# Dexamethasone Suppresses the Generation of Phenylhydrazine-Induced Anemia in the Rat (43385)

BURTON S. DORNFEST,*[,1] DAVID M. LAPIN,[†] SAMUEL ADU,* AND BRIAN A. NAUGHTON[‡]

*Department of Anatomy and Cell Biology,* State University of New York Health Science Center at Brooklyn, Brooklyn, New York 11203; Department of Biology,[†] Touro College, New York, New York 10036; and Department of Medical Laboratory Sciences,[‡] Hunter College School of Health Sciences, New York, New York 10010*

*Abstract.* **The immunoactive steroid dexamethasone (DXM) was administered to rats injected with a dose of phenylhydrazine (PHZ) known to induce anemia. PHZ treatment alone resulted in a hemolytic anemia that was most pronounced on Days 1–7 after injection. This anemia was accompanied by a leukocytosis that was greatest on Days 2–7 following PHZ treatment. Lymphocytes accounted for >75% of this incremental increase. In contrast, rats treated with PHZ as well as with DXM displayed erythrocyte counts and hematocrits within the normal range. Although the reticulocyte counts of these rats were higher than those of controls, they were significantly lower than those of animals receiving PHZ alone. In addition, DXM suppressed the leukocytosis and splenomegaly resulting from PHZ administration and inhibited the rise in plasma IgG titers induced by PHZ exposure. DXM also altered the ratio of peripheral blood T and B lymphocytes of PHZ-treated rats. DXM suppression of PHZ-induced anemia is further confirmation that this anemia is associated with immune activation.**

[P.S.E.B.M. 1992, Vol 199]

Phenylhydrazine (PHZ) is an antipyretic drug that induces hemolytic anemia in laboratory animals (1–3) and in humans (4). Recent studies in our laboratory have provided evidence that the anemia induced in the rat by PHZ is associated with the activation of the immune system (3, 5). This is characterized by a rapid rise in serum immunoglobulin titers, a marked increase in the B lymphocyte population of the peripheral blood and spleen, and an altered ratio of T helper to T suppressor cells. The enhanced serum IgG levels, prominent peripheral lymphocytosis, and the shifts in the lymphocyte subpopulations correlated well with the onset and degree of anemia induced by PHZ. In addition, histologic studies revealed the presence of blastogenic lymphoid cells in the lymph nodes and spleens of PHZ-treated rats (6). PHZ also induced the blastogenesis of isolated peripheral blood mononuclear cells (PBMC) of both untreated and PHZ-treated animals *in vitro* and potentiated the mitogenic effects of phytohemagglutinin and pokeweed mitogen on PBMC (5). Earlier light microscopic studies revealed an enhanced lymphopoiesis in the lymphoid tissues and bone marrows of PHZ-treated rats, and the appearance of atypical and blastic lymphoid cells in the peripheral circulation was verified by electron microscopic studies. In a related study, we reported that chronic PHZ administration caused an elevation in prostacyclin and prostaglandin $E_2$ levels and promoted a transient thrombocytopenia (7). These events have been associated with immune activation and the generation of drug-induced hemolytic anemia by immune mechanisms (3, 8, 9).

The work reported here was undertaken to study the effects of the synthetic glucocorticosteroid (GCS) dexamethasone (DXM) on the hemolytic anemia induced in the rat by PHZ. The effect of GCS in modulating immune responses is well documented (10–14). To confirm that the anemia induced by PHZ in the rat is associated with immune activation, we administered multiple injections of DXM to rats receiving PHZ at a dose known to induce anemia. To this end, DXM was

[1] To whom requests for reprints should be addressed at 96 Everett Road, Demarest, NJ 07627.

Received May 29, 1991. [P.S.E.B.M. 1992, Vol 199]
Accepted November 21, 1991.

0037-9727/92/1994-0491$3.00/0

given to rats prior to the injection of PHZ, since it has been shown that GCS administered to animals shortly before immunization can suppress the anticipated cellular (15) and humoral immune responses induced by antigenic challenge (15, 16). In contrast, when GCS is administered after antigenic stimulation, the inhibitory (suppressive) effects upon serum Ig levels are much less pronounced (15). Lymphocytic subpopulations were evaluated phenotypically using flow cytometry, standard peripheral blood differential counts were performed, and serum IgG levels were measured by radial immunodiffusion (17) in animals treated with PHZ and/or DXM. The effects of DXM on the anemia by PHZ were assessed by comparing changes in hematocrits (Hct) and red blood cell (RBC) counts of animals administered DXM or saline prior to the PHZ administration.

## Materials and Methods

**PHZ and DXM Administration.** Male Long-Evans rats weighing 225–250 g were used in all procedures. Three groups of experimental animals were employed. (i) Rats were injected subcutaneously with 0.3 ml of 0.9% sterile saline for 2 consecutive days and on the third day (designated Day 0) animals were injected subcutaneously with 0.3 ml of a 0.9% saline solution of PHZ (Sigma Chemical Co., St. Louis, MO) at a concentration of 4 mg/100 g body wt, followed 2 hr later by a third injection of 0.3 ml of saline. (ii) Rats were injected subcutaneously with 0.3 ml of a 0.9% saline solution of DXM sodium phosphate (Merck, Sharp, and Dhome, West Point, PA) at a concentration of 0.6 mg/100 g body wt for 2 consecutive days and on the third day (Day 0) injected subcutaneously with 0.3 ml of a 0.9% saline solution of PHZ at a concentration of 4 mg/100 g body wt, followed 2 hr later by a third injection of the same dose of DXM. (iii) Rats were injected subcutaneously with 0.3 ml of a 0.9% saline solution of DXM at a concentration of 0.6 mg/100 g body wt for 2 consecutive days and on the third day (Day 0) injected subcutaneously with 0.3 ml of 0.9% saline, followed 2 hr later by a third injection of the same concentration of DXM. A total of 35–40 rats were used in each experimental group.

These methods are schematized in Figures 1–4. Untreated rats were employed as controls; preliminary studies did not reveal significant differences in any parameters between saline-injected and untreated controls.

