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Phenylhydrazine Is a Mitogen and Activator of Lymphoid Cells*

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ABSTRACT

Rats were administered a single injection of phenylhydrazine (PHZ) which induced an hemolytic anemia that reached maximal levels two to four days following injection. This was accompanied by a leukocytosis which was most pronounced four to six days after injection; lymphocytes and monocytes accounted for 75 percent to 80 percent of the leukocyte count, respectively. All peripheral blood cell values, including the red cell count and hematocrit, returned to their pre-injection levels by the 11th post-injection day. Analysis by flow cytometry of peripheral blood mononuclear cells (PBMC) isolated from PHZ-treated rats by Ficoll-Hypaque gradient separation showed a marked increase in the B cell population of the peripherel blood. This was also seen in cultures of PBMC obtained from untreated rats following incubation with PHZ. Cultures of PBMC obtained from rats four to five days after PHZ injection which were incubated with pokewood mitogen (PWM) or phytohemagglutinin (PHA) showed significant increases in blastogenesis as indicated by [^{3}H] thymidine incorporation when compared to cultures of PBMC obtained from untreated rats incubated with these mitogens. Incubation of cultures of PBMC obtained from untreated rats with PHZ significantly increased blastogenesis in cultures of five day duration. Atypical and blastic lymphoid cells were evident in cytosmears of PBMC isolated from PHZ-treated rats and also in sections of PBMC pellets studied using the trans-

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mission electron microscope. Serum of the PHZ-treated rats contained elevated immunoglobulin titers as measured by radial immunodiffusion. The results show that PHZ stimulates lymphoid cell blastogenesis and can sensitize circulating lymphoid cells to PHA and PWM indicating that PHZ is capable of stimulating the immune system of the rat.

Introduction

Phenylhydrazine (PHZ) is an antipyretic drug[19] which induces hemolytic anemia in laboratory animals.[8,10,35] In addition, PHZ had at one time been used to reduce red blood cell (RBC) mass in patients with polycythemia vera.[6,19] The hemolytic action of PHZ has been reported to occur by a non-immune mechanism(s).[32] PHZ, among other actions, has been reported to directly lyse human erythrocytes *in vitro*.[26] However, it was found by us that rat erythrocytes incubated with PHZ for periods up to six hours showed no detectable hemolysis.[29] 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.[2] Phenylhydrazine is a potent oxidant and is reported to cause membrane damage in rat RBCs by thiol oxidation[15] which, in turn, results in rapid removal of these cells from the circulation by the reticuloendothelial system.[2]

In this connection, a recent study demonstrates that a lectin-like receptor on murine macrophages is involved in the recognition and phagocytosis of human red blood cells (RBC) oxidized by PHZ.[14] Recent studies in our laboratory also provide evidence that PHZ stimulates the immune system, thereby suggesting that immune mechanisms may contribute to the PHZ-induced hemolysis. It has been found that PHZ causes a marked leukocytosis in the rat, primarily owing to an increase in circulating lymphoid cells.[9] In addition, histologic examination of the lymph nodes, spleen, and thymus of rats injected with the drug reveals a broad lymphopoietic response. Of particular interest is the finding that PHZ-induced hemolysis is accompanied by an increase in circulating antibodies capable of reacting with homologous rat RBCs via the indirect Coomb's reaction.[28,29]

The work now reported was undertaken to study further the influence of PHZ on rat lymphocytes. To this end, the direct effect of PHZ on the proliferative responsiveness of peripheral blood mononuclear cells (PBMC) of untreated rats and the proliferative responsiveness of PBMC of PHZ-injected rats to the polyclonal mitogens PHA and PWM were assessed *in vitro*. Lymphoid cell population changes induced by exposure to PHZ both *in vivo* and *in vitro* were assessed by phenotyping with fluorochrome labelled monoclonal antibodies. To confirm further an immune response to PHZ *in vivo*, serum IgG levels were quantified. In addition, morphological alterations of peripheral lymphoid cells were studied with the transmission electron microscope.

Materials and Methods

Animals

Male six to seven weeks old Long-Evans rats weighing 150 to 200 g were used in all procedures.

PHZ Administration

Rats were injected subcutaneously with four mg PHZ per 100 g body weight* in 0.5 ml of sterile 0.9 percent saline. The PHZ was prepared immediately before use. Control rats were untreated or injected with an equivalent volume of saline.

Peripheral Blood Cell Counts

To establish that an anemic condition was induced, tail vein blood samples were taken just prior to injection (preinjection values) and on the 2nd, 3rd, 4th, 5th, 6th, and 11th days following injection. White blood cell (WBC) and red blood cell (RBC) counts were made using a Coulter electronic cell counter,† hematocrits (Hct) were measured by the micromethod, reticulocytes were counted as a percent of 1,000 total RBC's on blood smears treated with new methylene blue, and differential counts were performed on blood smears treated with Wright's stain.

Cell Separation

Peripheral blood mononuclear cells were isolated from blood obtained from PHZ-injected and untreated or saline-injected rats by aortic exsanguination into heparinized syringes on the 2nd, 3rd, 4th, 5th, 6th, and 11th postinjection days by Ficoll-Hypaque density gradient separation.‡ The PBMC were washed once with 0.87 percent ammonium chloride to lyse any contaminating RBCs and then washed again with phosphate buffered saline (PBS); the cells recovered were resuspended in PBS. The morphology and purity of the PBMC population was determined from smears treated with Wright's stain.

Cell Culture

The PBMC were cultured in 96 well flat-bottom culture plates.§ Each culture well contained 1×10^6 cells in 0.2 ml of RPMI-1640 supplemented with HEPES (25 mM), penicillin (100 μg per ml), streptomycin (100 μg per ml), glutamine (2 mM), and 10 percent heat inactivated fetal bovine serum ("complete medium").‖ Cultures were done in triplicate. The plates were incubated without shaking at 37°C for periods of three and five days in a humidified atmosphere of five percent CO_2 in air. All cultures were pulsed for the last 18 hr of incubation with [^{3}H] thymidine; ¶2 Ci per mM, 0.25 μCi per 20 μl per well), harvested on glass fiber filters with a Mash II multiple automated sample harvester§ and washed thoroughly with distilled water. The filters were allowed to dry and then added to five ml of Liquiscint** in a scintillation vial, mixed, and the radioactivity determined in a Beckman liquid scintillation counter and expressed as mean counts per min (cpm). The viability of the cultured cells was determined by trypan blue dye exclusion.

Electron Microscopy

Cultured cells were pelleted and fixed for one hr in 2.5 percent glutaraldehyde in 0.1 mol per L phosphate buffer (pH 7.3), washed with phosphate buffer, postfixed with oxmium tetroxide, dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Thin sections were cut on the LKB Ultamicrotome,†† stained with five percent uranyl acetate, and poststained with 0.4 percent lead citrate. Sections were examined with a JOEL 100C electron microscope.

* Sigma, St. Louis, MO.
† Coulter Electronics, Inc., Hialeah, FL.
‡ Histopaque-1007, Sigma, St. Louis, MO.
§ Microbiological Associates, Bethesda, MD.
‖ Gibco, Grand Island, NY.
¶ New England Nuclear Corp., Boston, MA.
** National Diagnostics, Manville, NJ.
†† LKB Instruments, Gaithersburg, MD.

