5b). It must be noted, however, that untreated control levels of colony counts could not be repeated from one assay day to the next (varying as much as by a factor of 5) and were therefore normalised with respect to each other by expressing them as 100% untreated control counts for that
day. Spleen colony counts on days 5 and 9 were unusually low, being < 10 colonies/petri dish. A high degree of error could therefore be expected by comparing treatment groups with untreated controls on those days. Assuming, for the sake of discussion, that these values do represent a true indication of the effect of GR1784 upon spleen CFU's in normal mice, then such an observation could be explained by an advanced maturation state of the colony forming cells (CFC's) that are measured by the CFU assay and their subsequent transport into the peripheral blood stream. Such an event would also predict elevated PBLC levels on these assay days. PBLC levels, however, remained unchanged or were even slightly depressed on these days (Fig. 2), as already discussed above. It was therefore concluded that GR1784 treatment had no consistent effect upon spleen CFU's in normal mice.

None of the other parameters showed any reproducible GR1784 treatment effects in normal mice. It is therefore concluded that GR1784 exhibits no direct immunoregulatory properties in normal mice consistent with its previously observed immunopotentiating effects in immunosuppressed animals.

With respect to the second question, the parameters chosen for study could be broken down into two broad categories: Those pertaining to early stem cell populations and those pertaining to relatively mature cell
populations. The parameters that measured early stem cell populations were bone marrow CFU's and bone marrow cellularity. The spleen is a visceral hematopoietic organ that has been shown to be particularly erythropoietic and granulopoietic following hematological insult (29, 30). The spleen CFU and cellularity parameters therefore referred to a committed granulocyte cell population that was at an intermediary stage of differentiation. The PBLC parameter and the splenic lymphocyte mitogenesis response referred to mature cell populations. The mitogenic parameter, however, is an indicator of mature lymphocyte function and will be discussed later, separately from the other hematopoietic parameters.

One possible mechanism of immunorestoration by GR1784 following X-irradiation or CY administration is a direct sparing effect upon mature cell populations. The parameters pertaining to such cell populations are PBLC, spleen weight, spleen cellularity and spleen CFU's.

Cy induced a pronounced depression of PBLC levels on days 2 and 5 that gradually disappeared through days 9 and 16 (Fig. 2). This leukopenia was in good agreement with the results of others which showed PBLC's in C57Bl/6 mice to be depressed to 27 - 33 % of control levels by 200 mg/kg Cy on days 1 to 5, with a gradual return to normal being observed by day 20 (21). GR1784 treatment had no effect on the rate of PBLC recovery following Cy administration.
Analysis of spleen weight and S.W.I. revealed the three phases of depression, rebound and normalisation that are characteristic of Cy cytotoxicity (Fig. 3a & 3b). These results were in good agreement with previous observations in C57Bl/6 mice treated with 200 mg/kg Cy, which demonstrated spleen weight depression on days 1 to 5, splenomegaly on day 9 and a return to normal on day 17 (12, 28). GR1784 treatment had no effect on the recovery of either of these parameters following Cy administration.

Spleen cellularity was shown to closely mirror spleen weight following Cy treatment (Fig. 4a). The spleen has been shown to function primarily as an erythropoietic and granulopoietic organ following hamatosuppression (29, 30). Therefore, the rebound phase represented an increase in the number of immature neutrophils that subsequently went on to repopulate the peripheral blood leukocyte pool. These findings were consistent with other reports which utilised both C57Bl/6 and BDF₁ mice and a Cy dose of 200 mg/kg (21, 31).

The enormous elevation of spleen CFU levels observed in Fig. 5b following Cy administration was in excellent agreement with previous reports, in which 200 mg/kg Cy caused elevations in splenic CFU's of 1200 % and 2400 % on days 7 and 9 respectively (12, 21). In corroboration of the findings with spleen weight and spleen cellularity, GR1784 treatment had no effect upon spleen CFU's following
Another possible mechanism of hematorestoration is an increased level of hematopoiesis in the bone marrow. The two parameters relevant to hematopoiesis are bone marrow cellularity and bone marrow CFU's.

In accordance with previous findings (12, 21, 31), bone marrow cellularity did not rebound in a similar manner to spleen cellularity following Cy administration, but rather returned to normal more gradually beginning at day 5 (Fig. 4b.). Hematopoietic recovery from the cytotoxic effects of Cy has been shown to be initiated in the bone marrow by pluripotent stem cells (19, 21, 31). These are less sensitive to Cy than the rapidly cycling colony forming cells (CFC's) and respond to the cytotoxic effects of Cy administration by rapidly repopulating the depleted CFC pool. As these pluripotent stem cells differentiate and expand they are transported out of the bone marrow to peripheral hematopoietic organs, such as the spleen, where they undergo clonal expansion in response to their new microenvironment (31). The rebound phase characteristically observed in the spleen cell compartment following Cy administration was therefore not observed in the bone marrow compartment, due to the continual export of this differentiating cell population. Again, there was no GR1784 treatment effect in Cy immunosuppressed mice.

The CFU parameter provides a measure of the number of
CFC's in the bone marrow (11, 31). This is a stem cell population of the macrophage-granulocyte lineage that has been shown to behave in a similar manner to another stem cell population, commonly referred to as endogenous colony forming units (e-CFU's). Recovery following X-irradiation and Cy administration has been correlated closely to the number of surviving e-CFU's in the bone marrow (32, 33, 34). Survival of these stem cells ensures their subsequent differentiation into mature functional elements of the reticuloendothelial system, where they help cope with the resurgence of endogenous pathogens that secondarily cause death 10 to 20 days following Cy treatment or X-irradiation (35).

