# Bone Marrow Restoration of Transplantation Immunity in the Leopard Frog *Rana pipiens*[1] (35062)

EDWIN L. COOPER[2] AND D. WILLIAM SCHAEFER[3]
(Introduced by Charles H. Sawyer)
*Department of Anatomy, School of Medicine, University of California, Los Angeles, California 90024*

In an anuran amphibian, the leopard frog, *Rana pipiens,* the thymus is necessary for restoring the morphological integrity of the spleen damaged by lethal doses of irradiation (1). A functional immune system could be restored at low doses (800 R) in the presence of thymus and bone marrow. The bone marrow was not apparently dependent upon the thymus for morphological or functional restoration at high doses (300 R). Studies of thymus–bone marrow–spleen interactions in mammals are usually based on results using young adults irradiated at relatively low dose levels. Poikilotherms, less commonly used vertebrates in comparative immunology, tolerate higher doses and the adult stages are usually not precisely equivalent to those of mammals. This study, an extension of our earlier one, confirms the independence of marrow at high doses of irradiation in refurbishing a damaged immune system in adult frogs.

*Materials and Methods. Animals.* Adult Wisconsin leopard frogs, *Rana pipiens,* were obtained from Brescia's Frog Farm, Compton 4, California, and placed initially in a 5% solution of Chloromycetin (Parke-Davis). They were kept in plastic containers in 1 in. of tap water at a temperature of 25 ± 1°. Ether was used as the anesthetic. They were fed meal worms 2 to 3 times each week.

*Blood cell counts.* Blood samples were taken from each frog approximately 3 times weekly at a site directly inferior to the tympanic membrane along the edge of the lower jaw. Blood smears were stained by the method of Wright and differential cell counts were determined.

*Irradiation.* Gamma irradiation was accomplished at a rate of 810 R/min with a total exposure of 5000 R by means of a 10,000 Ci cobalt-60 source in the Department of Nuclear Medicine at UCLA. Each group of frogs, including the nonirradiated (Group I) controls, consisted of 14 frogs. Group II received total body irradiation; for group III the right hind limb was shielded in the center of a block of lead which enclosed the extremity on all sides with 9 inches of protection and thus allowed less than 20 R exposure.

*Skin grafting.* The details of this technique have been described previously and will not be presented again (1). One of the two skin grafts from each animal served as an autograft, but allografts were exchanged between two frogs. Frogs were kept in individual translucent plastic containers for 5–7 days postgrafting to minimize interference with graft healing then transferred by groups to single covered plastic pans. Frogs were grafted 5 days postirradiation since the lymphocyte count in the peripheral blood drops to its lowest point by the seventh day after irradiation (2). Thus early healing of the graft occurred in the period corresponding to the lowest lymphyocyte level.

*Graft inspection.* The grafts were inspected every other day beginning 4 to 5 days postgrafting. The percentage of surviving melanocytes, a criterion for evaluating graft sur-

[1] Supported in part by funds from Grants Nos. E-492 and IN-3J of the American Cancer Society and Grant No. GB 17767 of the National Science Foundation.

[2] Guggenheim Fellow.

[3] The authors extend appreciation to Miss Helene Tournaire and Mrs. Ilse Spitz for their assistance in preparing the manuscript.

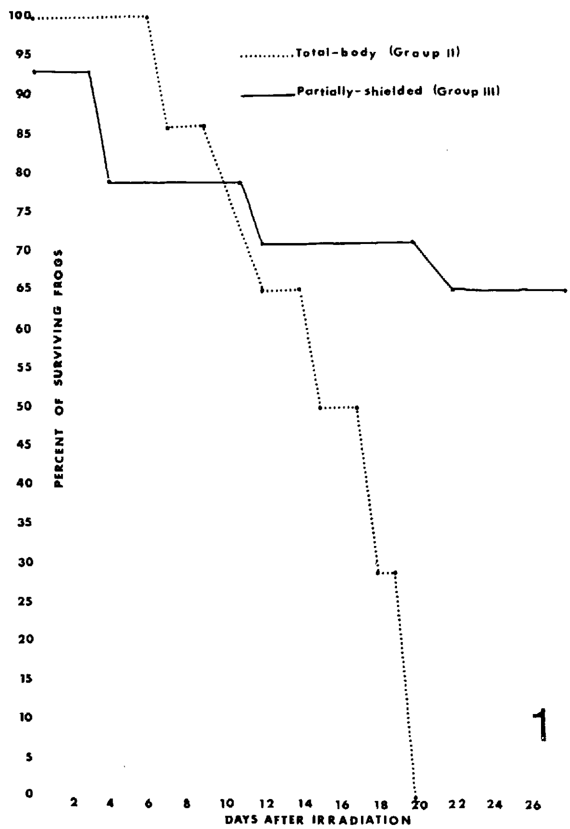


FIG. 1. Comparison of survival times of frogs receiving total body irradiation (5000 R) or with partial shielding.

vival, was approximated by periodic observation through a dissecting microscope. When no melanocytes remained allografts were considered totally rejected.