Immunoglobin Quantification

Serum antibody levels in blood obtained by aortic exsanguination from rats just prior to PHZ injection and on the 1st, 2nd, 4th, 5th, 7th, and 11th post-injection days were assayed using the Mancini radial immunodiffusion method.[23] Pre-prepared plates containing sheep anti-rat IgG (AAR 02K, 02S) and sheep anti-rat IgM (AAR 08K, 08S) were purchased along with the appropriate standards.‡‡ Standard (at 100 percent, 60 percent, and 20 percent concentration) and test serum per plasma from normal and PHZ-treated rats were placed in individual wells. Diffusion was allowed to proceed at room temperature until the ring surrounding the most concentrated standard was nine mm in diameter (45 to 50 hr). Results were then read from a standard curve plotted as the square of the diameter of the precipitation ring vs. dilution.

Analysis of Mononuclear Leukocytes by Flow Cytometry

Peripheral blood mononuclear cells isolated from normal and PHZ-treated rats were reacted with equal 100 μl volumes of the following monoclonal antibodies at 1:100 dilution: MRC OX-33, W3/25, MRC OX-8, MRC OX-54‡‡ or mouse IgG_1.† After washing two times with 0.5 percent bovine serum albumin (BSA) in PBS, the cells were resuspended and reacted with sheep anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) at a concentration of 1:100 for 30 min. Specimens were washed two times with 0.5 percent BSA in PBS and one ml of Immunolyse solution§§ was added to the eliminate erythrocytes. After two minutes of incubation, 250 μl of fixative were added to each tube. Samples were centrifuged for three min at 500 × *g*, the supernatant removed, the cells resuspended in one ml of PBS without Ca^{++} or Mg^{++}. All analyses were performed using the EPICS C flow cytometer.† The fluorescence wavelength was set at 488 nm and forward light scatter vs. 90 degree light scatter histograms were used to establish bitmaps for different cell populations. Analysis of log fluorescence of each sample was performed by setting the lower window to exclude the fluorescence of peripheral blood cells treated with mouse IgG_1-FITC alone or mouse IgG_1 followed by the secondary fluoresceinated antibody. The MRC OX-33 is a mouse monoclonal antibody which recognizes the leukocyte common antigen on rat B lymphocytes.[39] W3/25 reacts with rat helper T cells and macrophages.[4] MRC OX-8 recognizes rat cytotoxic/suppressor T cells and natural killer cells,[5] and MRC OX-54 labels rat T cells and thymocytes.[3] Log fluorescence was gated on the appropriate bitmaps to exclude those cells lacking the light scattering characteristics of lymphocytes. Cultured cells were also phenotyped using these antibodies.

Assessment of Mitogenic Activity

PHZ Stimulation. To assess the direct effect of PHZ on the proliferative responsiveness of PBMC of untreated rats, cultures of PBMC were incubated for periods of three and five days with PHZ. Activation of PBMC was determined by quantifying [^{3}H] thymidine uptake as an indicator of blastic transformation. A stock solution of PHZ was prepared for use immediately before each experiment by dissolving 100 mg PHZ in 25 ml of isotonic saline. Dilutions of $1:10^1$, $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$ of the stock solution were made with the "com-

† Coulter Electronics, Inc., Hialeah, FL.
‡‡ Serotec Inc., Cambridge, UK.
§§ Coulter Immunology, FL.

plete" culture media and 20 μl of each dilution as well as 20 μl of undiluted stock solution were added to the appropriate culture to yield final PHZ concentrations per well of 80 pg, 0.8 ng, 8 ng, 80 ng, 0.8 μg and 8 μg.

PWM and PHA Stimulation. To assess proliferative responsiveness of PBMC of PHZ-treated rats, [^{3}H] thymidine uptake was assessed after exposure of cultures of PBMC to the polycloncal activators PHA and PWM. The mitogens, PHA[¶¶] one μg per ml; or PWM[‖] 1:100 dilution of a five ml stock were incubated with cultures of PBMC of PHZ-injected rats and saline-injected or untreated rats for three and five days. In addition, PHA and/or PWM and PHZ were tested in combination *in vitro* to assess the responsivity of PBMC of both normal untreated and PHZ sensitized erythrocytes but no augmentative affects were found.

Statistical analysis. Levels of significance in this study were determined by using Student's T test.

Results

The Effects of PHZ on Peripheral Blood Cells in the Rat

A single injection of PHZ (four mg per 100g body weight) induced an anemia that was maximal during the second to fourth post-injection days, as evidenced by the RBC count and hematocrit (Hct) which were 68 percent and 74 percent of pre-treatment values, respectively (figure 1). In a previous study, this concentration of PHZ was established to be the optimal dose for inducing the peripheral cell responses now reported.[9] The reticulocyte count of the PHZ-injected rats was elevated, ranging from five percent to eight percent during the first three post-injection days, as compared to a normal count of approximately one percent in untreated rats. In addition to the changes in erythroid cell numbers, a marked leukocytosis with peak levels during the fourth to fifth post-injection days was observed (figure 1). Lymphocytes and monocytes accounted for approximately 75 percent to 80 percent of the total count in both PHZ-treated and untreated rats. By the 11th post-injection day, all peripheral blood cell values had returned to their pre-injection levels, at which time the rats were no longer screened. These findings are consistent with changes in the peripheral blood cell population in response to PHZ which were reported in detail in earlier studies.[8,9,29] Blood cell values for the saline-injected controls remained essentially unchanged.

Examination of smears of the PBMC obtained from the blood of PHZ-treated rats showed the presence of monocytes, lymphoblasts and normal as well as atypical lymphocytes from the second up to the 11th post-injection day (figure 2). Transmission electron microscopy of pelleted PBMC of PHZ-treated animals revealed a population which was rich in atypical and/or blastic lymphoid cells (figure 3). The histopaque preparation consisted of a population with an average of 99 percent mononuclear cells of which >90 percent appeared lymphoid. The trypan blue dye exclusion test indicated that the mean viability of the PBMC was 98 percent.

Analysis by flow cytometry revealed a significant decrease in the T to B cell ratio ($P < 0.02$) in the peripheral blood of PHZ-injected rats for up to 11 days following injection as compared to saline injected control rats indicating a marked increase in the B cell population (table I).

The Effects of PHZ on Serum Immunoglobulin Titers

Quantification of IgG levels by radial immunodiffusion revealed a significant increase in antibody titers by as early as

[¶¶] Wellcome, Beckenham, England.

