The Effect of the Amount of Isologous Bone Marrow Injected on the Recovery of Hematopoietic Organs, Survival and Body Weight after Lethal Irradiation Injury in Mice

By Paul Urso and C. C. Congdon

SURVIVAL of lethally irradiated animals and the thymus weight in sublethally irradiated mice were shown to be correlated with the amount of blood-forming tissue shielded or injected.[1-5] In the present report, the relation between the amount of isologous bone marrow injected intravenously and several criteria of recovery was determined in mice exposed to 900 r total-body x radiation. These criteria included 30-day survival; quantitative bone marrow response; peripheral blood leukocyte count; thymus, spleen, and body weights; appearance of the animals; and the histology of hematopoietic tissues.

Materials and Methods

Male and female BALB/c × $AF_1(CAF_1)$ mice about 3-4 months old were used in these experiments. They were housed ten to a cage, in which food and water were available *ad libitum*. The mice were weighed for 2 or 3 consecutive days prior to irradiation.

Ten mice were irradiated at each exposure in a partitioned rotating plastic drum 80 cm. beneath the x-ray target. The radiation factors were: 250 kvp; 3 mm. of Al added filtration; hvl, 0.55 mm. of Cu; dose rate in air, ~95 r/minute.

Within 4–6 hours after irradiation, experimental mice were injected intravenously with 0.5–1.3 ml. of a suspension containing measured amounts of bone marrow obtained from femurs of normal CAF_1 mice. The ends of a femur were sawed off and the bone marrow plug was pushed into sterile Tyrodes solution. The plug was flushed through a 26-gauge needle two or three times to separate the cells. Preliminary measurements showed that, on the average, the marrow from one normal mouse femur contained 12.5×10^6 nucleated cells. Therefore, further dilution of the bone marrow suspension with Tyrodes solution yielded a preparation in which the approximate number of cells was known. A more precise determination could be made if aliquots of the suspension were stained with Giemsa or methylene blue and the cells counted in a hemocytometer. Aliquots were preserved by formaldehyde.

In each experiment, one irradiated bone marrow-injected mouse and one irradiated uninjected mouse were sacrificed daily to determine the parameters reported in the results. Similarly treated animals were maintained for survival studies.

The quantitative bone marrow response was determined by a technique that gave the total number of nucleated bone marrow cells in an 8-mm. segment of the shaft of a femur. The ends were sawed off the shaft and the bone marrow was pushed out with a needle and syringe containing 0.6 ml. of Tyrodes solution with 1.5 per cent formaldehyde. Since it was difficult to separate the cells from one another in the bone marrow of irradiated mice, the marrow plug was flushed through a 27-gauge needle 4–6 times. Immediately after the last flushing, some of the suspension was drawn into small capillary tubes for volume (microhematocrit method) determinations; the remainder was diluted in Tyrodes-formaldehyde solution and stained with either Giemsa or methylene blue. When the total number of bone

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marrow cells was expected to be very low, the dilution was usually no higher than 5 ml; but when the count was expected to reach a million or more cells, the dilution was increased to at least 10 ml. During the shaking, an aliquot was taken from the stained preparation and was counted in a hemocytometer. The nucleated cells found in four of the large squares on either side of the chamber were counted, and a calculation of the total number of nucleated cells per femur was made. Since the counting technic is similar to the one used by Dews et al.[6], the calculation of the errors involved in the technic can be applied to this study. The number of red blood cells in the suspension from the bone marrow plug were also determined.

Additional data taken for further correlation with bone marrow cell dose included general appearance, peripheral blood leukocyte count, body weight at the time of sacrifice, and thymus and spleen weights of the mice. The left femoral bone shaft and other hematopoietic tissues were fixed in Zenker-formalin for histologic study. Similar data were obtained for normal animals.

