and both groups were markedly inferior to the non-shocked controls. Thyroid uptake measurements indicated that the basal metabolism of electrically shocked rats was increased over that of non-shocked animals, but no statistically significant differences existed between gentled and non-gentled groups.

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Circulating Rat Cells in Lethally Irradiated Mice Protected with Rat Bone Marrow.* (22421)

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Murphy(1) established that (a) embryos lacking ability to resist growth of heterologous tissue could be rendered as refractory as adults by introduction of an isologous adult spleen or bone marrow and (b) adults, capable of resisting growth of heterologous tissue, could be rendered as receptive as embryos by pretreatment with sublethal total-body X irradiation. Rapidly growing tumor tissues were used as donor material. Over 30 years later, Owen(2) demonstrated circulating erythrocytes genetically and phenotypically distinct from the animal's own, which arose from embryonic erythrocytopoietic transplants of the co-twin through placental anastomosis. Successful homologous and heterologous tissue and protein acceptance by embryos and X-irradiated adults followed by a prolonged or even lasting period of tolerance to these agents has been reported(3-8). Lindsley *et al.*(9) demonstrated erythrocyte mosaicism in sublethally X-irradiated rats that were subsequently injected with homologous rat bone marrow.

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Previous work(10) in this laboratory showed that C_3H x $101F_1$ mice receiving 950 r of total-body X radiation protected against radiation death by a single intravenous injection of an isologous bone marrow suspension responded relatively well to either rat or sheep RBC antigens 30 days later. However, lethally X-irradiated mice protected by an injection of heterologous bone marrow (Sprague-Dawley rats) failed to respond to rat RBC antigen and responded only very weakly to sheep RBC antigen 30 days later. The establishment of the degree of transplantation of rat hematopoietic tissue became necessarily important in an attempt to interpret the immune response of these heterologously protected mice.

Materials and methods. Normal 12-week-old C_3H x $101F_1$ mice, equally divided in sex, received 950 r and were subsequently protected with heterologous bone marrow suspension. Three types of control mice were run simultaneously: (a) nonirradiated normal, (b) irradiated (950 r) and protected with isologous bone marrow suspension, and (c) nonirradiated but injected with heterologous bone marrow suspension. The irradia-

tion conditions were: 250 kvp, 30 ma, 3 mm Al filter, at 80 cm, ~ 100 r/min. The bone marrow suspension, made to 1 ml in Tyrode solution, was injected intravenously within 4 hours after X irradiation. Heterologous rat bone marrow suspension was obtained from 1 femur and 1 humerus, and the isologous bone marrow from 1 femur. Thirty-four mice were used. The 6 controls and 8 heterologously protected mice were individually caged, body weights recorded, and blood samples from the tail vein taken at various intervals. The remaining heterologously protected mice were killed at intervals for serial study. The quantitative cell count method for an assay of isohemagglutinin reported by Wilkie and Becker(11) was modified to determine the RBC population of blood samples. Pooled mouse anti-rat RBC serum (titer, 128-256) and pooled rat anti-mouse RBC serum (titer, 512-1024) were heated at 56°C for 30 minutes. Blood samples (3-4 drops) taken from the tail vein of mice were collected in a solution containing a 1:3 mixture of 0.1 M versene (pH 6-7) and 0.85% NaCl. After washing one volume of packed cells 3 times with 200-300 volumes of saline, a 1-2% cell suspension was prepared in 0.85% NaCl. To four 10 x 75 mm test tubes, 1 volume (0.1 ml) of the well-mixed cell suspension was added; and (a) 1 volume of normal pooled mouse serum (tube A), (b) pooled mouse anti-rat RBC serum (tube B), (c) pooled rat anti-mouse RBC serum (tube C), and (d) a 1:1 mixture of the 2 antisera (tube D) was added to each of these tubes respectively. The tubes were capped with parafilm, shaken, incubated at room temperature for 10 minutes, shaken, centrifuged at 1000 r.p.m. for 1 minute, and the degree of agglutination determined by gently shaking the RBC "pellet" from the bottom of the test tubes. The tubes were then placed in a Kahn shaker set at 116 oscillations per minute for 20-30 minutes. The free RBC count was made of an aliquot from each tube with Hayem's solution as the diluent. It was decided to use both anti-rat and anti-mouse RBC sera to check the results of one against the other. Furthermore, use of both antisera should permit detection of the presence of possible "sensitized RBC" analogous to the "sensitized" Rh-positive cells, provided that the normal range of free cells in tube D is known. In theory, there should be no free cells in tube D, but in practice 5% may become free under the experimental condition, owing to the weak binding of cell antibodies and the physical shaking of test tubes. The following formulas were employed: (a) % cell recovery $= \frac{B + C - 2D}{A}$ x 100, (b) % rat RBC $= \frac{A + C - B}{2A}$ x 100, and (c) % mouse RBC $= \frac{A - C + B}{2A}$ x 100. The mean recovery of total RBC based on over 90 determinations was 96%. The method of albumin suspension of cells(12), the method of enzyme-treated cells(13), and the anti-gamma globulin method(14) were employed to test for the presence of possible "sensitized" red blood cells.