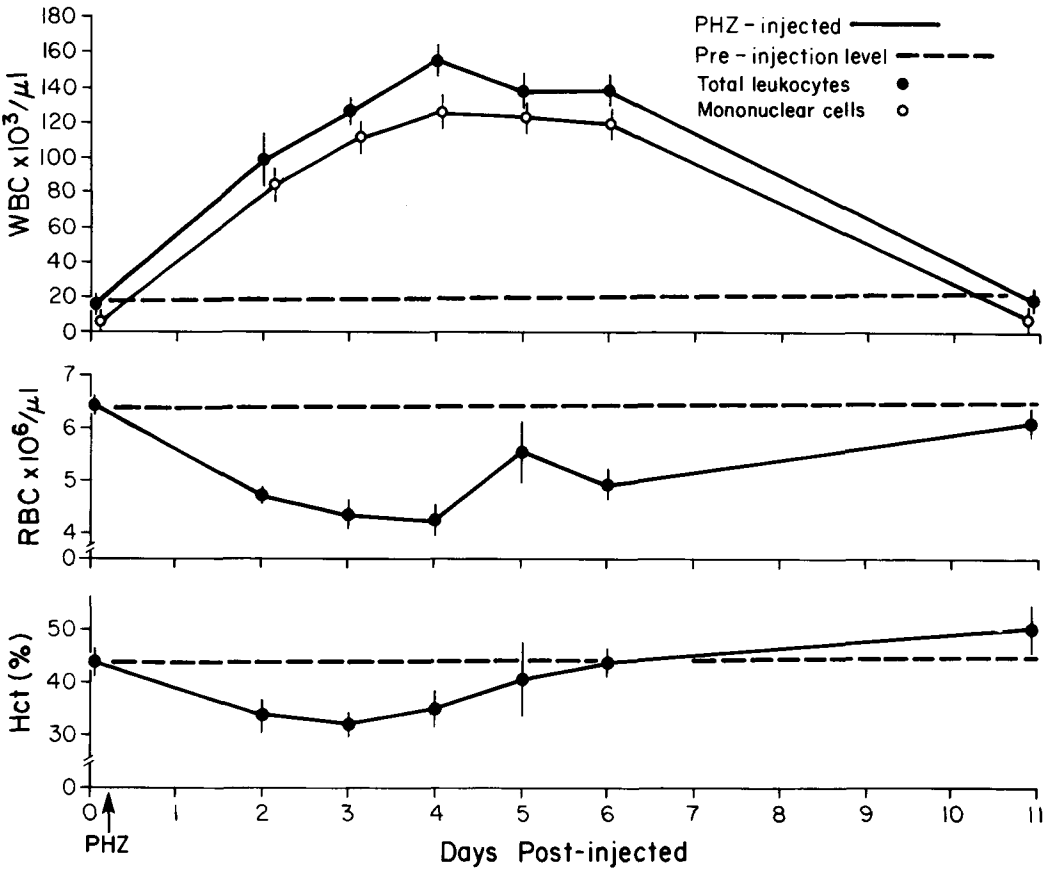

FIGURE 1. Alterations in white blood cell count, red blood cell count, and hematocrit induced in rats by a single injection of PHZ (4 mg per 100g body wgt). Mean values are shown; vertical bars indicate ± 1 SEM.

48 hr following PHZ injection and persisting to the 11th post-injection day (figure 4). However, there was no significant difference in IgG levels between the second and 11th post-injection days, although all of these values were significantly higher than pre-injection (control) levels.

PROLIFERATIVE EFFECTS OF PHZ IN CULTURE

[^{3}H] thymidine incorporation in cultures of normal rat PBMC was significantly elevated in both the three day and five day cultures ($P < 0.05$ and $P < 0.01$, respectively) following incubation with either PHA or PWM as compared to cultures to which no mitogen was added (figure 5) (The bar represents a mean of five to 14 rats). Exposure to PHZ over the five day incubation period at concentrations ranging from 80 pg to 80 ng per well resulted in significantly higher uptake of [^{3}H] thymidine ($P < 0.01$) when compared to cultures which did not receive PHZ or mitogen. However, the same concentrations of PHZ did not significantly increase uptake of [^{3}H] thymidine over the shorter (three day) incubation period. Concentrations of 0.8 μg and 8 μg of PHZ per well were found to be toxic as indicated by trypan blue dye exclusion. Otherwise, the dye exclusion test indicated that for the three day and five day PHZ-treated cultures the PBMC were 75 to 85 percent and 60 to 70 percent viable, respectively, when the cultures were harvested.

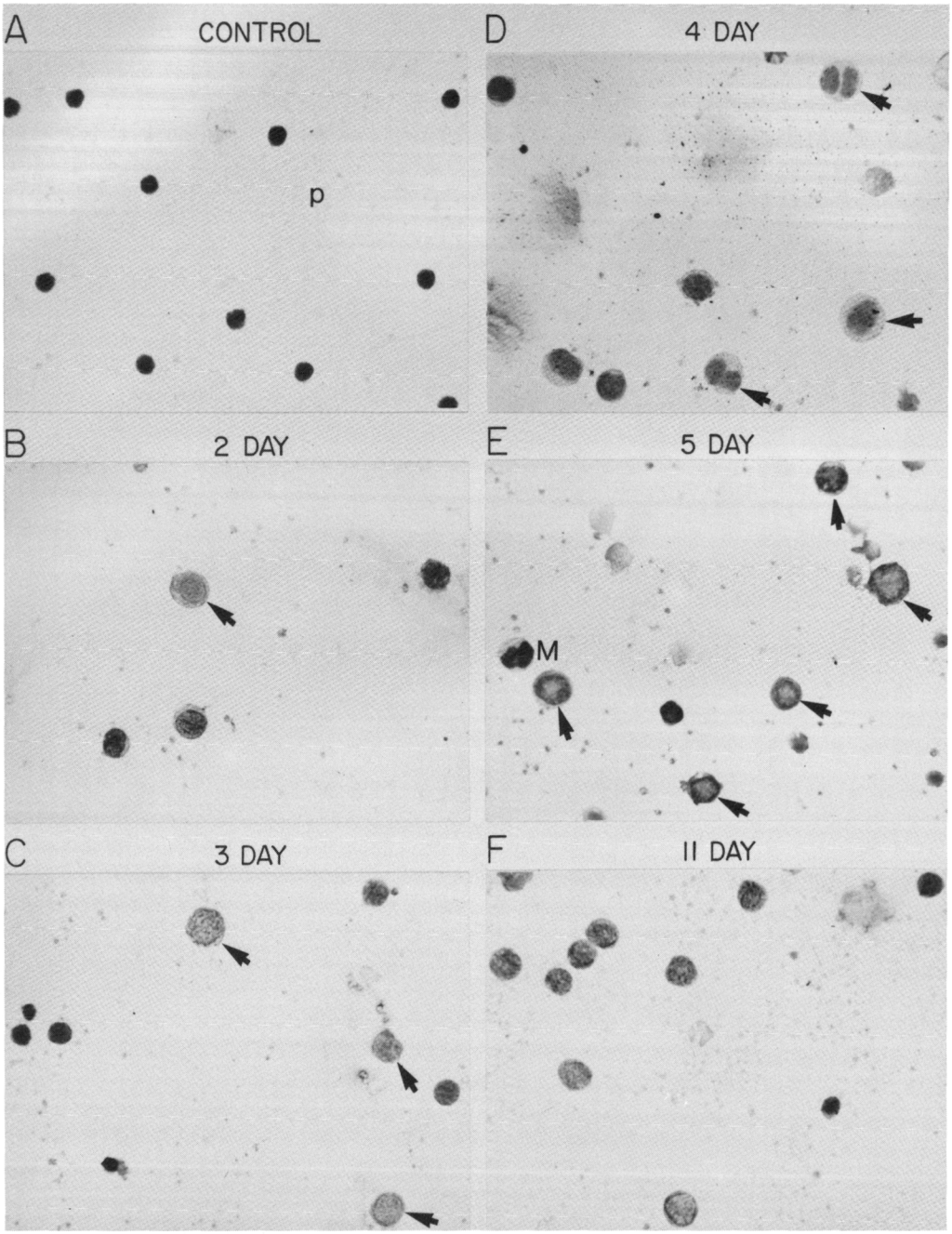

FIGURE 2. Smears of peripheral blood mononuclear cells (PBMC) (oil immersion, magnification = 1,000×) isolated by Ficoll-Hypaque gradient centrifugation. A. Control (non-injected) rat. Normal lymphocytes are seen. Preparation is rich in platelets (p) which are present in the background of all the prints. B. Two day phenylhydrazine (PHZ) post-injected rat showing a blastic lymphoid cell (arrow) and atypical lymphocytes. C. Three day PHZ post-injected rat showing blastic lymphoid cells (arrows). D. Four day PHZ post-injected rat showing binucleate, a blastic lymphoid cell (arrows) and atypical lymphocytes. E. Five day PHZ post-injected rat showing blastic lymphoid cells (arrows) and a monocytoid cell (M). F. Seven day PHZ post-injected rat showing blastic lymphoid cells with disaggregated chomatin.