Results

Eight different doses of bone marrow ranging from 0.007×10^6 to 237.9×10^6 nucleated cells were used to determine the dose response relation after 900 r of total-body irradiation. An increase in *30 day survival* occurred with an increase in the amount of bone marrow received (fig. 1 and table 1). None of the mice injected with minimal amounts of bone marrow survived for 30 days, whereas 100% survival was obtained with massive doses. Statistical analysis of this data

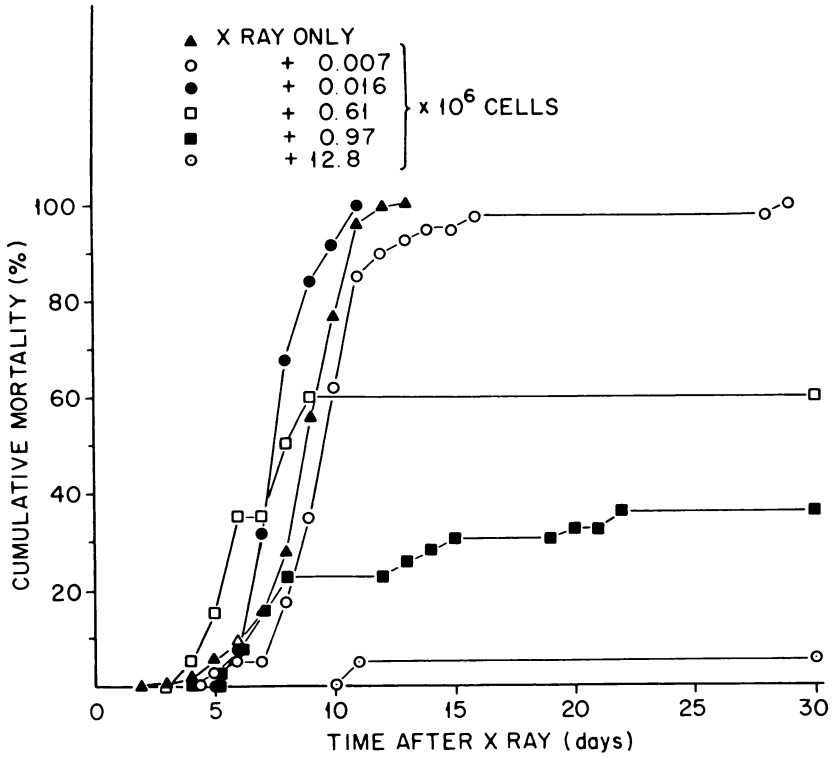

FIG. 1. The effect of bone marrow cell dose on cumulative mortality of irradiated (900 r) mice. Nucleated marrow cells were given intravenously immediately after irradiation.

TABLE 1.—*Effect of Bone Marrow Dose on Survival of Irradiated* (900 r) CAF_1 *Mice*

Expt.	Estimated No. of Cells Injected Per Mouse ($\times 10^6$)	Males			Females		
		Number of Mice	Survival %	Mean Day of Death	Number of Mice	Survival %	Mean Day of Death
1	237.9	2	100		3	100	
2	98.2	8	100				
3	64.4				10	100	
4	12.8	10	100		10	90	11.0
5	0.97	20	75	12.8	19	53	9.1
6	0.61	10	50	8.4	10	30	5.4
7	0.016	12	0	7.5	13	0	8.7
8	0.007	20	0	11.0	20	0	9.9
9	0	97	0	9.3	91	0	8.8

indicated that 0.42×10^6 (0.17×10^6, 0.92×10^6)* cells in the males and 1.06×10^6 (0.42×10^6, 2.5×10^6) cells in the females was sufficient to give 50% survival for 30 days. There was no statistically significant sex difference in survival although the implication was that males are able to recover with somewhat lower doses.