*Statistics.* Statistical treatment of the data was carried out according to the method of Litchfield (3, 4). From 4 to 14 frogs were used in computation of the means for blood cell percentages, depending upon the number available.

*Results. Survival of frogs.* Shielding of the right hind limb protected enough hemopoietic tissue to prolong survival time, to replenish the peripheral blood with lymphocytes, and to maintain factors responsible for skin allograft rejection (Fig. 1). Two of the three deaths occurring in the shielded group within 4 days postirradiation were perhaps due to infections. The third death was due to anesthesia, administered at the time of irradiation.

*Survival of autografts.* In only one case did an autograft fail to heal properly in a frog exposed to total-body irradiation. Otherwise autografts, self tissue, always healed and remaned permanently viable.

*Allografts. Control frogs—nonirradiated (Group I).* Grafts of control animals began to undergo rejection between the 3rd and 5th days. Thereafter, the percentage of surviving melanocytes rapidly decreased; the sharpest decline occurred between the 7th and 9th days until the last graft was totally rejected on the 20th day (Fig. 2). The MST of skin allografts in Group I was 14.25 days ± 1.5 (SD = 1.23).

*Allografts. Frogs exposed to total-body irradiation (Group II).* In frogs subjected to

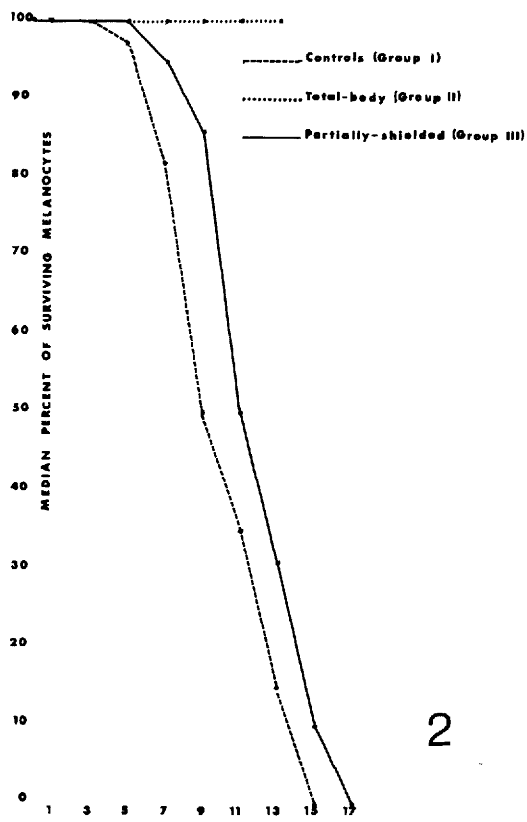


FIG. 2. Survival time of skin allografts in three groups of frogs. Note the parallel curves of control and partially shielded group.

TABLE I. Percentage of Leukocytes in Normal Frogs.

| Group | | Lymphocyte | Neutrophils | Basophils | Eosinophils | Monocytes |
|---|---|---|---|---|---|---|
| I | Controls | 80.6 | 15.5 | 1.6 | 1.1 | 1.2 |
| II | Prior to irradiation | 78.6 | 15.4 | 2.3 | 1.6 | 2.1 |
| III | Prior to irradiation | 77.7 | 16.4 | 2.2 | 2.4 | 1.3 |

total-body irradiation, graft survival was on the 15th day after grafting (20th day 100% at the time of death; the last frog died after irradiation). In this group, the initial death occurred 7 days after irradiation, and the MST of the animals was 15 days ± 2.3 (SD = 1.41).

*Allografts. Frogs exposed to total-body irradiation with partial bone marrow shielding (Group III).* Graft rejection (Fig. 1) occurred as in control animals, but with a delay of 2 days; this difference was not significant at the 19/20 probability level. For example, the median percent of surviving melanocytes in the control animals reached 50 on the 9th day; equivalent rejection occurred on the 11th day in the shielded group. Melanocytes declined sharply in the shielded group between the 9th and 11h days postgrafting (14th and 16th days postirradiation), Fig. 2. The MST of skin allografts in Group III was 16.8 days ± 1.2 (SD = 1.14).