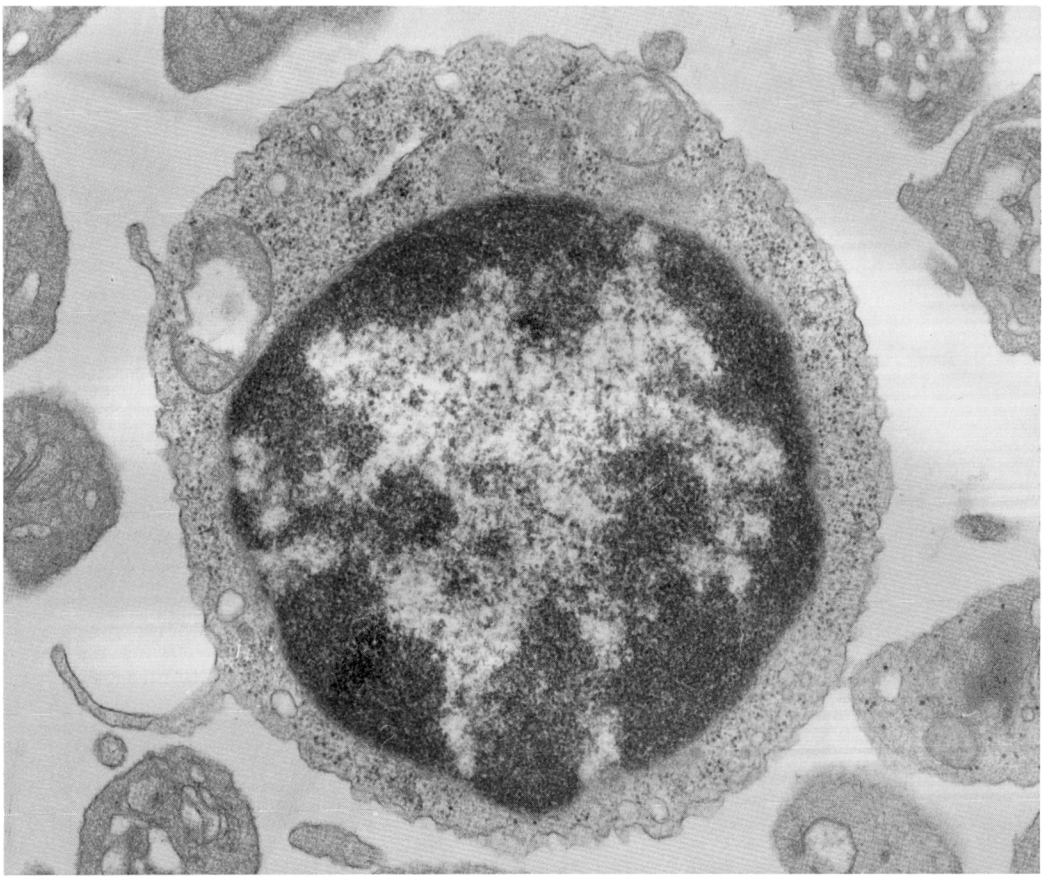

FIGURE 3A. Transmission electron micrograph of pelletted peripheral blood mononuclear cells (PBMC). A Lymphocyte in a pellet of PBMC cultured for five days without exposure to phenylhydrazine (PHZ) or other mitogens. The nuclear chromatin configuration is consistent with that of a normal, mature lymphocyte (8,300×).

Analysis of cultured cells by flow cytometry revealed a significant decrease in the T to B cell ratio ($P < 0.01$) in five day cultures of PBMC obtained from untreated rats that were incubated with a concentration of 8 ng of PHZ per well. T:B was determined for the 8 ng PHZ concentration since this quantity evoked the greatest response. Similar but lower responses were observed with the polyclonal mitogens PWM and PHA as compared to cultures of PBMC obtained from untreated rats which were not incubated with these agents. Mean T:B ± standard error of the mean (SEM) for cultures incubated with PHZ, PWM and PHA were 0.82 ± 0.36, 0.94 ± 0.42 and 0.29 ± 0.03, respectively, as compared to a T:B of 3.05 for untreated cultures.

PROLIFERATIVE EFFECTS OF PHA AND PWM IN CULTURE

In figure 6 is depicted [^{3}H] thymidine incorporation in three-day cultures of PBMC obtained from rats two to 11 days after injection with PHZ. Each data point represents the mean of five to 14 rats. [^{3}H] thymidine incorporation into lymphoid cells was increased significantly ($P < 0.05$) after incubation of PBMC of untreated (non-injected) rats with PWM or PHA when

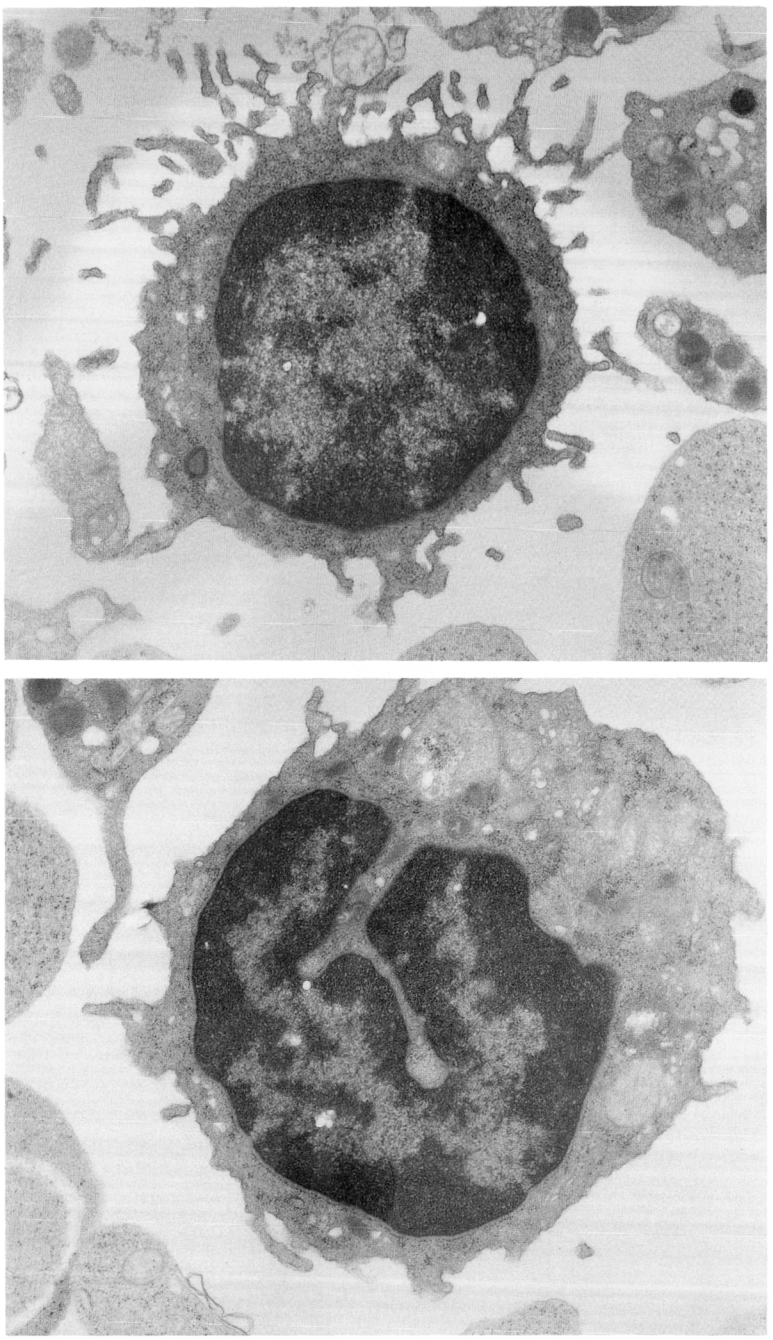

FIGURE 3B. Transmission electron micrograph of pelletted peripheral blood mononuclear cells (PBMC). Atypical lymphocytes in pellets of PMBC cultures four days after treatment with phenylhydrazine (PHZ). Cellular membrane alterations can be seen in the cell on the top (8,300×) and nuclear abberrations can be seen in the cell on the bottom (10,000×). Both lymphocytes appear mature.