The effect of varying the amount of bone marrow injected on the *bone marrow response* in the 8 mm. segment of the right femoral bone shaft of the injected animal is illustrated in figure 2. Each point on the curve for irradiated uninjected mice represents 2–8 mice. Points on all other curves represent one or two mice. The graph shows a significant variation in bone marrow response with the amount of bone marrow injected. All irradiated animals receiving bone marrow showed a decrease in nucleated bone marrow cells that, except for one experiment, followed temporarily the cytopenia observed in the irradiated controls. The time at which bone marrow reduction stopped depended on the amount of bone marrow injected. With the lowest doses (0.007×10^6 cells to 0.016×10^6 cells), reduction continued to follow x-ray control levels until 8–13 days, at which time slight recovery was observed. Mice receiving 0.61×10^6 to 0.97×10^6 bone marrow cells began to show an increase in bone marrow counts on the fifth or sixth day and reached the highest count at 14 days. For the next 5 days, the count remained slightly below normal in experiment 5 (table 1). A count taken on the thirty-fifth day showed that a normal level had been reached. Mice injected with massive doses of bone marrow (64.4×10^6 cells or more) and those receiving all the cells from one femur (12.8×10^6 cells) showed the earliest and most rapid bone marrow recovery, beginning on the second or third day and reaching normal levels by the fourth to the seventh day, depending on the dose. Histologically, the cellularity of the bone marrow paralleled the bone marrow count, although the time relation is not so sharply defined. The left femur of animals receiving a massive dose (237.9×10^6 cells) showed microscopically marked cellularity on the first 3 days after treatment, in contrast to the controls. Determinations obtained from the microhematocrit method on the suspension of bone marrow cells obtained from the 8 mm. segment of the right femoral bone shaft were erratic and did not correlate with bone marrow response. Red blood cell counts of the bone

* 95% confidence limits are given in parenthesis.

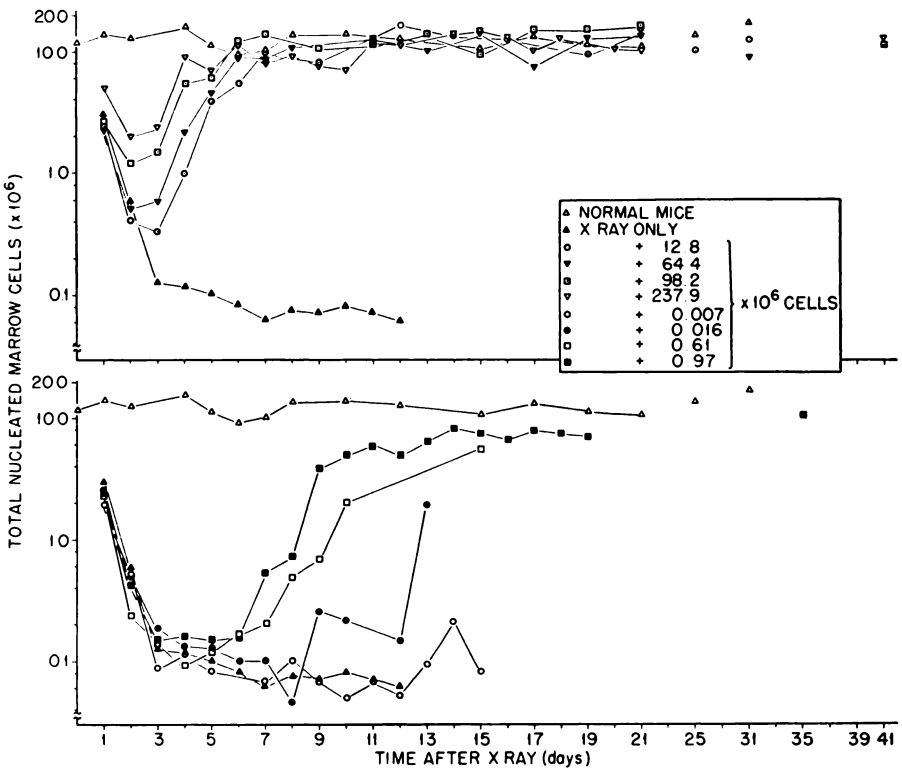

FIG. 2.—Effect of bone marrow dose on bone marrow cell response of the right femur shaft of irradiated (900 r) mice. Nucleated marrow cells were given intravenously immediately after irradiation.

marrow suspension showed an increase from the average values of 3.5–4.5 × 10^6 cells in normal bone marrow to an average value of 23.7 × 10^6 cells in x-ray control animals. This observation reflects the passive congestion that occurs in the bone marrow after irradiation. In the irradiated mice given bone marrow, the red blood cell content of the right femur returned to normal levels as bone marrow recovery occurred.