**Counting Procedures.** Differential counts were made of Wright's stain slides of tail vein blood samples and absolute leukocyte (WBC) and RBC counts were made using a Coulter Electronic cell counter (Coulter Electronics, Hialeah, FL). Hct were measured using the micromethod and reticulocyte counts were performed on blood smears stained with new methylene blue. Tail vein blood samples were collected immediately before each injection of PHZ, DXM, or saline.

**Blood Volume, Plasma Volume, and Circulating Red Cell Volume Determinations.** Rats from each of the three experimental groups were sacrificed for blood volume (BV), plasma volume (PV), and circulating red cell volume (CRCV) determinations 1 day prior to PHZ-injection in Groups 1 and 2 and 1 day prior to saline injection in Group 3. Measurements also were made on Day 0 and on the fourth, tenth, fifteenth, and twentieth days following PHZ (or saline) injection. Three to five rats were used for each time interval studied. These determinations were based on the *in vivo* dilution of a known activity of $^{51}$Cr-labeled RBC that were labeled by incubating 5 ml of heparinized blood collected from normal nontreated rats with 20 $\mu$Ci of $^{51}$Cr (200–316 mCi/mg) for 20 min at 37°C. The cells were washed twice with sterile 0.9% saline, the plasma and white blood cell layers were removed, and the plasma-saline supernatant fluid was counted for radioactivity to ensure that any excess $^{51}$Cr remaining unattached to the RBC was removed. The labeled RBC were resuspended with saline to 90–100% of the original volume, and 0.2 ml of the suspended cells with activity of 40–80 $\times$ $10^3$ cpm was injected into the femoral vein. The cells were allowed to circulate for 20 to 25 min, after which 0.2 ml of tail vein blood was withdrawn and radioactivity of the cells was counted in a well-type gamma scintillation counter (Beckman Instruments, Fullerton, CA). BV, CRCV, and PV were calculated as follows:

$$BV = \frac{\text{cpm injected}}{\text{cpm sample}} \times \text{volume of sample} - \text{injection volume} \quad \text{[Eq. 1]}$$

$$CRCV = BV \times Hct \quad \text{[Eq. 2]}$$

$$PV = \text{difference between [1] and [2]} \quad \text{[Eq. 3]}$$

**Analysis of Mononuclear Cells by Flow Cytometry.** PBMC were isolated by Ficoll-Hypaque (Histopaque 1077; Sigma) density gradient centrifugation of anticoagulated blood collected from rats by aortic exsanguination. Blood was collected from Group 1 rats on the sixth and tenth days following PHZ injection and from Group 2 rats on the first and second days of DXM treatment preceding PHZ injection and on the first, sixth, seventh, and twentieth days following PHZ injection. These time intervals for analysis of PBMC were chosen on the basis of WBC as well as RBC alterations observed in the peripheral blood of animals treated with PHZ and/or DXM (Fig. 1). Days 6 and 10 following PHZ injection of Group 1 rats were selected for flow cytometry evaluation, since the RBC counts of these rats were maximally depressed between the fourth and seventh days after PHZ injection. In addition, a

rebound in RBC counts and Hct was observed on the tenth day after injection (Fig. 1). Flow cytometry was performed for Group 2 animals (DXM-PHZ treated) on the first and second days of DXM treatment preceding PHZ administration to ascertain the effects of DXM on the ratio of T to B lymphocytes to establish baseline values, and on the first, sixth, and seventh days following PHZ injection, since alterations in WBC, RBC, and Hct were at or near maximal at these times. By Day 20 after PHZ treatment, these values had returned to near normal levels. Blood was also collected from untreated (control) rats. Following separation, the PBMC were washed once with 0.87% ammonium chloride to lyse any contaminating RBC and washed again with phosphate-buffered saline (PBS), and the cells were resuspended in PBS. The morphology and purity of the PBMC population was verified from Wright's stain cytosmears of the preparations. The PBMC were reacted with equal 100-$\mu$l volumes of the following mouse monoclonal antibodies at a 1/100 dilution: MRC OX-33, which recognizes the leukocyte common antigen on rat B cells (18); MRC W3/25 (CD4), which binds to $T_4$ lymphocytes and some macrophages (19); MRC OX-8 (CD8), reacting with $T_8$ lymphocytes and natural killer cells (20); MRC OX-54 (CD2), which recognizes epitopes common to all rat T lymphocytes and thymocytes (21) (Serotec Inc., Cambridge, UK); or mouse $IgG_1$, (Coulter Immunology). After washing twice with 5% bovine serum albumin in PBS, the cells were resuspended and incubated for 30 min with a 1/100 dilution of sheep antimouse $IgG_1$ conjugated to fluorescein isothiocyanate. Samples were washed twice in 5% bovine serum albumin-PBS and fixed with 250 $\mu$l of 4% neutral buffered formalin. After centrifugation for 3 min at 500*g*, the supernatant was removed and cells were resuspended in 1 ml of PBS without $Ca^{2+}$ or $Mg^{2+}$. All analyses were performed using an EPICS C flow cytometer (Coulter Electronics) tuned to a fluorescence wavelength of 488 nm. Forward light scatter versus 90° light scatter histograms were used to establish windows for lymphoid cell populations. The percentages of cells displaying fluorescence ≥98% of the controls (PBMC treated with mouse $IgG_1$-fluorescein isothiocyanate alone or mouse $IgG_1$ followed by the secondary fluoresceinated antibody) were determined. Log fluorescence was gated to exclude cells with light scatter characteristics different than lymphocytes. The ratio of T to B lymphocytes was calculated from the percentages of cells positive for MRC OX-54 or MRC W3/25 and MRC OX-8 versus MRC OX-33, as described previously (3, 5).

**Immunoglobulin Quantification.** Serum IgG levels in aortic blood collected from rats in the three experimental groups were measured at periodic intervals using the Mancini radial immunodiffusion method (17). Pre-prepared plates containing sheep antirat IgG (AAR 02K, 02S) and sheep antirat IgM (AAR 08K, 08S) were obtained from Serotec, along with the appropriate standards. Standards (5 $\mu$l) (at 100%, 60%, and 20% concentrations) or test plasma from untreated, PHZ-treated, or DXM- and PHZ-treated rats was allowed to proceed at room temperature until the ring surrounding the most concentrated standard was 9 mm in diameter (~40–50 hr). Results were then read from a standard curve plotted as the square of the diameter of the precipitation ring versus dilution.