compared to cultures of PBMC of untreated rats which were not incubated with mitogen (Day 0, figure 6). Incubation of PBMC of either four to five day post-injected rats with PWM or PHA, respectively, resulted in a significant increase in [^{3}H] thymidine incorporation ($P < 0.01$) when compared to cultures of PBMC of untreated rats which were incubated

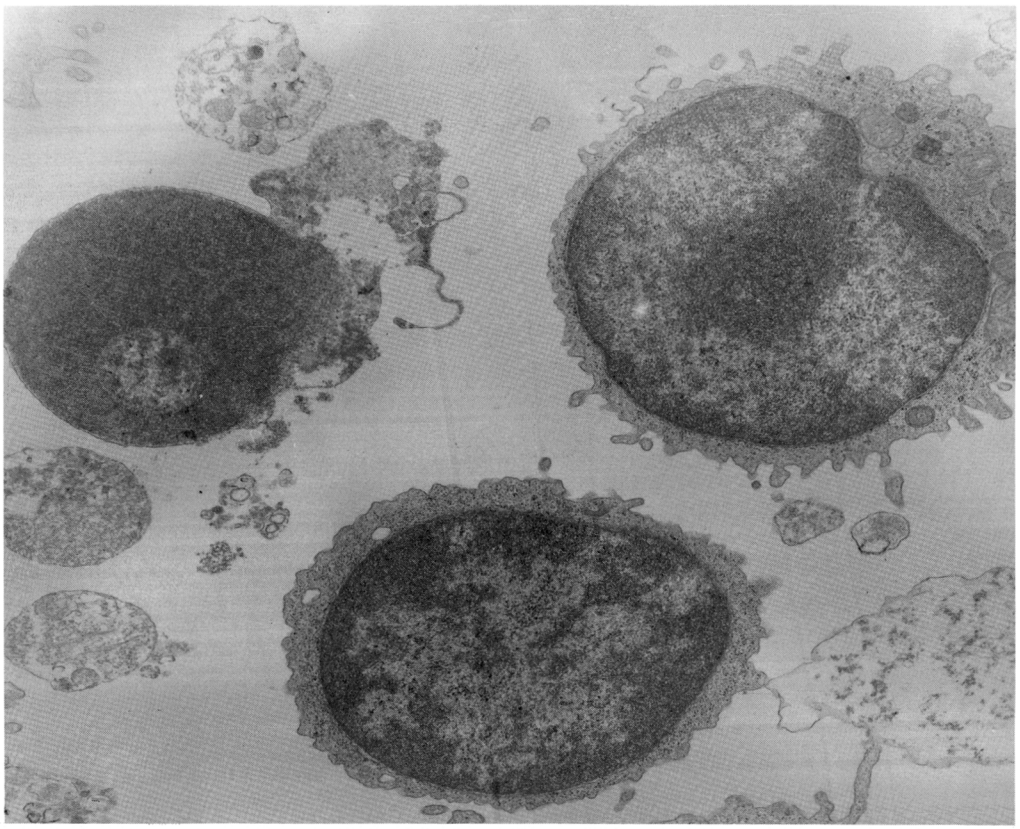

FIGURE 3C. Transmission electron micrograph of pelletted peripheral blood mononuclear cells (PBMC). Blastic lymphoid cells in PBMC cultures five days after incubation with phenylhydrazine (PHZ) (8,300×).

with PWM and PHA, or cultures of PBMC of four to five day post-injected rats which were not incubated with mitogen.

Cultures of PBMC obtained from rats four days after PHZ injection showed a significant increase in uptake of [^{3}H] thymidine when incubated with PWM but not with PHA. The highest increase in radio-uptake was noted after incubation of cultures of PBMC obtained from rats five days after PHZ injection with either PWM or PHA, with PHA showing the most pronounced increase. Radio-uptake in cultures of PBMC of PHZ-treated rats with no mitogens added was not higher than in cultures of PBMC of rats not receiving PHZ (day 0). As judged from a trypan blue dye exclusion test, 75 percent to 85 percent of the PBMC were viable when the cultures were harvested. [^{3}H] thymidine uptake into lymphoid cells of PBMC of saline-injected vs untreated rats was essentially the same.

Analysis by flow cytometry revealed a significant decrease in the T to B cell ratio ($P < 0.01$; table II) in three day cultures of PBMC obtained from rats four to five days after injection with PHZ that were incubated with PWM and PHA, as compared to cultures of PBMC obtained from non-injected control rats which were not incubated with mitogen. A significant difference in T:B was also found between cultures of PBMC of five day, but not four day

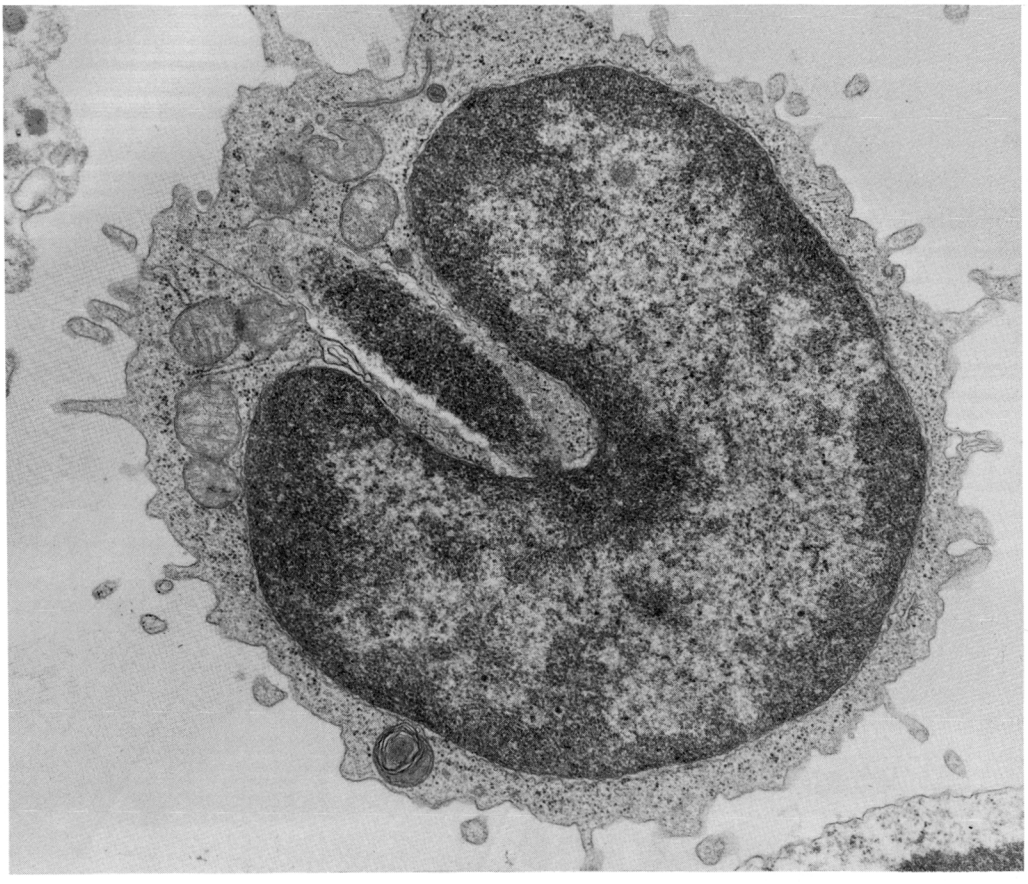

FIGURE 3D. Transmission electron micrograph of pelletted peripheral blood mononuclear cells (PBMC). Atypical mononuclear cell, putatively lymphoid, in a peripheral blood monouclear cells (PBMC) culture five days following phenylhydrazine (PHZ) exposure (10,000×).

