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CHANGES IN PERIPHERAL BLOOD CELLS IN MICE AFTER INJECTION WITH BENZO(A)PYRENE DURING PREGNANCY

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ABSTRACT

Pregnant C3H/Anfcum mice were injected i.p. with 150 ug benzo(a)pyrene (BP)/g body weight at the second trimester (12 days). Quantitative and differential changes were assayed in peripheral blood leukocytes and erythrocytes at various times before and after mating and treatment. Within 5 days after injection, a 2- to 3-fold reduction in leukocytes was observed when compared to controls (corn oil vehicle-injected or untreated pregnant females) which persisted into the 10th postpartum day. The erythrocytes were also significantly reduced but not to the same degree (1.2- to 1.5-fold). Depression in white blood cells is attributed to lymphocyte depletion since the granulocytes were virtually unchanged and the lymphocyte to granulocyte ratio, ordinarily >2 was 1 or less than one. No change in monocytes was observed and none of the cell populations, including the erythrocytes, appeared to be abnormal (e.g., no increase in reticulocytes). A moderate reduction (1.5-fold) in these elements also occurred in the controls (vs virgin females). The effect of pregnancy also led to transient decreases (shortly after mating into about 3 days before parturition) in medium sized lymphocytes and 3- to 4-fold increases in small lymphocytes. These results show that although pregnancy depresses the leukocyte profile, exposure to BP exacerbates this change, and preferentially affects the lymphocytes. These blood profile changes may have important health consequences in the mother and her progeny.

INTRODUCTION

Benzo(a)pyrene (BP), an ubiquitous environmental pollutant and energy by-product of coal pyrolysis, is a potent immunosuppressive and tumorigenic agent (27, 35). Exposure of progeny

in utero by injection of from 4 to 6 mg into the pregnant mother during the second trimester of pregnancy not only leads to extensive neoplasias of different types in the offspring (7, 30), but also increases sensitivity for immunosuppression (30) and tumorigenesis (22, 30). Furthermore, cell-mediated immune responses of the offspring are suppressed early in life (1-4 wk) and sustained for 18 mo (31). The doses used were equivalent to single or cumulative exposures (inhalation) to individuals in certain industrial occupations and in domestic situations including: in steel mills at topside of coke ovens and in wall tarring operations (12) (where concentrations can reach values of from 300 to 6000 ug/m^3 of air); in small living enclosures under primitive cooking conditions where women can be exposed to 9.2 ug/m^3 (1).

In the course of assaying T cell ontogenesis in the progeny during fetal life (32, 33), we noted significant reductions in thymus and spleen weights of the BP-injected mother during pregnancy and postpartum (up to 1 wk) (34). Briefly, evaluation of the T cell population in maternal lymphoid tissue revealed; a) in the thymus, significant exacerbated and sustained reductions in T cells, Lyt 1^+ and Lyt 2^+ subsets, and b) in the spleen the T and Lyt 1^+ cells were reduced during pregnancy followed by postpartum enhancement, while the Lyt 2^+ cells were

dramatically enhanced at 15 to 19 days of pregnancy, returned to control levels and became elevated on the 5th postpartum day (15, 34).

It is well documented that lymphoid cells including lymphocytes of experimental animals and humans have an active aryl hydrocarbon hydroxylase (AHH) enzyme system that is capable of metabolizing polycyclic aromatic hydrocarbons including BP to suspected carcinogenic intermediates (e.g., 7, 8-diol-9, 10-epoxide) (2, 4, 6). In view of the importance of the circulating lymphocyte pool in host defense, and since BP leads to striking changes in the maternal lymphoid tissue, we decided to investigate the peripheral blood cell profiles in injected pregnant mice. Our purpose was to determine the relative sensitivity of the lymphocyte, granulocyte and erythrocyte elements after exposure to BP. Such determinations are important not only as regards the disruptive influence of carcinogenic compounds on normal blood profiles essential for physiologic and immunologic activity, but also may be valuable in developing a model for assaying cancer risk assessment in individuals under conditions of chronic (1) or acute (13) exposure to this dangerous environmental pollutant.