**Spleen Weights.** Rats from the three experimental groups were sacrificed at periodic intervals and the spleens were excised and weighed. Weights are expressed as g/100 g body wt.

**Statistical Analysis.** Levels of significance in this study were determined by using Student's *t* test. *P*-values of ≤0.05 were considered to be significant.

## Results

An increase in the leukocyte count was observed by as early as 1 day after the initial injection of PHZ into rats that were not pretreated with DXM (Group 1). The count continued to increase, reaching a maximum level of $135 \times 10^3$ cells/$\mu$l on the fourth day following the injection, with lymphocytes accounting for approximately 75% of the total enhancement (Fig. 1). By the sixth to seventh postinjection days, a decline in leukocyte numbers was observed, and counts returned to the normal range of $8–10 \times 10^3$ cells/$\mu$l by 14 days after injection. An increase in the leukocyte count in PHZ-treated rats receiving DXM (Group 2) was also observed at 1 day after the injection of PHZ, but this increment was significantly lower ($P \leq 0.01$) than that observed for PHZ-treated rats that did not receive DXM. For these rats (Group 2), the leukocyte count was above the normal range by the second day after the injection of PHZ and reached a maximal level of $60 \times 10^3$ cells/$\mu$l on the seventh postinjection day. By 14 days following PHZ injection, the count for this group also returned to the normal range. The maximal leukocyte count reached in the PHZ-treated rats receiving DXM was less than half that observed in the PHZ-treated rats that were administered saline alone (Fig. 1). Lymphocytes accounted for about 55% of the total increment; most of the other cells were polymorphonuclear neutrophils. The lymphocyte counts of Group 2 rats declined markedly and the polymorphonuclear neutrophil counts increased for the first 2 days after DXM treatment and continued for 24 hr following the subsequent PHZ injection; by the first day following PHZ injection, the polymorphonuclear neutrophils accounted for about 80% of the total leukocyte count. Lymphocyte counts did not return to the normal range until 12 days after PHZ injection and, at that point, represented about 80% of the total count. The leukocyte counts of rats in Group 2 were highly variable, with

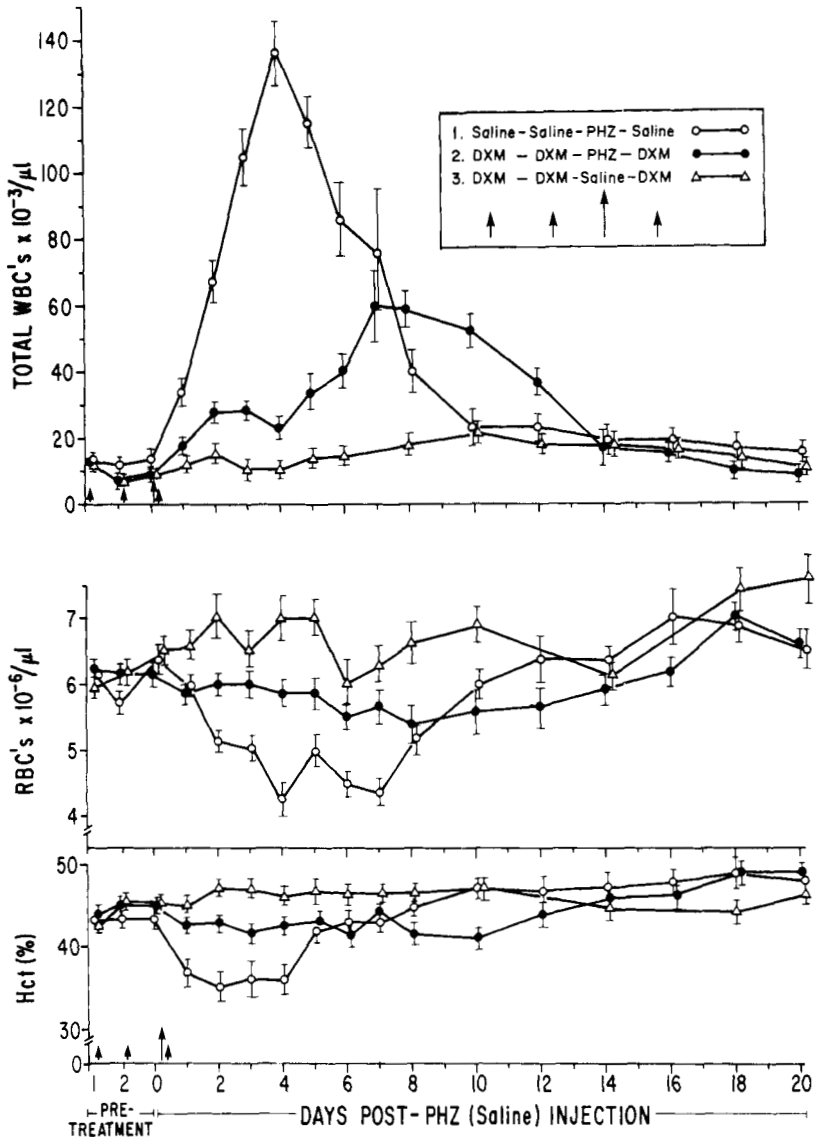


**Figure 1.** The effect of PHZ, DXM, or PHZ and DXM on white and red blood cell counts and hematocrits. The pretreatment level (control) for each group is the value shown at Time 0 prior to the initial injection of test agent. During the pretreatment, rats were given two injections of either saline or DXM. *X*-axis arrows correspond to the treatment schedule. Vertical bars through the means indicate ± 1 SE.

some animals showing an almost complete suppression of the PHZ-induced leukocytosis (10–12% of the treated rats).