While this work was in progress, attention was called to the USNRDL report of Nowell *et al.*(15) who showed that, histochemically, rat granulocytes gave positive alkaline phosphatase reaction unlike the mouse granulocytes. They demonstrated by this method the presence of alkaline phosphatase positive cells in lethally X-irradiated mice protected with rat bone marrow suspension. This test was undertaken by employing the method of Rabinovitch and Andreucci(16). A comprehensive report on the double serum-agar diffusion method has been published by Oudin (17), Jennings(18), and Wilson and Pringle (19). Serum samples obtained on the 6th, 13th, 17th, 19th, 27th, 39th, and 72nd days were tested simultaneously by this method against rabbit anti-rat serum with an interfacial titer of 4^9 and against rabbit anti-mouse serum with an interfacial titer of 4^{10}. Mouse and rat sera mixed in proportions of 1:0, 3:1, 2:1, 1:1, 1:2, 1:3, and 0:1 were tested simultaneously against both antisera. These served as positive controls.

Results. None of the individually caged lethally X-irradiated and heterologously protected mice died during the first 30 days. On

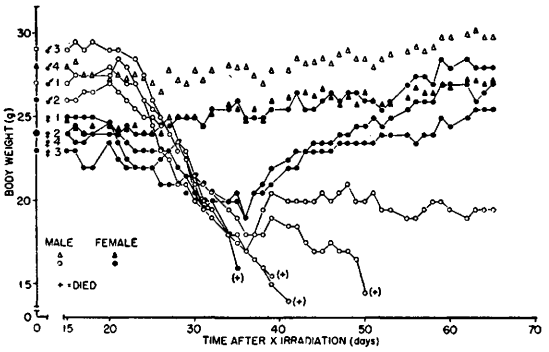

FIG. 1. Effect of rat bone marrow on body wt of lethally X-irradiated (950 r) mice. Control (triangles): avg of nonirradiated mice, irradiated injected with rat bone marrow, and irradiated injected with mouse bone marrow. Experimentals (circles): irradiated mice injected with rat bone marrow.

the 14th day, the heterologously protected mice could not be differentiated from the three types of control mice by gross inspection, and this was also borne out in the distribution of the body weight as shown in Fig. 1. The primary weight loss that occurred about a week after X irradiation was rapidly regained within the following week (heterologously protected males, 26.0-29.0 g; females, 23.0-25 g; control males, 27.0-29.5 g and females, 24.0-25.5 g). Starting on about the 21st day, the heterologously protected mice, except in the case of 1 female, began losing weight precipitously, reaching a minimum on about the 36th day. During this period abundant fecal deposit was observed in