MATERIALS AND METHODS

Animals, Mating Procedures and Treatment

The animals used in these experiments were inbred C3H/Anfcum (Cumberland View Farms, Clinton, TN) mice. These mice are considered responders whose level of AHH activity is increased dramatically by pretreatment with polycyclic aromatic hydrocarbons which is genetically controlled by dominant alleles at the Ah (aromatic hydrocarbon) loci (20). The genotype of such animals is designated $Ah^{b/b}$ or $Ah^{b/d}$ (hybrids), while those showing a relative inefficiency in AHH induction are non-responders genotypically designated as $Ah^{d/d}$ (e.g., DBA/2). The animals were mated at 12 wk of age (2 females per male) and observed daily for vaginal plugs. When the plug was detected (day 0) the females were injected on the 12th day of pregnancy with 150 ug BP/g body weight i.p. as described earlier (30) and then isolated. All mice before or after treatment were housed under similar conditions with free access to food and water.

Experimental Groups

Animals were separated into 3 groups: Those injected with BP (Aldrich Chemical Co., Milwaukee, WI) dissolved in corn oil (experimentals); those injected with corn oil vehicle only in volumes similar to those receiving BP (controls), and a third group was constituted of uninjected age-matched virgin females.

Quantitative assay of peripheral blood elements

Total leukocyte and erythrocyte determinations were initiated on the 22nd day before mating and performed at 5 to 10 day intervals until the experiment was terminated (at 55 days). Determination on the amounts of these cells was accomplished by drawing blood from the lateral tail vein into a white blood cell pipette (for leukocytes) and diluted 1/20 with Turk's solution, or into a red blood cell pipette (for erythrocytes) and diluted 1/200 with Hayem's solution (29). The pipettes were previously coated with innocuous sigmacote solution (Sigma, St. Louis, MO) to prevent clotting. Cells were counted in a hemocytometer and quantified in terms of cells/mm^3 of blood.

Differential white blood cell determinations

To determine total cells, a small volume of blood from the tail vein was lightly touched on a glass slide, smeared, rapidly air dried and cells fixed in methanol. The smears were stained with Wright's solution and the cells differentiated into lymphocytes (large >10 μ ; medium 8 to 10 μ ; small <8 μ), granulocytes (neutrophils, eosinophils and basophils) and monocytes (9).

Two hundred nucleated cells were enumerated at random. Cells were identified as monocytes if they were > 10 μ, had a characteristic folded nucleus, (unlike the large lymphocytes

which were differentiated on the basis that the nucleus was either round or with a slight indentation), and a slightly basophilic cytoplasm (9). The frequency (%) of nucleated cells (e.g., granulocytes and/or lymphocytes) was determined and this value was multiplied by the number of cells to quantify total numbers for each cell type per unit volume (mm^3) of blood. The quantity of cells for each animal (5 to 6 animals per group) at the time interval assayed is the mean of 2 smears (i.e., 400 or more cells per animal).

Statistics

Differences between experimentals and controls were determined by a one- (e.g., between BP-exposed or corn oil-injected mice) or two- (i.e., when comparing all three groups) way analysis of variance, and/or by the Student's t-test for unequal sample sizes (26).

RESULTS

Quantitative changes in leukocytes and erythrocytes

Injection of BP led to remarkable and striking reductions in leukocytes (figure 1). As can be seen the quantity of leukocytes before and after mating and up to the time of injection with the carcinogen remained at control levels and was not appreciably different from the virgin females. On the 5th day after BP injection, however, the quantity of leukocytes in

controls was significantly lower than those of the virgins ($p<0.01$). In the BP-injected mothers reduction in these cells was dramatically exacerbated, being from 2- to 4-fold lower than that of the controls ($p<0.0001$ maximum). These subnormal amounts lasted up to the 10th postpartum day when the experiment was terminated. Litter size is most likely not a factor in the observed depressions since the mean number of pups born for the experimentals and controls was not significantly different (30). Although similar changes occurred in the amount of circulating red blood cells, these were not as dramatic as for the leukocytes (figure 2). Further, we did not observe morphologic abnormalities in these cells (e.g., no change in the amount of reticulocytes) and in the lymphocytes or leukocytes (data not shown).