*Peripheral blood. Control frogs—nonirradiated (Group I included).* Control numbers were obtained not only from control Group I, but also from the preirradiation differential cell counts taken from Groups II and III (Table I).

*Peripheral blood. Total-body irradiation (Group II).* Some hematologic effects of total-body irradiation of 5000 R are shown in Figs. 3, 4, and 5. Lymphocytes were the most vulnerable of all the leukocytes as indicated by the drop in mean percentage within the first 24 hr after irradiation. This value continued to decline, with some fluctuation, to the 14th day, at which time a slight recovery began. The decline was accompanied by a sharp increase in the mean percentage of neutrophils, a value which may not have necessarily represented an increase in the absolute number, but merely an increase of the neutrophil percentage relative to other leukocytes. The relative mean percentages of basophils, eosinophils, and monocytes are shown in Fig. 5. No remarkable effects of irradiation on these cells were apparent and all of the percentages fluctuated within a range of 0 to 6%. The separation of total lymphocyte types into small, medium, and large revealed no important influence of irradiation on percentages of these cells within the lymphocyte samples. Quantities of small lymphocytes remained greater than medium and large lymphocytes throughout the period of investigation.

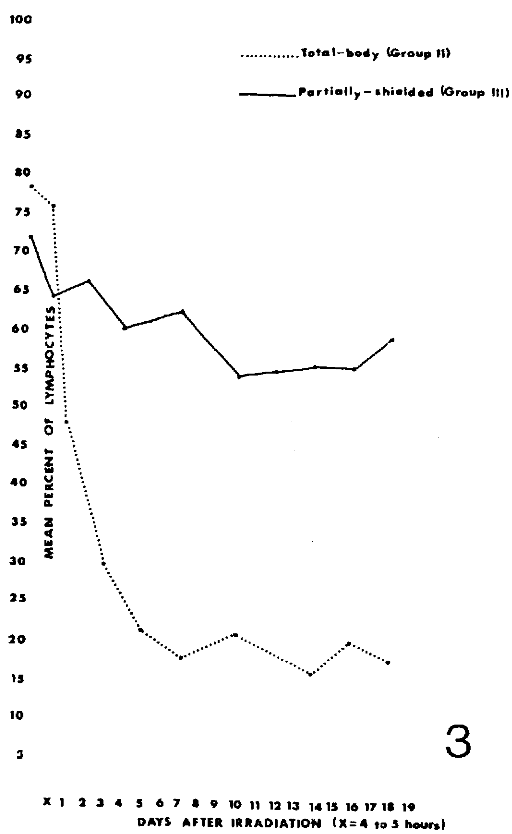


FIG. 3. Curves show difference in mean percent of peripheral blood lymphocytes in both the total-body irradiated and partially shielded group.

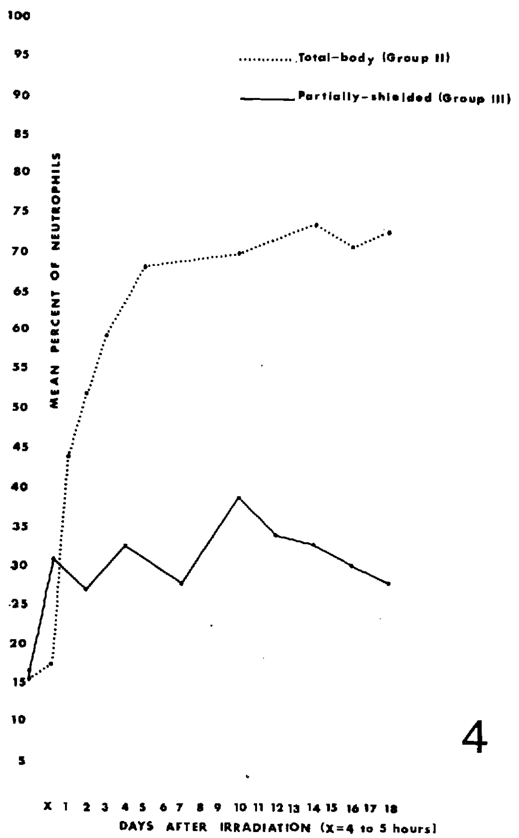


FIG. 4. Neutrophils increased in the total-body irradiated group when compared with the partially shielded group.

*Peripheral blood. Frogs irradiated with partial bone marrow shielding (Group III).* Shielding of the right hind limb was effective in preventing the precipitous decline in the percentage of circulating lymphocytes, although the number stayed below normal control counts (Fig. 3). However, the mean neutrophil percentage increased (Fig. 4). As was observed in animals irradiated without shielding, no remarkable effects were seen in the mean percentages of basophils, eosinophils, and monocytes (Fig. 5).