Before each animal was sacrificed, a *peripheral blood leukocyte* count was made to determine whether there was a correlation between leukocyte count and the amount of bone marrow injected. The data demonstrate that a dose response effect on total leukocyte count occurred (fig. 3). Only animals receiving very low doses of bone marrow showed a prolonged leukopenia corresponding to that shown by the controls but with slight recovery about the twelfth day. Recovery of leukocyte levels in higher dose experiments was more rapid and occurred at an earlier time, especially in the experiments where eight or more femurs (64.4 × 10^6 cells or more) were used. However, for these very high doses, no variation of leukocyte response occurred. The recovery of peripheral blood leukocytes usually began 1 to 2 days after the initial recovery of the bone marrow.

In an attempt to correlate the response of the other parameters with the amount of bone marrow injected, the effect of the eight different doses of bone

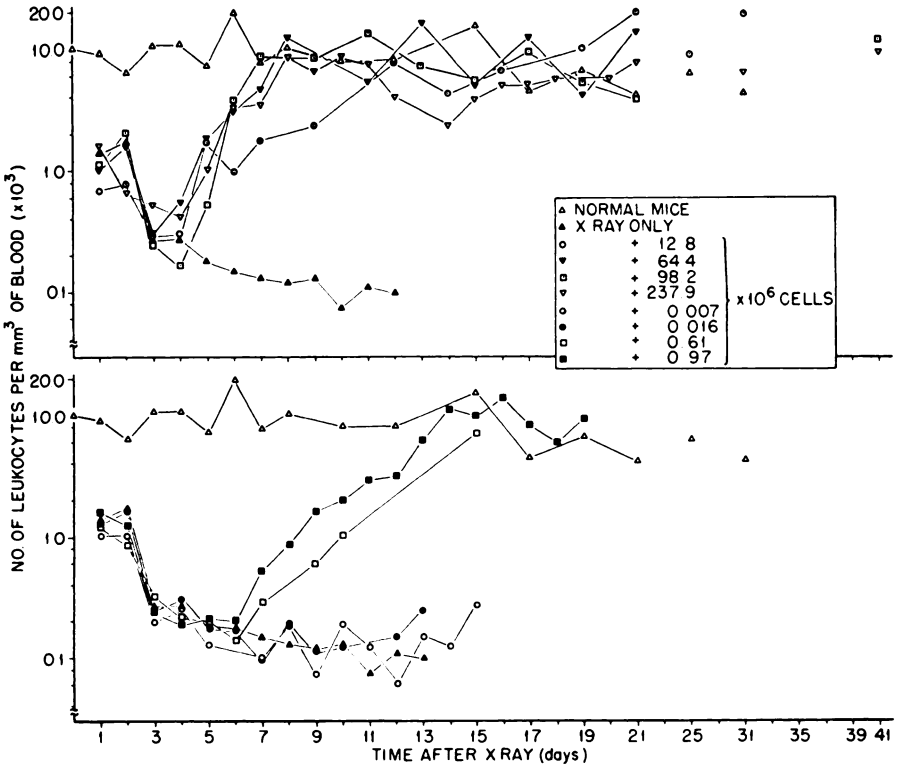

FIG. 3.—Effect of bone marrow dose on peripheral blood leukocyte response of irradiated (900 r) mice. Nucleated marrow cells were given intravenously immediately after irradiation.

marrow on *thymus*, *spleen*, and *body weights* in irradiated mice was studied. It was found that the response of these end points correlated within certain limits with the amount of bone marrow received (fig. 4, 5, 6). Thymus weight response remained essentially the same from 64.4 × 10^6 to 237.9 × 10^6 bone marrow cell doses. A similar observation was made for body weight response. The beginning of recovery of thymus weight was delayed, in contrast to recovery of bone marrow and peripheral blood leukocyte after the same treatment. The recovery for body weight was also delayed, even though the weight loss for the higher doses of bone marrow (12.8 × 10^6 cells or more) was smaller than for the lower doses. The responses of the thymus weight to 12.8 × 10^6 bone marrow cells was not significantly different from the 0.97 × 10^6 cell dose. Animals responding to the bone marrow injections never completely regained normal body weight during the period of observation, and those receiving 12.8 × 10^6 bone marrow cells or an LD_{50} attained normal thymus weight somewhere between 21 and 40 days after treatment. Histologically, thymus regeneration showed the same dose-response relation as did thymus weight. Generally, however, regeneration began earlier than increase in thymus weight. Many granulopoietic cells were seen in the thymus during this period. In contrast to thymus weight, the weight of the spleen recovered in about the same manner as the peripheral blood leukocytes (fig. 5).