The leukocyte counts of rats injected with DXM alone (i.e., no PHZ treatment) (Group 3) remained within the normal range of 8–20 × $10^3$ cells/$\mu$l throughout the 20-day test period. Lymphopenia and neutrophilia were evident in Group 3 rats for up to 14 days following the injection of saline (as a substitute for PHZ), at which time a normal level of ~80% lymphocytes was observed. In animals receiving PHZ alone, Hct and RBC counts declined to their lowest levels during the first to fourth days and second to seventh days after the injection of PHZ, respectively. The Hct was approximately 80% of the pretreatment values, whereas RBC counts were 68–80% of those of the untreated control animals. Hct and RBC counts returned to normal levels by 10–12 days after PHZ injection. PHZ-treated rats that received DXM (Group 2) did not display this dramatic decrease in either Hct or RBC count. In this regard, the lowest RBC count noted was ~90% of the pretreatment value on the eighth day following PHZ injection, whereas Hct values showed no significant diminutions. Hct and RBC counts of rats in both Group 1 and Group 2 returned to pretreatment levels by 12–14 days following PHZ injection. Hct and RBC counts were higher than the pretreatment values by 14 days after PHZ injection. Rats in Group 3, which were given DXM but not PHZ, displayed elevated Hct values and RBC counts after the first of the three daily administered DXM injections. These values, with mi-

nor variation, remained elevated for the 20-day period that the animals were assessed (Fig. 1).

The reticulocyte counts of PHZ-treated rats became elevated regardless of whether or not they had prior DXM treatment (Fig. 2) (Groups 1 and 2); reticulocyte counts of PHZ-treated rats not receiving DXM (Group 1) reached maximal levels of ~40% by 6–7 days after PHZ injection. This was significantly higher ($P \leq 0.01$) than the reticulocyte counts of rats injected with PHZ after prior treatment with DXM (Group 2). The reticulocyte counts in rats treated with PHZ after receiving DXM reached maximal levels of 10–17% at 4–10 days after PHZ injection. Rats receiving only DXM (Group 3) showed no meaningful changes in reticulocyte counts. PHZ did not alter the growth rate of Long-Evans rats or induce any weight loss as compared with untreated control animals (Group 1). DXM, however, induced weight loss in all animals, including those treated with PHZ. Rats that were administered DXM alone (Group 3) displayed weight loss by the first day after the initial DXM injection. These losses were most pronounced at 1 day after the last of the three DXM injections, averaging about 12% of the pretreatment weight. Rats in Groups 2 and 3 showed weight gains by the third day after the last DXM injection. PHZ induced an enhancement of the weights of spleens of rats, which were significantly increased ($P \leq 0.01$) by the first day after PHZ administration and reached maximal levels of approximately 1.8 g/100 g body wt (absolute mean value of 4.0 g) by 4 days after injection (Fig. 3). Spleens of rats that were treated with DXM and PHZ or DXM alone manifested significant decreases in weight during the 3 days of DXM administration as compared with the spleen weights of untreated rats. DXM pretreatment significantly diminished ($P \leq 0.01$) the splenomegaly observed following PHZ administration. Thus, spleens of DXM- and PHZ-treated animals displayed weight increases that were highest by the fifth to sixth day after the injection of PHZ, averaging about 0.58–0.64 g/100 g of body wt (absolute mean values of 1.4–2.0 g), as compared with 1.8 g/100 g body wt with PHZ alone. Spleen weights of rats in all groups returned to pretreatment levels by the twentieth day after PHZ injection.

The total BV, CRCV, and PV of the rats in Group 1 (PHZ-saline injected) and Group 2 (PHZ-DXM treated) showed no significant differences from values for the untreated control animals. In this regard, control rats exhibited BV, CRCV, and PV of 5.7 ml, 2.3 ml, and 3.4 ml per 100 g body wt, respectively. For Groups 1 and 2, BV ranged from 5.5 to 6.2 ml, CRCV from 2.2 to 2.7 ml, and PV from 3.5 to 3.7 ml per 100 g body wt. The values for Group 3 rats (DXM-saline) were generally within the same ranges as those in Groups 1 and 2, except for the tenth and twentieth days following the final DXM injection, when the values for BV, CRCV, and PV were 7.2–7.6 ml, 2.8–3.1 ml, and 4.1–4.4 ml per 100 g body wt, respectively. These changes were only observed in Group 3 rats only on the tenth and twentieth days after PHZ injection and occurred after the most substantial alterations in cell counts induced by PHZ and/or DXM treatment (Fig. 1). Moreover, these higher values could not be correlated with differences observed in the peripheral blood counts among any of the experimental groups. These findings indicate that blood volume changes could not account for the pronounced alterations in cell numbers observed among and within the experimental groups.

Analysis of serum IgG titers revealed that DXM suppressed IgG synthesis in untreated rats and significantly inhibits (range, $P \leq 0.01$–$0.05$) the rise in IgG titers resulting from PHZ injection (Fig. 4). This trend was observed for all time periods that were studied. We reported previously that PHZ treatment resulted in a decrease in the ratio of T to B lymphocytes (T:B) in rat peripheral blood, bone marrow, and spleen (3, 7).

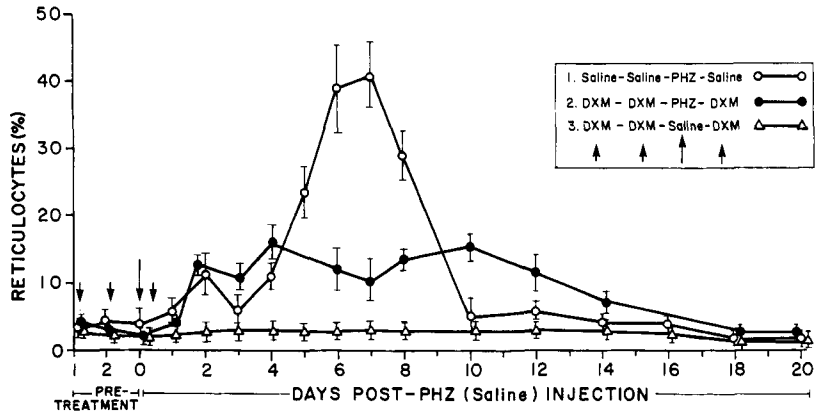


**Figure 2.** Alterations in reticulocyte counts induced by PHZ or PHZ and DXM administration as compared with those of groups receiving DXM alone. The untreated Long-Evans rat reticulocyte count was ≤3%. Vertical bars through the means indicate ± 1 SE. Arrows above the *x* axis represent dose schedules.