The early destruction of bone marrow CFC's reported previously (10, 12, 21) was not observed in this study, most likely because the Cy insensitive pluripotent stem cells had already repopulated the bone marrow CFC population by the time of the first assay point on day 2 (Fig. 5a.). In keeping with previous findings (10, 22), the elevated bone marrow CFU levels on day 2 returned back to normal by day 5, concomitant with the transport of CFC's out of the bone marrow to secondary hematopoietic organs. No significant elevation of bone marrow CFU's consistent with a hypothesis of hematorestoration via increased hematopoiesis was observed in immunosuppressed mice treated with GR1784 (Fig. 5a). These results were consistent with
a similar lack of treatment effect observed for bone marrow cellularity (Fig. 4b).

Most radiation protectants are only effective when administered 1 day prior to irradiation (7, 34, 36, 37, 38). This has been correlated with the time required to stimulate the differentiation of progenitor cells into a less radiation sensitive intermediate (40). GR1784, however, has been shown to exert radioprotective properties with treatment beginning 1 day post irradiation (Gordon, P. and Wepsic, T., 1985, unpublished data). A potential mechanism of action is therefore more likely to be an increased functional responsiveness of surviving leukocyte compartments, rather than the protection of pluripotent stem cell compartments.

The polyclonal mitogens, Con A and LPS, have been shown to stimulate relatively mature clones of B & T lymphocytes respectively (41). The proliferative response of splenic lymphocytes towards these mitogens therefore constitutes a measure of mature cell function.

In the Cy dose titration experiment, T and B lymphocyte mitogenesis were found to be dose dependently suppressed through 9 days following Cy administration, although T lymphocyte mitogenesis was found to be less suppressed on days 3 and 6 than on day 9 (Fig. 6). Such findings were in excellent agreement with previous reports that showed B lymphocytes to be more susceptible to low
doses of Cy than T lymphocytes (23, 24, 25, 26). The data was also in accord with previous work, which showed T and B lymphocyte mitogenesis to be suppressed for at least 10 days at 200 mg/kg Cy (27). The 150 and 200 mg/kg doses of Cy were both shown to induce adequate suppression of T & B lymphocyte mitogenesis over the 9 study days. In accordance with the guidelines set out in the Rationale, the lower of the two doses was selected for use in subsequent experiments examining the immunomodulating capacities of GR1784 in immunosuppressed mice. This dose has also been shown to cause > 50 % inhibition of bone marrowcellularity and granulocyte-macrophage CFU (GM-CFU) in C57Bl/6 mice by day 4, values returning to normal by day 10 (21). Furthermore, at this dosage another drug (the biologic response modifier MVE-2) was able to restore depressed bone marrow cellularity and GM-CFU values (9).

The administration of Cy at 150 mg/kg in the GR1784 immunomodulation experiments resulted in a suppression of splenic lymphocyte mitogenesis that was more profound in the case of B lymphocytes than T lymphocytes (Fig 6). This finding confirmed a similar observation made in the Cy dose titration experiment and was also in agreement with previous reports (23, 24, 25, 26, 27). Although GR1784 was not observed to have any effect upon B cell mitogenesis (Fig. 6a), it significantly elevated T cell mitogenesis on day 2 post Cy administration (Fig. 6b). This elevation was
not maintained to a significant degree through the next assay points on days 9 and 16. It is concluded that this partial restoration of a component of the cellular immune response might play a part in suppressing the proliferation of endogenous pathogens at a critical time following irradiation injury. Recovery from irradiation injury has been correlated with a gradual return to normal of PBLC's, beginning on day 10 following irradiation (13). The provision of even a small measure of protection from endogenous pathogens during the first 10 days following the radiation event may therefore contribute towards enhanced irradiation survival.

On the basis of these results, useful future experiments might include an elaboration upon the small GR1784 treatment effect observed on T cell mitogenesis, using various doses of Cy and more time points between days 0 and 9. Due to variability in radiation induced death in F344 rats, it might also be useful to repeat the radiation protection data in the more standard C57Bl/6 mouse model, with an additional focus upon the immunological responsiveness of the surviving lymphocytic compartment.
GR1784 is an experimental nucleoside drug that has been shown to restore suppressed hematological parameters in rats following either X-Irradiation or Cyclophosphamide (Cy) administration and to provide significant protection towards lethal X-Irradiation in rats.

Experiments were conducted in normal and Cy immunosuppressed C57Bl/6 mice, in order to further our understanding of the immunological mechanism of action of this drug. These demonstrated no immunostimulating effects of GR1784 in normal mice, assessing a number of hematological parameters and also mature lymphocyte responses towards B and T cell mitogens. GR1784 was found to best demonstrate its action within a damaged immune system, like another immunomodulator of this family of nucleoside drugs, Isoprinosine.

Previously observed hematorestorative properties could not be explained by a direct protective effect on pluripotent progenitor cells in the bone marrow or relatively mature cell populations in the peripheral blood, spleen or bone marrow, following Cy administration. A colony forming unit (CFU) assay was employed in order to measure the number of colony forming cells (CFC's) in the
spleen and bone marrow. This failed to demonstrate any potentiating effects of GR1784 on hematopoiesis following hematological insult by Cy.

A small but significant elevation of depressed T lymphocyte mitogenesis in response to Con A was observed on day 2 following Cy administration. It is suggested that such an increase in responsiveness of the surviving T lymphocyte compartment might play a role in warding off the surgence of endogenous pathogens that secondarily causes death following X-Irradiation and might therefore contribute towards GR1784's radioprotective properties.
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