Differential changes in white blood cells

In the mother injected with BP we observed a striking and sustained reduction in lymphocytes (e.g., on the 5th postpartum day, $p<0.001$ between the BP and control cells), particularly in medium sized cells (figure 3 and table 1) which was sustained up to the 10th postpartum day. At the same interval, the small lymphocytes increased 3- to 4-fold (table 1). In contrast there was little, if any, reduction in the granulocytes (this included neutrophils, eosinophils and basophils). No significant changes

in monocytes occurred (data not shown). The ratio of lymphoctes to granulocytes (L/G) remained greater than 2 in the controls similiar to the virgin mice. In the experimental mice this ratio was essentially reversed, i.e., 1.00 or <1.00 from 5-7 days posttreatment to the 5th postpartum day (results recorded for the 10th day were similar).

DISCUSSION

The results reported here show that the carcinogen, BP, can mediate reductions in peripheral blood leukocytes of the mouse reflected primarily by exacerbated and profound depression of lymphocytes. The amounts of some other blood elements were also reduced but not to the same extent. Thus, it appears that this carcinogen, at the concentration used, preferentially affects lymphocytes.

It is very likely that the damaging effect of BP is related to the route of exposure. Severe reductions in peripheral blood leukocytes have been reported after daily oral administration of BP (3 to 4 mg/mouse) for 10 to 50 days in non-responder DBA/2 ($Ah^{d/d}$) mice, while in (C57Bl/6xDBA/2) F1 responders ($Ah^{b/d}$) only mild disruptions occurred (21). This is in contrast to the depletions seen in our mice also considered to be responders, i.e., $Ah^{b/b}$ (16), which received a dose of BP equivalent in magnitude to a single oral administration given to the DBA/2

mice. It appears that after oral administration to responder mice, BP is more actively metabolized in the bowel limiting the availability of toxic intermediates to the hemopoietic tissues (16), while after i.p. injections more BP reaches the lymphocytes for metabolic activation that probably leads to a depletion of these cells. After inhalation, one would expect depletion of lymphocytes since BP-diol-epoxide-DNA-adducts in these cells have been detected (presumably from metabolic activation) in coke oven and foundry workers (24, 13), and since the majority of ^{3}H-BP is cleared (in the rat) by direct absorption into the blood (28)

It is likely that the effects we have seen occur from a direct action on these blood elements since mouse lymphocytes (splenic) and human peripheral blood lymphocytes are capable of metabolizing BP to its toxic intermediates (2, 6). Although it is well documented that erythrocytes and granulocytes have active monooxygenases (characteristic of the P-450 system) (5, 18) metabolism of BP by these cells has not been well documented. However, there is some evidence that polymorphonuclear leukocytes (of the guinea pig) do not metabolize BP (4). Thus, the effect on erythrocytes and granulocytes may be due to toxic factors which co-valently bind to molecular components of these blood elements [e.g., on the hemoglobin of the

erythrocytes, (25)].

Since murine bone marrow and spleen cells can metabolilze BP (and other polycyclic aromatic hydrocarbons), an effect on hemopoietic precursors probably also occurs (see e.g., 21). However, depletions that occur in these organs may only account for a small part, if any, in the dramatic reductions we have observed for the lymphocytes. Such an effect may be contributory beyond the experimental period analyzed (>10 days postpartum) provided recovery does not occur.

It is reasonable to assume that the status of the progeny immune system would be influenced by factors (migrating lymphocytes, humoral substances) from the mother (14, 19). In this context, it is known that the immunologic capability and the amount of circulating lymphocytes of the progeny from the thymectomized mothers are depressed (23, 17). It is also known that maternal lymphocytes can be found in the fetus and newborn either by their ability to traverse through the placenta (10) or from the colostrum (i.e., during suckling) (36). In a syngeneic system migrating cells would maintain residence (especially in immunocompromised mice) for several weeks and even months similar to that described for semi-allogeneic systems (36). Since Lymphocytes are depleted, the numbers migrating would be reduced and thus they would be less effective in influencing

progeny immune status. Furthermore, the markedly depleted thymus of the mother (34) (which may also reflect reductions in the peripheral blood lymphocytes) may also signal limited absorption of humoral factors that may be important in immunologic development. It is well documented that humoral substances from the thymuses (thymic hormones) in diffusion chambers implanted into mice thymectomized as neonates can restore immune status (23).