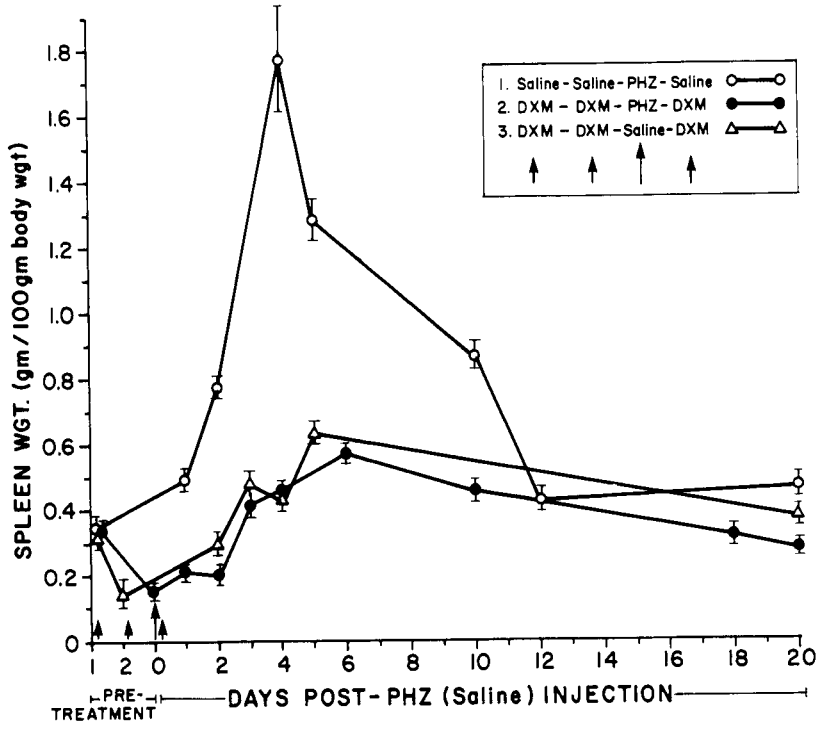


**Figure 3.** The influence of PHZ, DXM, or PHZ and DXM on spleen weights. Vertical bars through the means indicate ± 1 SE. *X*-axis arrows correspond to the treatment schedule.

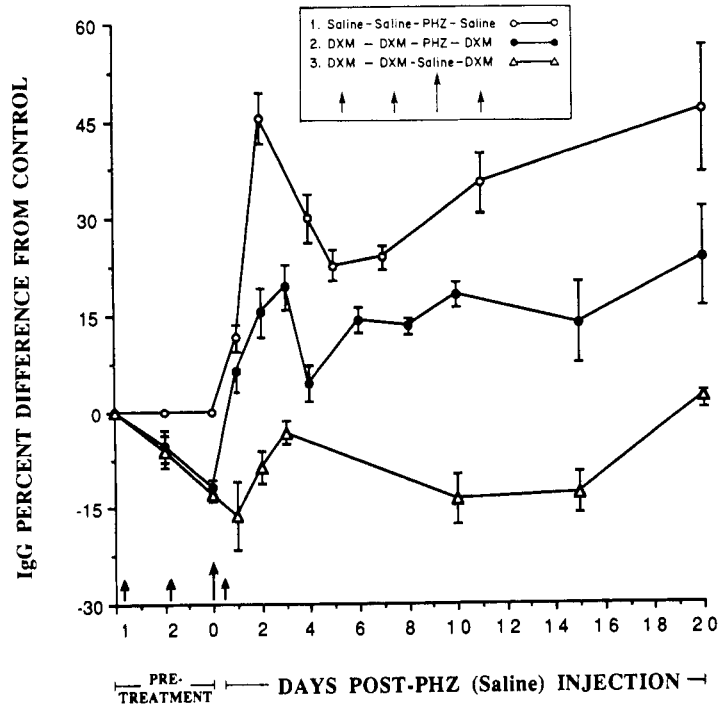


**Figure 4.** Percentage of differences of plasma IgG levels from untreated or saline-treated controls resulting from the administration of PHZ, DXM, or PHZ and DXM. Vertical bars through the means indicate ± 1 SE. For the saline-saline-PHZ-saline group, the pretreatment values and the Day-0 value are identical.

DXM, when administered in either a single or double dose, also significantly depressed the T:B ($P \leq 0.01$) (Fig. 5). In addition, this agent accentuates the decrease in T:B resulting from PHZ treatment.

## Discussion

The immunosuppressive influences of GCS such as DXM are well documented (10, 13, 15, 22–24). GCS have been shown to elicit a wide range of effects on the

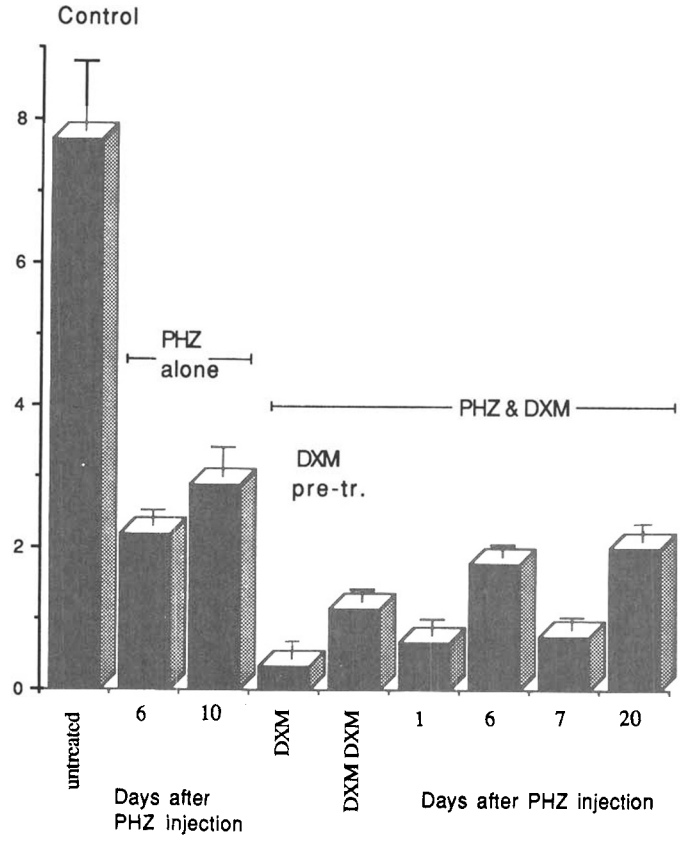


**Figure 5.** Mean ratio of the numbers of T to B lymphocytes in the peripheral blood of rats receiving PHZ, DXM, or PHZ and DXM as compared w th untreated controls. The DXM pretreatment group depicts the influence on T:B of one versus two injections of DXM according to the treatment schedule as described in the Materials and Methods. Vertical lines through the tops of the bars indicate ± 1 SE.