post-PHZ injected rats which were not incubated with mitogen, as compared to the cultures of PBMC derived from saline-injected controls which did not receive mitogen. However, there was no significant difference in the T to B cell ratio found between three day cultures of PBMC obtained from PHZ-injected rats four to five days after treatment incubated with PWM and PHA as compared to cultures of PBMC of four to five day post-injected rats to which no mitogen was added (table II). This lack of significance is not unexpected since the PBMC collected from the PHZ-injected rats were already primed by previous exposure to PHZ *in vivo*. However, the T:B in both cases were lower relative to the control.

A significant difference was found between the T:B of PBMC recovered from the peripheral blood of saline-injected (control) rats (7.73 ± 0.96) and the T:B of PBMC recovered from the peripheral blood of untreated rats following three days in culture to which no mitogen had been added (3.05 ± 0.22) (tables I and II). The lower T to B cell ratio of the PBMC resulting from culture may be explained on the basis of either a selective proliferation of B cells versus T cells or a selective destruction of T cells under culture conditions.

TABLE I

Mean Percentage of Peripheral Blood Mononuclear Cells Displaying Positive Fluorescence and Mean T:B* of Untreated Versus Rats Injected With Phenylhydrazine

	OX-33	*W3/25*	*OX-8*	*T:B*
Untreated Control	*3.29 ± 0.49*	*14.92 ± 3.67*	*13.16 ± 3.90*	*7.73 ± 0.96*
Time (days) post PHZ injected				
1	7.59 ± 1.41 ($p < 0.01$)†	12.17 ± 2.71	11.67 ± 1.78	3.46 ± 0.79 ($p < 0.02$)
2	6.07 ± 0.73 ($p < 0.01$)	9.75 ± 1.88	14.51 ± 2.35	3.97 ± 0.60 ($p < 0.02$)
4	19.21 ± 2.81 ($p < 0.01$)	32.08 ± 3.66	29.97 ± 3.50	3.38 ± 0.56 ($p < 0.02$)
5	8.24 ± 1.49 ($p < 0.01$)	8.88 ± 1.14	9.23 ± 0.68	2.40 ± 0.43 ($p < 0.01$)
11	16.54 ± 1.74 ($p < 0.01$)	30.40 ± 2.91	34.59 ± 4.73	4.03 ± 0.43 ($p < 0.01$)

*Calculated as the ratio of the sum of percent positive fluorescent events within the lymphocyte window of W3/25 and OX-8 to the percent positive fluorescent events within the same window for OX33.

†P values were determined using Student's T test and represent the level of significance between untreated versus rats treated with phenylhydrazine.

Discussion

The use of PHZ as a tool for studying hemolytic anemia in mammals[10,11] as well as the mechanism(s) by which the drug may induce RBC hemolysis[2,15,20,21,27,30,36] has been the subject of numerous publications. The mode of action of PHZ in inducing hemolysis has not been fully elucidated but has been

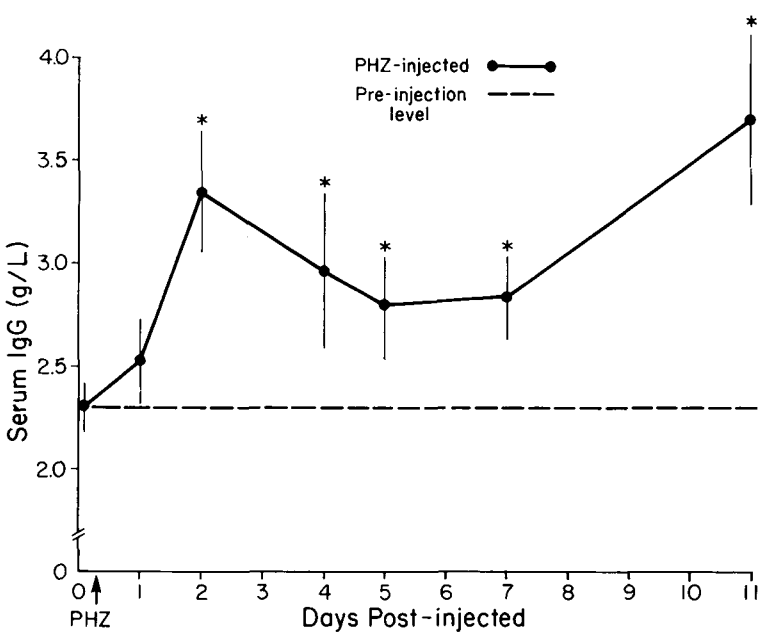

FIGURE 4. Mean Immunoglobulin G concentrations in the serum of rats at various time intervals following PHZ administration as determined by radialimmunodiffusion. Vertical lines through the means indicate ± 1 SEM.