*Symptoms of irradiation sickness. Total-body irradiated group (Group II).* All frogs ate satisfactorily prior to irradiation, but during the first few days later, most of them began to refuse food. Force-feeding was then instituted and continued until the day of death. By approximately the 10th day postirradiation discrete and diffuse reddened areas appeared on the skin; the most prominent lesions were seen on the pectoral region, the abdomen, the pelvic region, and the medial aspects of the hind limbs. Many frogs showed tonic and clonic spasms of musculature or generalized convulsions just prior to death. They died in a supine position with the limbs extended and rigid. When blood samples were taken after the first 4 or 5 days postirradiation, experimental frogs bled from the puncture wound longer than controls, in which clotting occurred within a few moments after puncture. Three frogs showed generalized edema at 6 and 8 days postirradiation, which increased markedly until death. Control frogs never showed edema.

*Partially-irradiated group (Group III).* In this group the same change in eating habits was observed as in the total-body irradiated group except that the shielded animals no longer required forced feedings after approx-

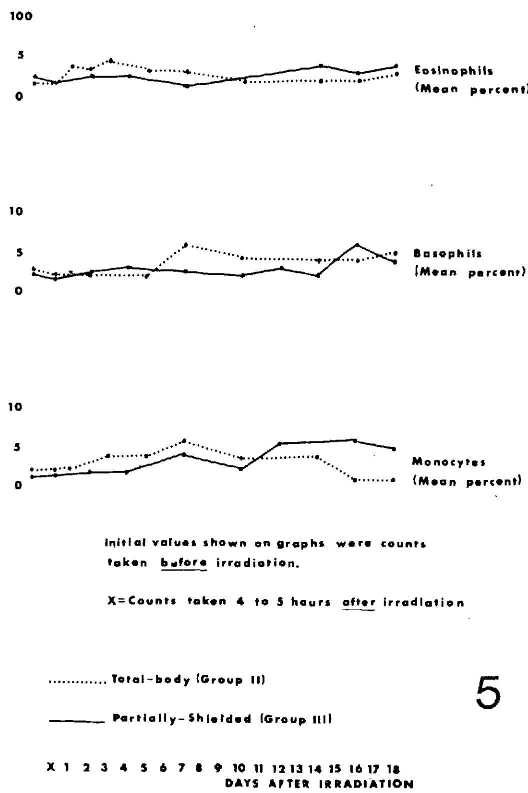


FIG. 5. Differences were less obvious in the eosinophil, basophil, and monocyte counts.

imately the 2nd week postirradiation. Only 1 frog showed skin lesions and some edema. We observed no remarkable difference in the clotting time from puncture wounds between the shielded and control groups.

*Infections. Rana pipiens* is host to many organisms but is able to live with some without untoward effects. Protozoa, bacteria, and helminths in wild populations of frogs are common. In the present investigation, none of the animals showed any gross signs of infection at the time the experiment was begun. Later, however, *Trypanosoma sp.* and the sporazoan, *Haemogregarina sp.*, were found in the blood of a few animals.

*Discussion.* Bone marrow is important in restoring a damaged immune system. Shielding of the right hind limb of *Rana pipiens* apparently protected enough bone marrow for some replenishment of peripheral blood cells. When frogs were irradiated without shielding at 5000 R, deaths occurred earlier than in the partially shielded and control groups, but allograft rejection was prevented. These results are essentially in agreement with the findings of Cooper (6), who concluded that X-irradiation of the killifish *Fundulus heteroclitus* at 2000 and 3000 R produced longer graft survival times concomitant with an increased number of deaths when compared with controls.

The mean lymphocyte percentage declined sharply, a value less apparent in the partially-shielded group, which rejected allografts almost as rapidly (within 2 days) as the controls. Most of the lymphocytes were undoubtedly derived from bone marrow, and are crucial in the alloimmune response to tissue antigens. Hildemann and Haas (7) observed allograft tolerance in normal bullfrog larvae as old as 36 days, and found a transition from tolerance to immune competence at approximately 40–60 days posthatching. Small lymphocytes were absent during the tolerant state but increased about 10-fold during the important period of 40–50 days of age.

Changes in the percentages of peripheral blood lymphocytes and neutrophils generally agreed with observations of Stearner (3) and Sturges and Levin (8). In the present investigation slight recovery of lymphocyte percentages occurred later since our dose of irradiation was considerably higher. In the Sturges and Levin experiment, frogs were irradiated for 45 min with a Coolidge tube, 7 mA, 9-in. spark gap, and a 5-in. focal distance; he gave no roentgen value. Stearner administered only 600 R to her frogs.