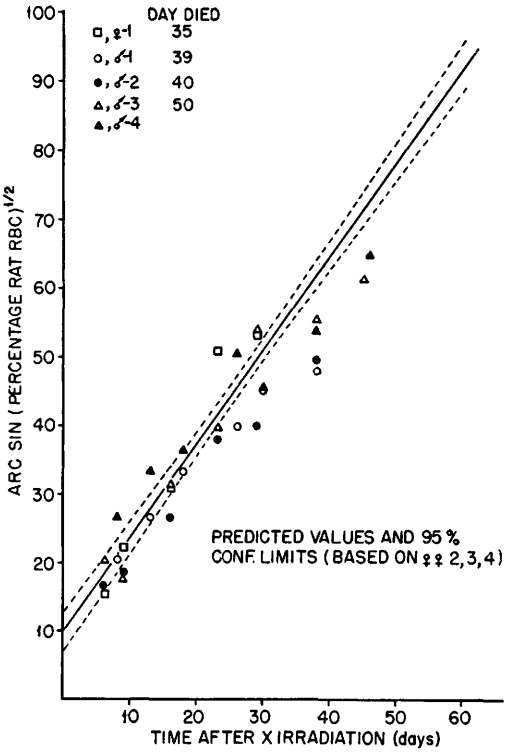

FIG. 3. Rate of percentage rat RBC increase ($y = 10.496 + 1.4027x$).

the cages of these animals. Two of the 4 survivors gradually recovered from the 36th day; one of the 4 showed only partial recovery; the fourth showed no ill effect throughout the 72 days' observation.

The results of the immunohematological study are presented in Fig. 2. The appear-

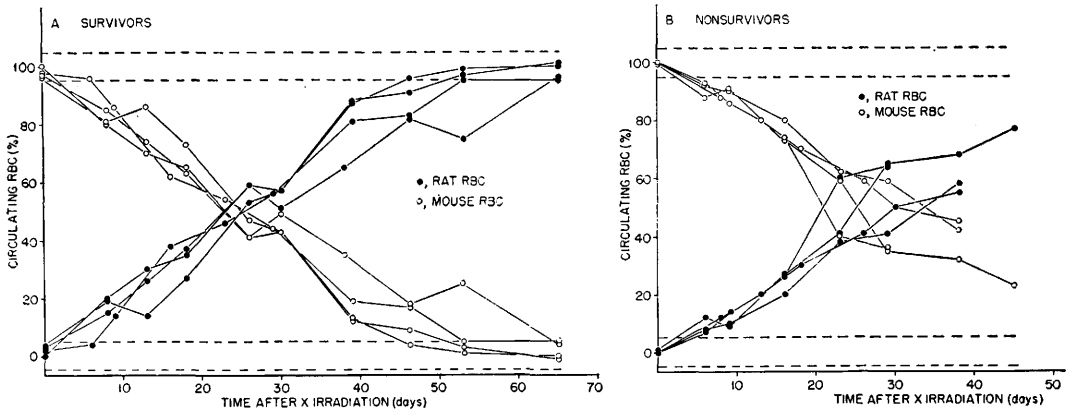

FIG. 2. Appearance of circulating rat RBC in lethally X-irradiated (950 r) mice injected with rat bone marrow. Control: avg of nonirradiated mice, nonirradiated injected with rat bone marrow, and irradiated injected with mouse bone marrow.