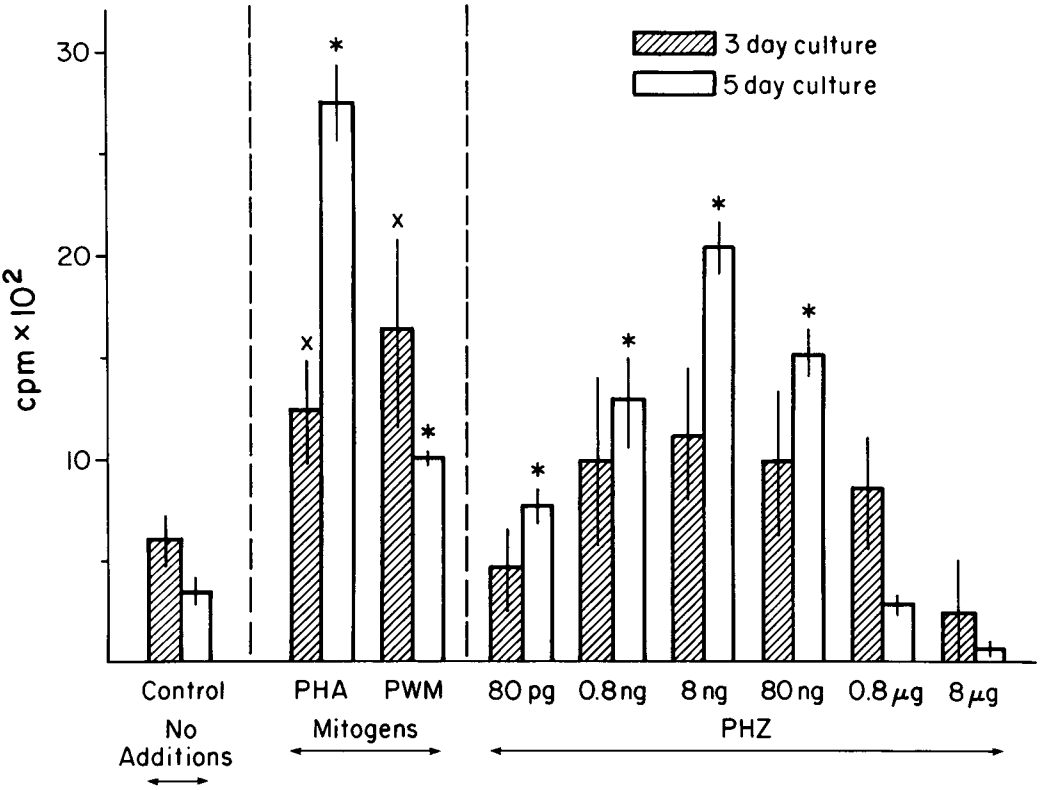

FIGURE 5. Effects phytohemagglutin (PHA), pokeweed mitogen (PWM), and phenylhydrazine (PHZ) on the uptake of [^{3}H] thymidine in three day and five day cultures of PBMC obtained from untreated (non-injected) rats. Results are expressed as the mean counts per minute (cpm) ± 1 SEM of triplicate cultures. Asterisks indicate five day cultures incubated with PHA, PWM, or PHZ in which [^{3}H] thymidine uptake was significantly higher ($P < 0.01$) when compared to uptake in five day cultures to which there was no addition of mitogen or PHZ. The X's indicate three day cultures incubated either with PHA or PWM in which [^{3}H] thymidine uptake was significantly higher ($P < 0.05$) when compared to uptake in three day cultures to which there was no addition of mitogen or PHZ.

proposed to result from a direct action on hemoglobin resulting in denaturation and Heinz body formation. In addition, exposure of RBCs to PHZ has been found to cause clustering of antigenic band 3 protein.[20] This predominant membrane protein has been identified as the senescent cell antigen which reportedly binds to autologous IgG and induces erythrophagocytosis.[16] Recognition and opsonization of this modified band 3 protein by autologous specific IgG, present at sub-tolerance levels in the serum, results in the sequestration and destruction of these cells by macrophages via F_c receptor mechanisms.[16,20] In this regard, PHZ has been shown to promote the binding of autologous IgG to RBCs.[20,21]

It has also been reported that PHZ exposure causes a marked decrease in the spectrin of human erythrocytes, resulting in gross morphological aberrations, most notably echinocyte formation.[31] In this connection, it has been reported that red cell membrane alterations resulting in the formation of spikes is associated with immunohemolytic anemia.[33] Other possible explanations for PHZ destruction of RBCs include biochemical modifications such as ATP decay,[27] calcium ATPase inhibition,[36] and increased potassium

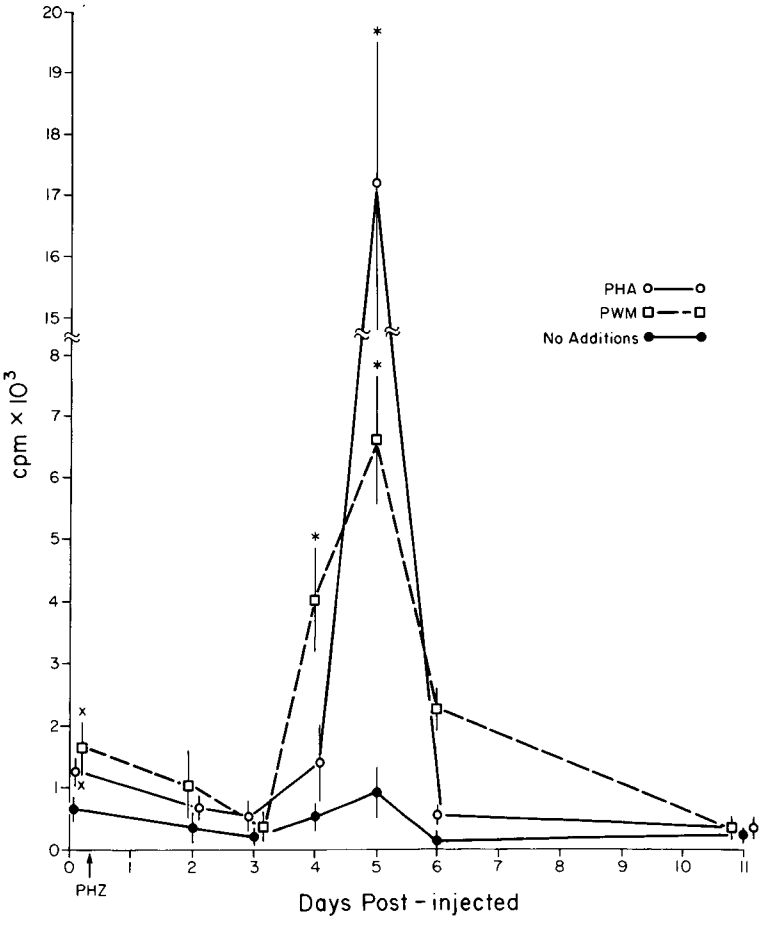

Figure 6. Effects of phytohemagglutin (PHA) and pokeweed mitogen (PWM) on the uptake of [^{3}H] thymidine in three day cultures of peripheral blood mononuclear cells (PBMC) obtained from rats two to 11 days after injection with phenylhydrazine (PHZ) (arrow). Results are expressed as the mean cpm ± 1 SEM of triplicate cultures. Asterisks indicate cultures incubated with either PHA or PWM in which [^{3}H] thymidine uptake was significantly higher ($P < 0.01$) when compared to uptake in cultures of PBMC obtained from untreated (non-injected) rats to which mitogen was added (day 0). The Xs indicate that there was a significantly higher ($P < 0.05$) uptake of [^{3}H] thymidine in cultures of PBMC of untreated rats incubated with either PHA or PWM compared to uptake in cultures of PBMC of untreated rats to which there was no addition of mitogen.

permeability,[30] which result in diminished RBC deformability followed by intravascular hemolysis.

Reports which implicate the immune system as having any direct role in the anemia induced by PHZ are sparse. It has been observed that protective immunity to malarial infection in rats and mice can be significantly enhanced by PHZ administration and that this effect generates memory, can be transferred with spleen cells, and that PHZ can act to supress as well as to enhance immune responsiveness in malaria infected mice.[7,34] No definitive explanation was offered in these studies for the difference in immune reactivity to the drug, but our data suggest that the exposure time of lymphoid cells to PHZ in the circulation relative to infection may be important in modulating immune responsiveness. In this connection, the exposure time of lymphoid cells to PHZ may be contingent on the metabolism of the drug. Very little has been documented on the metabolism of PHZ in mammals. It has been reported that PHZ is rapidly metabolized in warm-blooded animals since its presence in the blood or urine could not be demonstrated shortly after its administration.[18]

In one in-depth study in rabbits given [^{14}C] labelled PHZ orally, it was found that 50 percent of the dose was excreted in the urine four days after administration, the rest being found mainly in the

TABLE II

Mean Percentage of Positive Fluorescent Events Using Antibodies to Rat T Lymphocytes (OX-54)* and Rat B Cells (OX-33)†