immune system, the mechanism(s) of which is not completely understood. GCS have been found to exert profound effects on circulating lymphocytes and, in species like the mouse, rat, hamster, and rabbit, cause a marked hypocellularity in the spleen, lymph nodes, and, particularly, the thymus (10, 25). The lymphopenia observed in rats, mice, and guinea pigs following GCS administration has been attributed to the redistribution of lymphocytes to certain lymphoid tissues and the bone marrow at the expense of the vascular compartment (10, 23). In addition, GCS are capable of directly affecting the functions of most classes and subclasses of lymphocytes (12) as well as macrophages (22) and have been found to be directly cytotoxic to thymocytes and immature lymphocytes (24). Some of the effects of GCS on the immune system are mediated through inhibitory actions on the synthesis and secretion of cytokines released by activated T cells. GCS can also suppress the production and biologic activity of certain inflammatory mediators, such as leukotriene $B_4$ (13). GCS have been found to modulate the immune functions of macrophages and T and B lymphocytes by inducing changes in the phenotypic expression of various cell surface molecules (26). It is commonly accepted that cell-mediated immune reactions are more susceptible to GCS than humoral responses. Certain subpopulations of T lymphocytes may be more sensitive to GCS than B cells (27). DXM has also been found to promote B cell immunoglobulin secretion by inducing the production of a B cell-inducing factor by T cells (14). In addition to producing peripheral lymphopenia, GCS induces neutrophilia and eosinopenia (28). In this regard, it has been reported that GCS, among their other effects on neutrophils, induce a granulocytosis by decreasing the rate of egress of cells from the peripheral circulation and increasing the influx of cells from the bone marrow (29). The effectiveness of GCS on immune responses has been shown to be contingent upon the route of administration as well as the dose schedule. Thus, GCS have been found to be more effective when administered prior to immunization rather than after antigen exposure (15, 16). We injected rats with DXM prior to PHZ treatment, since preliminary studies revealed that administration of DXM after PHZ was relatively ineffective in suppressing the anemia induced by this agent. We also found multiple injections of DXM to be superior to single injections in suppressing PHZ-induced anemia.

Under the conditions employed in this study, DXM significantly suppressed the generation of anemia

resulting from PHZ injection. Supportive of these findings are earlier studies that demonstrated that cortisone and ACTH can mitigate or abrogate the hemolysis in many cases of acquired hemolytic anemia (30, 31). In addition, PHZ administered to adrenalectomized rats was found to cause a significantly greater destruction of RBC than it elicits in normal animals; the resulting hemolysis could be prevented by cortisone administration (32). In this same study, ACTH was found to almost completely prevent PHZ-induced anemia in intact rats. In this connection, GCS, and DXM specifically, have been reported to exert an indirect stimulatory effect on erythropoiesis (33, 34), which may, in part, account for the increased red cell levels that we observed in rats treated with DXM alone. Although the potential involvement of the immune system was not investigated in these earlier studies, the findings are consistent with our observations that DXM inhibits the generation of anemia and the splenomegaly induced by PHZ. The anemia caused by PHZ administration was substantially less severe in rats receiving DXM (Fig. 1). This effect was not mediated via alterations in plasma volume, which was essentially unchanged by any of the treatment regimens we employed in this study. Differential and absolute leukocyte counts indicated that DXM substantially reduced and, in some instances, completely abrogated the leukocytosis and lymphocytosis resulting from PHZ treatment. We interpret these findings as further evidence for the involvement of immunologic mechanisms in the generation of anemia by PHZ. The amelioration of PHZ-induced splenomegaly by DXM can be attributed to the diminished destruction of erythrocytes by the spleen.

A rise in plasma IgG concentrations was noted shortly after PHZ injection and became significantly higher than untreated controls by as early as 48 hr afterward (Fig. 4). In a recent study, we reported that PHZ-treated rats produce IgG antibodies capable of opsonizing rat erythrocytes sensitized by PHZ exposure *in vivo* or *in vitro* (3). The titers of these antibodies were highest in the first week following PHZ treatment and dropped substantially thereafter, but remained significantly higher than control levels for the entire 6-week experimental period (3). Similar patterns of IgG enhancement in response to PHZ were observed in the current study. The anemia, which is most pronounced in the first week after PHZ treatment, gradually became compensated, even though weekly PHZ treatments continued (1, 3). The mechanism(s) for this compensation has not been defined. However, treatment with DXM prior and subsequent to PHZ injection significantly inhibited the expected rise in IgG titers resulting from exposure to PHZ, although IgG levels still remained higher than those of untreated controls. The findings that PHZ-treated animals display elevated IgG titers (i) after the compensation of anemia in the chronically treated rats and (ii) in rats in which the generation of this anemia was prevented by DXM may be attributed to generalized stimulatory effects of PHZ on lymphoid cells. In this regard, nanogram quantities of PHZ were found to stimulate lymphocyte blastogenesis *in vitro* (5). The correlation of the inhibition of IgG production, diminished anemia, and drops in leukocyte (lymphocyte) counts is further confirmation that immune activation is an important mechanism in the generation of anemia in the rat by PHZ.