In any investigation using noninbred animals, factors such as sex, temperature, and genetic constitution should be considered. Eichwald and Silmser (9) demonstrated in mice that isografts exchanged between sexes were rejected. We therefore used equal numbers of males and females in each group, and allografts were exchanged only between members of the same sex. A definite correlation between temperature and survival of *Rana pipiens* after irradiation was established by Patt and Swift (10). Frogs maintained at 5° after X-irradiation, did not die following dosages as high as 6000 R but did when removal from the cold environment. Temperature in the present study was maintained constantly at 25 ± 1°, and whatever variations occurred were encountered by all animals. When a field population is used, genetic constitution of individuals cannot be controlled; all animals, however, including an appropriate number of controls, were obtained from the same stock. Future studies of amphibians should be better controlled later due to the pioneer efforts of Nace in developing defined strains (11).

The general effects of total-body irradiation in the leopard frog revealed some interesting similarities to those found in man (12 13). The two typical forms of the acute radiation syndrome in man are gastrointestinal and hemopoietic. Frogs in Group III refused to eat voluntarily for a period of approximately 2 weeks after irradiation, and those in Group II required force-feeding throughout the post irradiation period. All frogs ate well prior to irradiation. The hematologic effects resulted from damage to immature cells in the bone marrow and to lymphocytes in other sites. However, shielding the right hind limb protected enough bone marrow to maintain life during the critical early postirradiation period; the long-range effects would only result from prolonged follow-up investigations, perhaps for years.

Irradiation abrogated the allograft reaction until death of all frogs. Partial shielding resulted in allograft survival almost equivalent to controls. Splenectomy does not affect the alloimmune response in *Fundulus heteroclitus* (Goss, 14) and *Rana pipiens* (Vogel, 15). This is in agreement with the present observation that the bone marrow probably served to replenish a damaged or incompetent system which included the spleen at lower dose levels (1).

*Summary and Conclusions.* The effects of cobalt-60 gamma irradiation at 5000 R on the survival of skin allografts in adult *Rana pipiens* (leopard frog) were observed with the changes in the peripheral blood at 25 ± 1°. Graft survival was assessed by estimating the survival of melanocytes. Autografts healed and survived permanently. In nonirradiated control animals, median survival time (MST) of allografts was 14.25 days ± 1.5. In the group receiving total-body irradiation no graft rejection was observed at the time the frogs died, at the earliest on the 7th day postgrafting and the latest on the 20h day. By contrast, skin allografts in the partially irradiated and shielded group were rejected almost as rapidly (within 2 days), as those observed in unirradiated controls. The mean percent of lymphocytes in the peripheral blood declined sharply, but less in the partially shielded group. Little change was noted in the percentage of basophils, eosinophils, or monocytes but neutrophils seemed to increase. Bone marrow was sufficient to maintain the alloimmune mechanism and life itself.

1. Mandell, M. L., and Cooper, E. L., Transplantation, 1970, in press.

2. Globerson, A., and Feldman, M., Transplantation **2**, 212 (1964).

3. Stearner, P., J. Exp. Zool. **115**, 251 (1950).

4. Litchfield, J. T., J. Pharmacol. Exp. Ther. **97**, 399 (1949).

5. Litchfield, J. T., and Wilcoxon, F., J. Pharmacol. Exp. Ther. **96**, 113 (1949).

6. Cooper, E. L., Transplantation **3**, 446 (1964).

7. Hildemann, W. H., and Haas, R., J. Immunol. **83**, 478 (1959).

8. Sturges, M. M., and Levin, L., Proc. Soc. Exp. Biol. Med. **18**, 295 (1920).

9. Eichwald, E. J., and Silmser, C. R., Transplant. Bull. **2**, 148 (1955).

10. Patt, H. M., and Swift, M. N., Amer. J. Physiol. **155**, 388 (1948).

11. Nace, G. W., and Richards, C. M., "Biology of Amphibian Tumors." Springer-Verlag, New York (1968).

12. Miale, J. B., "Laboratory Medicine-Hematology," p. 630. Mosby, St. Louis (1962).

13. Davidson, H. O., "Biological Effects of Whole-Body Gamma Radiation on Human Beings." Johns Hopkins Press, Baltimore (1957).

14. Goss, R. J., Biol. Bull. **121**, 162 (1961).

15. Vogel, H. H., Jr., J. Exp. Zool. **85**, 437 (1940).

Received June 10, 1970. P.S.E.B.M., 1970, Vol. 135.