Untreated Control (no additions)	*OX-33* 21.61 ± 1.47	*OX-54* 64.35 ± 2.27	*T:B* 3.05 ± 0.22
Time (days) post PHZ injection of the PBMC donor			
No additions			
4	24.01 ± 4.73	50.43 ± 7.54 ($p < 0.05$)§	2.46 ± 0.54
5	28.99 ± 3.94	40.78 ± 4.66 ($p < 0.01$)	1.58 ± 0.24 ($p < 0.01$)
PWM addition‡			
4	58.56 ± 6.37 ($p < 0.01$)	62.93 ± 4.54	1.07 ± 0.04 ($p < 0.01$)
5	31.44 ± 8.16	34.28 ± 7.63 ($p < 0.01$)	1.13 ± 0.11 ($p < 0.01$)
PHA addition‡			
5	26.99 ± 4.50	38.22 ± 7.03 ($p < 0.01$)	1.38 ± 0.21 ($p < 0.01$

*A pan T marker.
†Ratio of the log fluorescence accumulation with OX-54 to the accumulation of OX-33 between these subpopulations in peripheral blood mononuclear cells (PBMC) of untreated versus injected rats which are incubated for three days in culture with either pokeweed mitogen (PWM) or phytohemagglutinin (PHA).
‡Refers to PBMC of rats four or five days post phenylhydrazine (PHZ) injection which were cultured with either PWM or PHA.
§P values were determined using Student's T test and represent levels of significance between PBMC cultures of untreated rats to which no mitogen was added versus PHZ injected rat PBMC with or without the addition of mitogen.

circulating RBCs which retained 10 percent of the dose for a period of at least 10 days.[26] It has also been reported that virgin mice and rats made anemic by bleeding or PHZ-treatment showed an increase in immunoglobulin secreting spleen cells.[25] The mechanisms proposed in this study to account for this effect implicate the resulting anemia as causative in inducing IgG secretion. On the other hand, our findings suggest that PHZ-induced anemia is due to an immune response induced directly by the drug. Also reported is that PHZ-induced anemia results in a greatly decreased incidence of Rauscher leukemia virus disease in mice. One possible explanation offered for this effect was that the drug, through stimulation of the reticuloendothelial system, induces an antibody reaction against the virus.[37]

Little information exists concerning the effects of PHZ on circulating lymphocytes and monocytes. Administration of PHZ to the guinea pig, in a dosage which induces anemia, also results in a significant monocytosis characterized by the presence of atypical DNA-synthesizing cells in the peripheral blood with counts reaching more than three times normal by the fifth post-injection day.[12] Administration of PHZ to the mouse is reported to cause a sharp rise in the peripheral lymphocyte count as early as four hours after treatment, with the count showing a four-fold increase on the fifth day after the first of three successive daily injections.[13] In this same study, there was a significant increase in the spleen colony forming cell (CFU-S) population which was maximal on the fifth day following PHZ injection.

In a more recent experiment in the murine system, PHZ has been found to stimulate production of T and B lymphocytes as well as erythroid progenitors.[17] The results of these studies in the guinea pig and mouse are similar to our findings in the rat. A single injection of PHZ sufficient to cause anemia induced a lymphocytosis accompanied by the appearance of atypical and blastic cells in the circulation which was maximal four to six days following administration of the drug. When PBMC collected from untreated rats was incubated with PHZ, [H^3] thymidine incorporation was significantly higher than controls in five day cultures. This latter finding is of particular interest since it indicates that there is no requirement for lymphoid cells to be previously activated in order to proliferate in response to PHZ since PHZ can act as a mitogen *in vitro*. This finding suggests that PHZ has the ability to stimulate lymphoid cells directly in the peripheral blood and lymphoid tissues. Supportive of this hypothesis is our finding that unseparated PBMC collected from rats receiving a single injection of PHZ were sensitized to PWM and PHA in culture. Thus, the PBMC of four and

five day post-PHZ injected animals incubated with PWM and five day post-PHZ injected animals incubated with either PHA or PWM showed significantly increased cellular incorporation of [^{3}H] thymidine when compared to the PBMC of control rats incubated with these mitogens.

The finding that [^{3}H] thymidine uptake in cultures of PBMC of PHZ-treated rats with no mitogens added was not higher than in cultures of PBMC of PHZ-untreated rats indicates that although the cells of PHZ-treated rats were primed and had undergone blastogenesis *in vivo* in response to the drug, the cells, although sensitized, required exposure to mitogen *in vitro* to undergo further blastogenesis. The data therefore suggest that different receptor sites may exist and/or different cell populations may be present that respond to PHZ as compared to PHA or PWM.

In PHZ-induced anemia, erythropoiesis is enhanced to compensate for the hemolytic effects of the drug.[10,28,29] Erythropoiesis is controlled by the glycoprotein hormone erythropoietin, which is elevated in the serum of PHZ injected animals.[10,11,29] In this and earlier experiments, it has been found by us that the rat immune system also is stimulated by PHZ and that upon exposure to this agent, rats generate antibodies capable of opsonizing erythrocytes and inducing their destruction by the RES.[28,29]

In the present study, it has been found that a single injection of PHZ significantly increased serum IgG levels within 24 hr following exposure to this drug. The finding that IgG titers were still elevated by the 11th post-injection day is indirect evidence that IgG production was multiple, since these antibodies are apparently not associated with the hemolysis which was noted by as early as 24 hr after PHZ treatment. No detectable hemolysis was observed on the 11th day. Specific IgG in the serum of PHZ-treated rats collected during periods of active hemolysis was capable of inducing anemia in normal recipients after passive transfer.[28] It appears that this antibody is specific for rat red cells exposed to PHZ either *in vivo* or *in vitro*. Phenylhydrazine may induce anemia by simultaneously altering erythrocyte band 3 protein and stimulating the production of the autologous IgG which binds it.

In contrast, whereas rats chronically injected with PHZ rapidly develop a pronounced anemia, this gradually becomes compensated with time.[8,10,28,29] The mechanism underlying this phenomenon is unknown, although it is possible that the animal metabolizes and eliminates PHZ more efficiently with continued exposure. Alternatively, evidence exists that lymphoid cells have a modulatory role in erythropoiesis.[1,22,24,38] What if any relationship exists between the enhanced erythropoiesis observed during the compensation of PHZ-caused hemolytic anemia and the induction of lymphoid blastogenesis by this agent remains to be elucidated. It is conceivable that PHZ exposure results in a cascade of events including erythrocyte membrane damage, enhancement of autologous IgG synthesis, opsonization of sensitized red cells and sequestration by the RES, and some as yet undefined stimulation of other lymphoid cells whose products may accelerate the replacement of cells lost to the anemia.

Summary

In addition to the well established effect of PHZ in inducing hemolytic anemia, the present as well as our other recent studies in the rat have shown that PHZ can induce a lymphocytosis[9,29] induce the appearance of atypical and blastic lymphoid cells in the peripheral circulation,[9] cause an increase in the

number of circulating B lymphocytes, morphologically alter cells of the lymph nodes, spleen, and thymus, and stimulate immunoglobulin formation.[9,28,29] The rapid rise in serum IgG levels following PHZ administration is consistent with the activation of a secondary immune response. Thus, the effects of PHZ are at least two-fold in that it (1) activates B lymphocytes in inducing a amnestic response and (2) induces membrane damage rendering erythrocytes susceptible to recognition and destruction by macrophages. In addition, it has been found in this study that PHZ stimulates lymphocyte blastogenesis *in vitro* in the absence of mitogen, enhances lymphocyte sensitivity to PWM and PHA *in vitro*, and alters the ratio of T to B lymphocytes both *in vitro* as well as *in vivo*, indicating that B cell proliferation and/or egress of these cells into the circulation is stimulated by PHZ. Thus, these findings show that in addition to its recognized hemolytic effect, PHZ strongly enhances the responsiveness of the rat immune system indicating an immune involvement in the resultant anemia.

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