We have shown, for the first time, that pregnancy changes the peripheral lymphocyte profile. Thus, regardless of BP exposure, medium sized lymphocytes are depleted shortly after mating extending to just before parturition, the small lymphocytes increase 3- to 4-fold, while the large lymphocytes are unchanged. Reasons for this directional change are not apparent at this time.

Information characterizing the sensitivity of mouse T and B cells (or their subsets) to the toxic effects of BP is limited. Others have reported defects in splenic T or B cell function (3, 8) and an increase in T suppressors (not phenotyped) (3). In situations where considerable cigarette smoke is inhaled (heavy smokers, >1 pack/day) in which BP is a prominent constituent, circulating $OKT4^{+}$ cells (helper/inducers) are decreased and the OKT4/OKT8 ratio decreased indicative of relative increases

in OKT8^{+} cells (suppressor/cytotoxic) (11). In mothers we have shown that splenic Lyt 2^{+} cells are dramatically increased during pregnancy and at 1 wk postpartum, and that changes in the mixed lymphocyte response (depression during pregnancy and postpartum) parallels the splenic profile for Lyt 2^{+} cells (34). Currently, we are assessing persistence of immunologic dysfunction (depressed mixed lymphocyte response) (34) in the mothers including quantitative and qualitative disturbances in the peripheral blood T cell population (i.e., beyond postpartum day 10) [e.g., T helper (Lyt 1^{+}) or T suppressor (Lyt 2^{+}, I-J^{+}) activity]. Persistence of such modifications, using a dose-response analysis, particularly at lower doses, may serve as a valuable indicator for the assessment of risk to cancer in individuals exposed to this carcinogen (those exposed at high levels) and may supplement evaluations being performed for this purpose on presence of BP-DNA adducts in human lymphocytes (e.g., in coke oven workers) (24, 13).

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FOOTNOTES

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TABLE 1

Changes in peripheral blood lymphocytes of female mice injected with benzo(a)pyrene.

Treatment[b] Group	Time after start of experiment	Mean Relative Amounts of Lymphocytes (%) ± SD[a]		
		LL[c] (> 10μ)	ML[c] (8-10μ)	SL[c] (< 8μ)
BP	Pre-mate (day 1-5)	5.5±1.2	90.0±3.2	4.7±1.5
CO		4.9±0.6	85.1±1.8	6.5±2.0
V		5.0±1.5	84.3±3.4	10.8±2.3
BP	Post-mate (day 5)	4.7	62.3±2.6	30.3±3.1
CO		5.2	54.2±5.1	38.5±5.2
V		4.6	81.0±4.2	9.2±3.0
BP	Pre-treat (day 2)	4.8	68.5±6.2	25.8±4.8
CO		4.9	70.5±4.7	24.2±4.3
V		3.5	90.1±5.1	6.4±2.6
BP	Post-treat (days 5-7)	4.7	90.6	7.8
CO		2.3	90.8	7.0
V		8.6	89.1	8.5
BP	After Birth (day 1)	3.8	89.3	7.1
CO		2.5	87.1	9.1
V		ND	ND	ND

a. SD = Standard Deviation (Student's t-test)
b. BP = Benzo(a)pyrene; CO = Corn oil; V = Virgins
c. LL = Large Lmphocytes; ML = Medium Lymphocytes; SL = Small Lymphocytes

FIGURE LEGENDS

Figure 1. Changes in peripheral blood leukocytes after injection of benzo(a)pyrene. ○, control (corn oil-treated or untreated pregnant mice); ●, Experimental (benzo(a)pyrene-treated pregnant mice); △, virgin females. Rx = treatment. Horizontal arrow pointing toward the right under straight line of abscissa indicates days before or after birth. Vertical arrows under dashed line of abscissa represent time periods after start of experiment. PBL = peripheral blood leukocytes. Vertical lines from symbols represent standard deviations from the means.

Figure 2. Changes in peripheral blood erythocytes after injection of benzo(a)pyrene. ○, control ●, experimental; △,virgin females. RBC = Red Blood Cells. Remainder of legend as in figure one.

Figure 3. Changes in lymphocytes (L) and granulocytes (G) after injection of benzo(a)pyrene. ▨, Control; ■, Experimental; □, Virgin females. PBL = peripheral blood leukocytes. Vertical lines from bars represent standard deviations from the means.