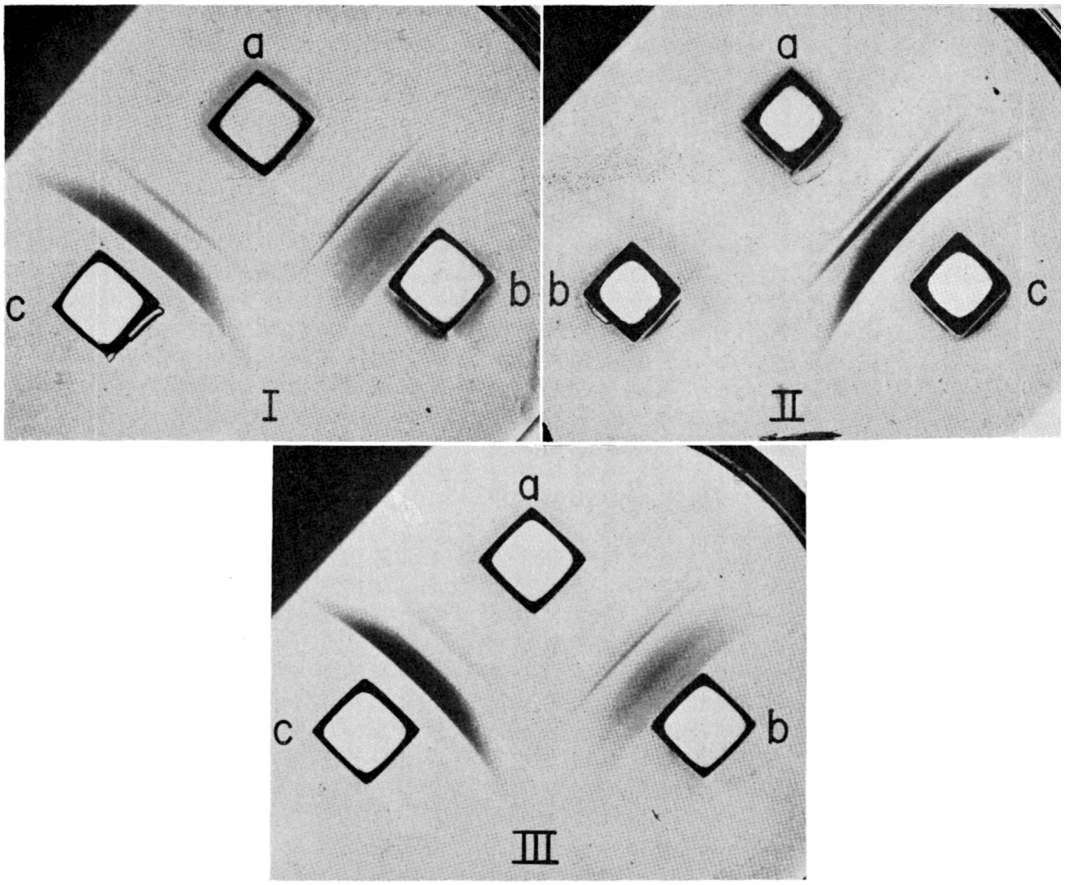

FIG. 4. Serum-agar diffusion pattern after 7 days' incubation. I: a, Normal mouse serum; b, anti-mouse serum; c, anti-rat serum. II: a, Normal rat serum; b, anti-mouse serum; c, anti-rat serum. III: a, Mouse serum 72 days after X-irradiation and rat bone marrow injection; b, anti-mouse serum; c, anti-rat serum.

ance of rat RBC in the circulation became apparent about a week after X irradiation and injection of rat bone marrow. A gradual increase in percentage of rat RBC can be seen in all 8 mice. On about the 25th day, the 50% level was attained. The 95-100% level in rat RBC was approached shortly after the 50th day in the 3 surviving healthy mice, and this level was maintained on the 65th day. With a marked loss in body weight, there was a concomitant decreased rise in percentage of rat RBC. This relation between the body weight change and percentage of rat RBC was evident in comparing the percentage of rat RBC of the 4 mice that died and the surviving male, which did not fully recover, with that of the 3 fully recovered survivors (Fig. 3). No circulating rat RBC could be detected in the control mice throughout the experiment.

Histochemical alkaline phosphatase tests were performed on smears obtained on the 45th and 63rd days. Blood smears from all the heterologously protected mice were positive; those from all the controls were negative. No difference between smears of the normal rats and these heterologously protected mice could be observed. Differential WBC count on smears obtained on the 63rd day showed a relative decrease in the agranulocytic count† from an average of 76% in the controls to 44% in the heterologously protected survivors.

Rat proteins could not be detected by the

† The term "agranulocytic cells" includes lymphocyte, monocyte, and plasmacyte.

double serum-agar diffusion method in serum samples obtained from the 6th to the 72nd days. A typical result is shown in Fig. 4. On the 72nd day, serum samples included the control mice and the 4 surviving heterologously protected mice.