An antierythrocyte antibody has been postulated as mediating the destruction of senescent red cells (35–37). This autologous IgG reacts with membrane sites on erythrocytes that have been altered by age and facilitates their removal by splenic macrophage Fc receptor mechanisms (35). The antigenic site has been identified as Band 3 protein (38). In this regard, PHZ has been reported to cross-link Band 3 proteins into aggregates that are recognized by the senescent red cell antibody (37). Hence, there is evidence that PHZ may cause red cell destruction by aging the cell, thereby exposing nascent antigen to circulating antibody. In addition, PHZ also enhances the level of sensecent red cell antibody, which presumably accumulates preferentially on older erythrocytes. Although a flow cytometry study indicates that IgG accumulates on erythrocyte membranes with age (39), there is little information concerning the quantity of membrane-associated IgG that is needed to trigger macrophage erythrophagocytosis or the effects of PHZ on RBC of various ages. Compensation of the anemia caused by PHZ or similar drugs, even during chronic exposure, may reflect a shift toward decreasing age in the red cell population, rendering the cells less susceptible to autologous IgG recognition, and possibly to the oxidizing effects of PHZ as well. The rapid enhancement of IgG titers, lymphocytosis, and diminished ratios of T to B lymphocyte numbers in the peripheral blood and lymphoid organs that were noted in this and previous studies (3, 5) is consistent with a secondary immune response. PHZ may act on at least two aspects of this response to generate anemia: by augmenting the quantity of autologous IgG capable of binding to older RBC, thereby accelerating their destruction, and by inducing senescence-like changes on younger RBC, rendering them recognizable by these antibodies. The continued stimulation of erythropoiesis by erythropoietin-dependent mechanisms during chronic PHZ exposure (1) also contributes to the phenomenon of compensation, ostensibly by increasing the percentage of reticulocytes and other young erythrocytes in the peripheral blood. In contrast to studies associating IgG accumulation with RBC aging, one group, using a sensitive enzyme-linked immunosorbent assay method to quantify IgG associated with erythrocyte membranes, reported that in rabbits, IgG accumulation on aged RBC was only

slightly higher than on younger erythrocytes (40). Since no positive controls (Coomb's $^{+}$RBC) were included in this experiment, it is unclear whether membrane-bound IgG was lost during some aspect of the assay procedure or whether IgG accumulation is not part of the normal senescence process for erythrocytes in rabbits.

Other investigators have reported that PHZ-induced hemolytic anemia was associated with nonimmune mechanisms. Thus, PHZ has been reported to directly lyse human erythrocytes *in vitro* (41). However, we found that rat erythrocytes that were incubated with PHZ for periods up to 6 hr showed no detectable hemolysis (42). Supportive of the latter finding are experiments performed *in vivo* demonstrating that erythrocytes, although not directly lysed by PHZ, are rapidly removed from the circulation by macrophages after being exposed to this drug. This event occurs primarily in the spleen, although the liver becomes increasingly more important in the sequestration of erythrocytes altered by higher doses of PHZ (43). PHZ has been reported to cause membrane damage in rat RBC by oxidation of membrane-associated thiol groups (44), which, in turn, promotes their rapid removal from the circulation by reticuloendothelial cells (43, 45). In this regard, a lectin-like receptor on murine macrophages is reported to be involved in the recognition and phagocytosis of human RBC exposed to PHZ (45).

In the present study, we found that DXM influences the ratio of T to B cell numbers in a manner similar to PHZ (Fig. 5) (5). In fact, the drop in T:B in PHZ-treated rats is accentuated by DXM. Although the precise reasons for this are not clear, the total WBC and lymphocyte counts are substantially higher in the PHZ-treated groups than in those animals injected with DXM and PHZ or DXM alone (Fig. 1). Therefore, the dramatic increase in the numbers of circulating and organ-associated B cells noted during PHZ treatment (3, 7) was suppressed by DXM. The enhancement in T:B by DXM reflects to a greater extent a reduction in T cells rather than an increase in B lymphocytes. Preliminary studies indicating that DXM suppresses $T_4$ lymphocytes more than $T_8$ cells (B. A. Naughton and B. S. Dornfest, unpublished observations) may have implications in the generation of a humoral response by PHZ. T cells have been shown by others to be more sensitive to GCS than B lymphocytes (27). In summary, DXM suppresses the hemolytic anemia induced by PHZ in the rat. This action was associated with the suppression of a number of events associated with PHZ exposure *in vivo*, including lymphocytosis, splenomegaly, increased serum IgG titers, and increased numbers of circulating and organ-associated B lymphocytes. These results confirm and extend our previous studies, indicating that immunologic mechanisms contribute to the generation of anemia in rats by PHZ.

This work was supported in part by Grant 7-70984 to the Research Foundation of the City University of New York from Marrow Tech Inc., La Jolla, CA, and PSC CUNY Grant 6-63200.

1. Dornfest BS, Naughton BA, Johnson R, Gordon AS. Hepatic production of erythropoietin in a phenylhydrazine-induced compensated hemolytic state in the rat. J Lab Clin Med **102**:274–285, 1983.
2. Erslev AJ. The role of erythropoietin in the control of red cell production. Medicine **43**:661–665, 1964.
3. Naughton BA, Dornfest BS, Bush ME, Carlson CA, Lapin DM. Immune activation is associated with phenylhydrazine-induced anemia in the rat. J Lab Clin Med **116**:498–507, 1990.
4. Brown GE, Giffin HZ. The treatment of polycythemia vera (erythremia) with phenylhydrazine. Arch Int Med **38**:321–345, 1926.
5. Dornfest BS, Bush ME, Lapin DM, Adu S, Fulo A, Naughton BA. Phenylhydrazine is a mitogen and activator of lymphoid cells. Ann Clin Lab Sci **20**:353–370, 1990.
6. Dornfest B, Lapin DM, Naughton BA, Adu S, Korn L, Gordon AS. Phenylhydrazine-induced leukocytosis in the rat. J Leukocyte Biol **39**:37–48, 1986.
7. Naughton BA, Moore E, Bush ME, Lapin DM, Dornfest BS. Hemostatic alterations associated with phenylhydrazine-induced anemia in the rat. J Med **20**:305–330, 1989.
8. Harris JW. Studies on the mechanisms of drug-induced hemolytic anemia. J Lab Clin Med **47**:760–775, 1956.
9. Schwartz R, Costa N. Autoimmune hemolytic anemia: Clinical correlations and biological implications. Semin Hematol **3**:2–26, 1966.
10. Claman HN. Corticosteroids and lymphoid cells. N Engl J Med **287**:388–397, 1972.
11. Fauci AS. Mechanisms of corticosteroid action on lymphocyte subpopulations. I: Redistribution of circulating T and B lymphocytes to the bone marrow. Immunology **28**:669–680, 1975.
12. Fauci AS, Dale DG. The effect of in vivo hydrocortisone on subpopulations of human lymphocytes. J Clin Invest **53**:240–246, 1974.
13. Goodwin JS, Atluru D, Sierapowski S, Lianos EA. Mechanism of action of glucocorticosteroids. Inhibition of T cell proliferation and interleukin 2 production by hydrocortisone is reversed by leukotriene $B_4$. J Clin Invest **77**:1244–1250, 1986.
14. Jeong G, Nakoinz I, Ralph P. Independent regulation of B-cell inducing factor and IL-2 production by T lymphocytes and direct and indirect promotion of immunoglobulin secretion by glucocorticosteroid. Cell Immunol **103**:199–206, 1986.
15. Gabrielsen AE, Good RA. Chemical suppression of adaptive immunity. Adv Immunol **6**:91–229, 1967.
16. Elliott EV, Sinclair NR. Effect of cortisone acetate on 19S and 7S haemoglobin antibody. A time course study. Immunology **15**:643–652, 1968.
17. Mancini G, Carbonara AO, Heremans JF. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry **2**:235–254, 1965.
18. Woollett GR, Barclay AN, Puklavec M, Williams AF. Molecular and antigenic heterogeneity of the rat leukocyte-common antigen from thymocytes and T and B lymphocytes. Eur J Immunol **15**:168–173, 1985.
19. Billett EE, Gunn L, Mayer RJ. Characterization of two monoclonal antibodies obtained after immunization with human liver mitochondrial membrane preparations. Biochem J **221**:765–776, 1984.
20. Brideau RJ, Carter PB, McMaster WR, Mason DW, Williams AF. Two subsets of rat T lymphocytes defined with monoclonal antibodies. Eur J Immunol **10**:609–615, 1980.