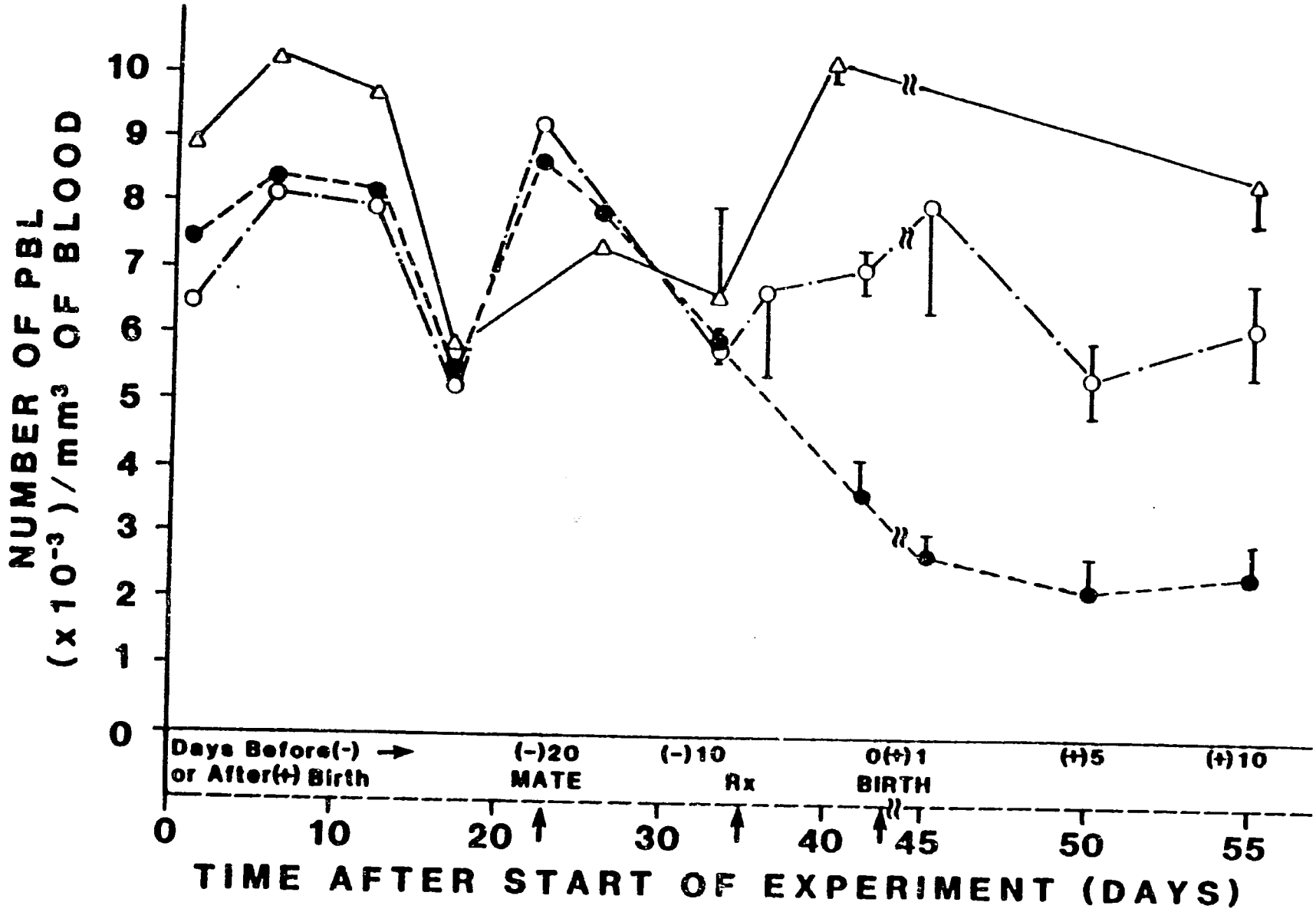
NUMBER OF PBL
(x 10^{-3})/mm^3 OF BLOOD
10
9
8
7
6
5
4
3
2
1
0
Days Before(-) →
or After(+) Birth
(-)20
MATE
(-)10
Rx
0(+)1
BIRTH
(+)5
(+)10
0
10
20
30
40
45
50
55
TIME AFTER START OF EXPERIMENT (DAYS)