All tests for the presence of "sensitized" RBC were negative. A weakly positive reaction was observed with the trypsin method, but control trypsin tested rat RBC gave a similar result.

Autopsies performed on the 4 mice that died showed no gross evidence of infection or hemorrhage. The most pronounced changes were extreme weight loss and atrophy of lymphatic tissue. The bone marrow in two animals examined microscopically showed about 75% of the normal cellularity in the femoral bone shaft and sternum.

Discussion. It has been shown that approximately 50% of the mice receiving lethal X irradiation and a subsequent intravenous injection of rat bone marrow died in 30 days (10). There were 5-10 mice in each cage. Others(20,21) have obtained similar results since Congdon and Lorenz(22) first demonstrated the protective ability of heterologous bone marrow. Results presented here show that none of these heterologously protected mice died during this period when they were individually caged. In 2 weeks, recovery from the critical X-irradiation effect occurred, as expressed by their gross appearance and body weight, but 1 week later, these mice began losing weight and continued to do so to the 5th and 6th weeks. Associated with the loss in weight was observed an abundant daily firm fecal deposit indicating normal or greater than normal food intake. During this critical period after the 4th week, 3 of 4 males and 1 of 4 females died. A period of general recovery then ensued. Surviving females appeared to be fully recovered by the 8th week and could not be differentiated from the controls. The surviving male, however, did not fully regain its original body weight.

Quantitative immunohematological studies showed that in 8 of 8 mice, transplantation of rat erythrocytopoietic cells took place. A week after X irradiation and a subsequent injection of rat bone marrow, rat RBC first appeared in the circulation and by the 25th day contributed 50% of the circulating RBC. On the 65th day in 4 of 4 surviving mice, 100% rat RBC was present in the circulation, and this level was already approached on the 53rd day in the 3 healthy females.

That rat granulocytopoietic cells also transplanted was shown by the positive alkaline phosphatase test performed on blood smears obtained on the 45th and 63rd days. A similar result was obtained by Nowell *et al.* (15). Differential WBC count on blood smears obtained on the 63rd day showed a relative decrease in the agranulocytic cells. Double serum-agar diffusion tests on serum samples showed no rat serum proteins. The presence of the rat gamma globulin would probably have been indicative of transplantation of plasmacytopoietic cells, which are also found in the bone marrow. It has been demonstrated by many workers that plasmacytic cells are involved in the production of antibody, a modified gamma globulin(23-27).

It can be concluded that injection of rat bone marrow to lethally X-irradiated mice permitted transplantation of erythrocytopoietic and granulocytopoietic cells as expressed in the appearance of the rat RBC and granulocytes in the circulation. The failure of transplantation of rat agranulocytopoietic cells is suggested indirectly by the absence of rat serum globulin and the relative decrease in the agranulocytic count of blood smear. Additional evidence for this concept will depend on the establishment that the host cells, rather than the donor cells, are the antibody producers. The failure to respond to rat RBC antigen by these mice(10) is caused by a complete replacement of the RBC production by the transplanted rat erythrocytopoietic cells. The sequential correlation of (a) loss in weight, (b) death-time relation, (c) immune response to rat and sheep RBC antigens, and (d) the activity of transplanted cells strongly suggests that the delayed death pattern is caused by an *in vivo* antigen-antibody reaction. A report on the delayed death pattern of these mice undertaken by Congdon of this Laboratory will follow(28).

Summary. 1. Mice receiving 950 r can be protected from the 30-day irradiation death by injecting rat bone marrow and caging them individually. 2. Transplantation of rat erythrocytopoietic and granulocytopoietic cells has been demonstrated by the appearance of rat RBC and granulocytes in the circulation. On the 65th day, 100% rat RBC was present in the circulation of all the surviving heterologously protected mice. 3. Rat serum proteins could not be detected in these mice by the serum-agar method.

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