21. Barclay AN. The localization of populations of lymphocytes defined by monoclonal antibodies in rat lymphoid tissue. Immunology **42**:593–600, 1981.
22. Balow JE, Rosenthal AS. Glucocorticoid suppression of macrophage migration inhibitory factor. J Exp Med **137**:1031–1041, 1973.
23. Chung HT, Samlowski WE, Daynes RA. Modification of the murine immune system by glucocorticoids: Alterations of the tissue localization properties of circulating lymphocytes. Cell Immunol **101**:571–585, 1986.
24. Cohen JJ, Duke RC. Endogenous endonuclease and thymocyte death. J Immunol **132**:38–42, 1984.
25. Dracott BN, Smith CE. Hydrocortisone and the antibody response in mice. I: Correlation between serum cortisol levels and cell numbers in thymus, spleen, marrow and lymph nodes. Immunology **38**:429–435, 1979.
26. Warren M, Vogel SN. Opposing effects of glucocorticoids on interferon-2-induced murine macrophage $F_c$ receptor and Ia antigen expression. J Immunol **134**:2462–2469, 1985.
27. Slade JD, Hepburn B. Prednisone-induced alterations of circulating human lymphocyte subsets. J Lab Clin Med **101**:479–487, 1983.
28. White A, Dougherty TF. Effect of prolonged stimulation of the adrenal cortex and adrenalectomy on the numbers of circulatory erythrocytes and lymphocytes. Endocrinology **36**:16–23, 1945.
29. Bishop CR, Athens JW, Boggs DR, Warren H, Cartwright GE, Wintrobe MM. Leukokinetic studies. XIII: A non-steady kinetic evaluation of the mechanism of cortisone-induced granulocytosis. J Clin Invest **47**:249–260, 1968.
30. Dameshek W, Rosenthal MC, Schwartz L. The treatment of acquired hemolytic anemia with adrenocorticotrophic hormone (ACTH). N Engl J Med **244**:117–127, 1951.
31. Gardner FH, McElfresh AE, Harris JW, Diamond LK. The effect of adrenocorticotrophic hormone (ACTH) in idiopathic acquired hemolytic anemias as related to the hemolytic mechanisms. J Lab Clin Med **37**:444–457, 1951.
32. Feldman J, Rachmilevitz M, Stein O, Stein Y. The adrenal cortex and hemolysis. I: Phenylhydrazine. Blood **8**:342–348, 1953.
33. Gordon AS, Mirand EA, Zanjani ED. Mechanisms of prednisolone action on erythropoiesis. Endocrinology **81**:363–368, 1967.
34. Malgor LA, Torales PR, Klainer E, Barrios L, Blane CC. Effects of dexamethasone on bone marrow erythropoiesis. Horm Res **5**:269–277, 1974.
35. Kay MMB. Aging of cell membrane molecules leads to appearance of an antigen and removal of senescent cells. Gerontology **31**:215–235, 1985.
36. Kay MMB, Goodman SR, Sarenson K, Whitfield CF, Wong P, Zaki L, Rudloff V. Senescent cell antigen is immunologically related to band 3. Proc Natl Acad Sci USA **80**:1631–1635, 1983.
37. Low PS, Wangh SM, Zingke K, Drenckhahn K. The role of hemoglobin denaturation and band 3 clustering in red blood cell ageing. Science **227**:531–533, 1985.
38. Lutz HV, Stringars-Wipf G. Senescent red cell-bound IgG is attached to band 3 protein. Biomed Biochim Acta **42**:117–121, 1983.
39. Magnani M, Papa S, Rossi L, Vitale FM, Fornaini G, Manzoli FA. Membrane-bound immunoglobulins increase during red cell aging. Acta Haematol **79**:127–132, 1988.
40. Dale GL, Daniels RB. Quantitation of immunoglobulin associated with senescent erythrocytes from the rabbit. Blood **7**:1096–1099, 1991.
41. McIsaac WM, Parke DV, Williams RT. The metabolism of phenylhydrazine and some phenylhydrazones. Biochem J **70**:688–697, 1958.
42. Naughton BA, Dornfest BS, Gordon AS. Extrarenal erythropoiesis. In: Fisher JW, Ed. Kidney Hormones. New York: Academic Press, Vol **3**: pp475–521, 1986.
43. Azen EA, Schilling RF. Extravascular destruction of acetylphenylhydrazine damaged erythrocytes in the rat. J Lab Clin Med **63**:122–133, 1964.
44. Jacob JS, Jandl JH. Effects of sulfhydryl inhibition on red blood cells. II: Studies in vivo. J Clin Invest **41**:1514–1523, 1962.
45. Horn S, Gopas J, Bashan N. A lectin-like receptor on murine macrophages is involved in the recognition and phagocytosis of human red cells oxidized by phenylhydrazine. Biochem Pharmacol **39**:775–780, 1990.