Figure 1

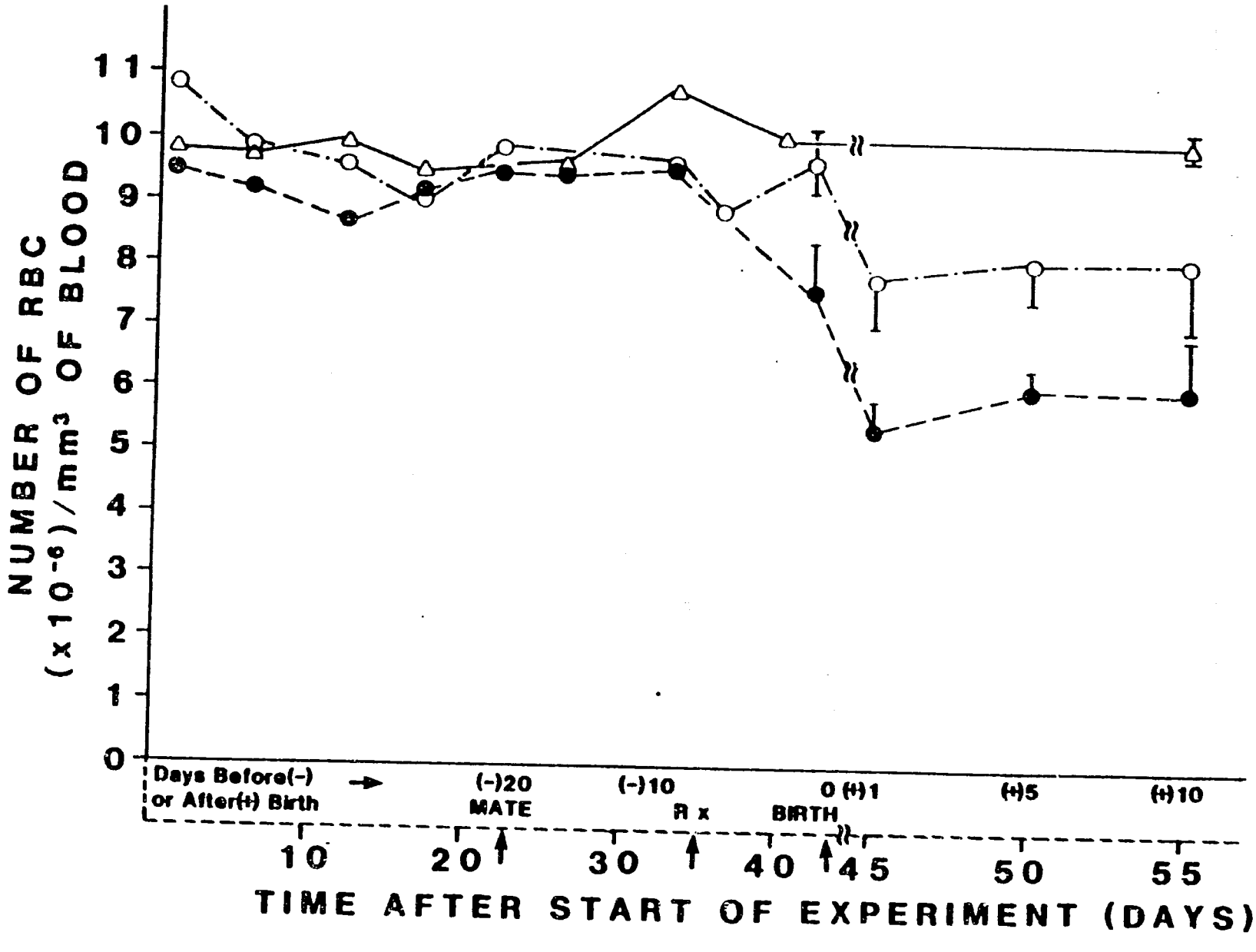
NUMBER OF RBC
(x 10⁻⁶)/mm³ OF BLOOD
0
1
2
3
4
5
6
7
8
9
10
11
Days Before(-)
or After(+) Birth
(-)20
MATE
(-)10
R x
0 (+)1
BIRTH
(+)5
(+)10
10
20
30
40
45
50
55
TIME AFTER START OF EXPERIMENT (DAYS)

FIGURE 2

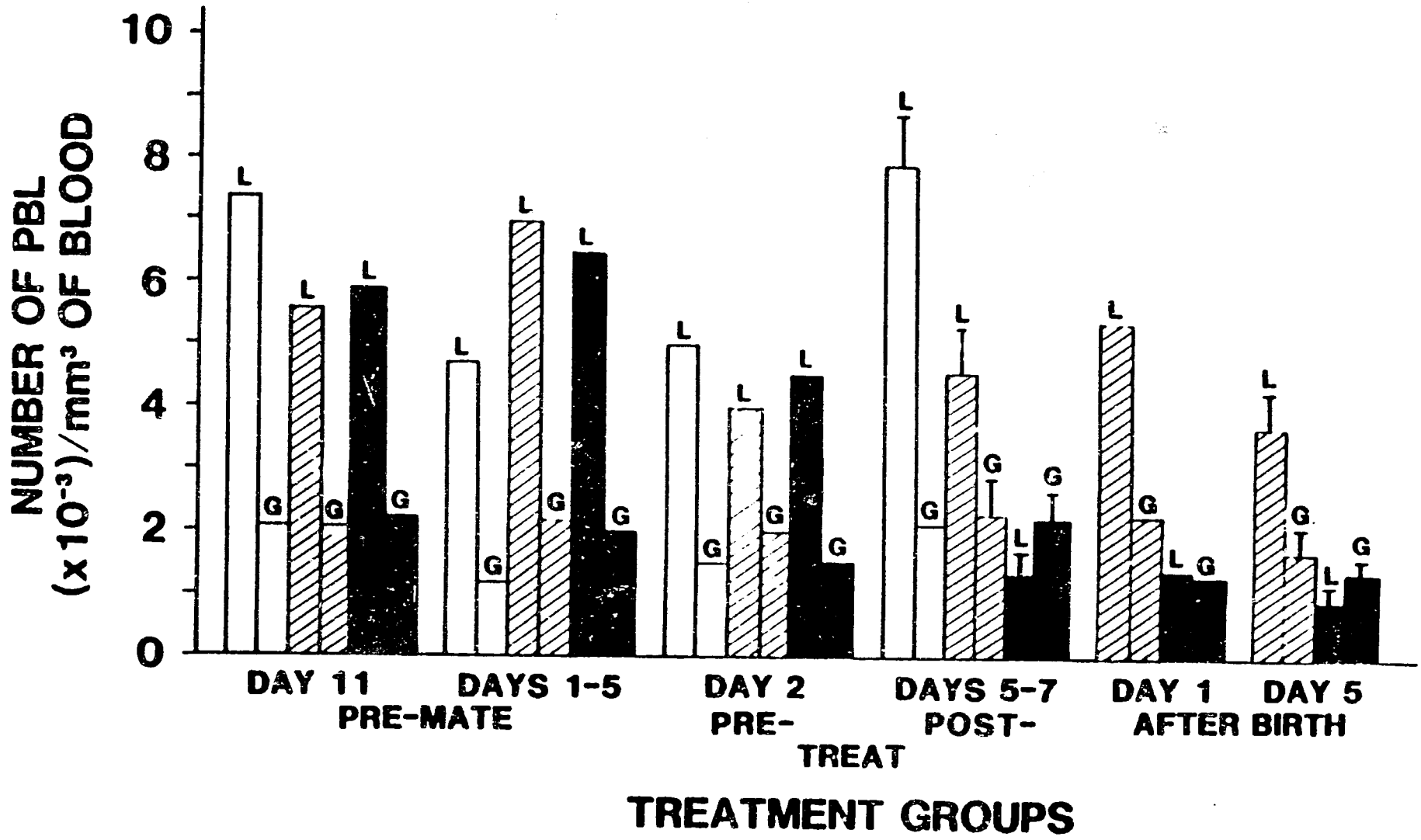
NUMBER OF PBL
(x10⁻³)/mm³ OF BLOOD
10
8
6
4
2
0
L
G
DAY 11
PRE-MATE
DAYS 1-5
DAY 2
PRE-
TREAT
DAYS 5-7
POST-
DAY 1
AFTER BIRTH
DAY 5
TREATMENT GROUPS

FIGURE 3

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