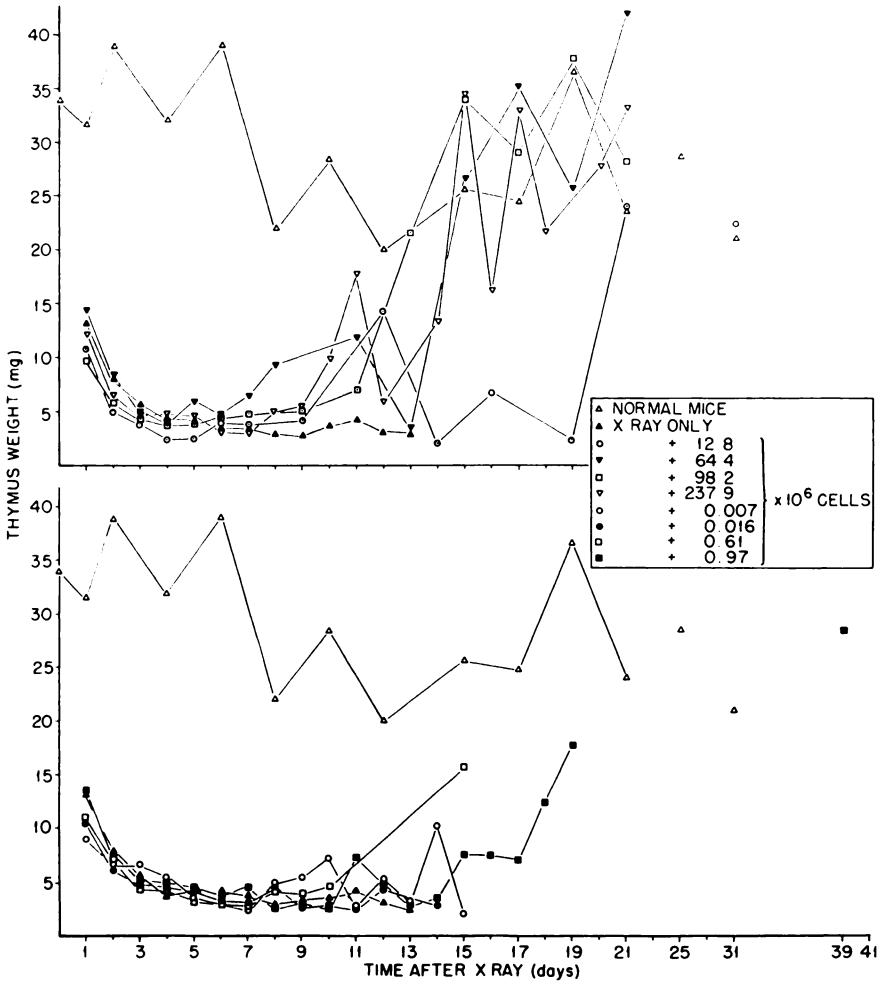

FIG. 4.—Effect of bone marrow dose on thymus weight response of irradiated (900 r) mice. Nucleated marrow cells were given intravenously immediately after irradiation.

At the time the leukocytes reached normal levels, however, spleen weight was far greater than the weight of a normal spleen and began to decrease and return to normal only after maximum weight was reached. After the initial destructive effect of radiation, the histology of the spleen showed that hematopoietic activity in the red pulp increased in time and in amount relative to the amount of bone marrow injected.

The *appearance and behavior* of the irradiated mice receiving massive doses of bone marrow (64.4 $\times$ 10^6 to 237.9 $\times$ 10^6 cells) was normal; there was no ruffling of hair or reduced activity. Those that received the lower doses of bone marrow went through a period of abnormal appearance and behavior, with ruffled hair, reduced activity, and body weight loss. At extremely low doses of bone marrow (0.12 $\times$ 10^6 to 0.97 $\times$ 10^6 cells) where some 30-day survival was obtained, the appearance of the surviving mice was abnormal, the hair being ruffled and the

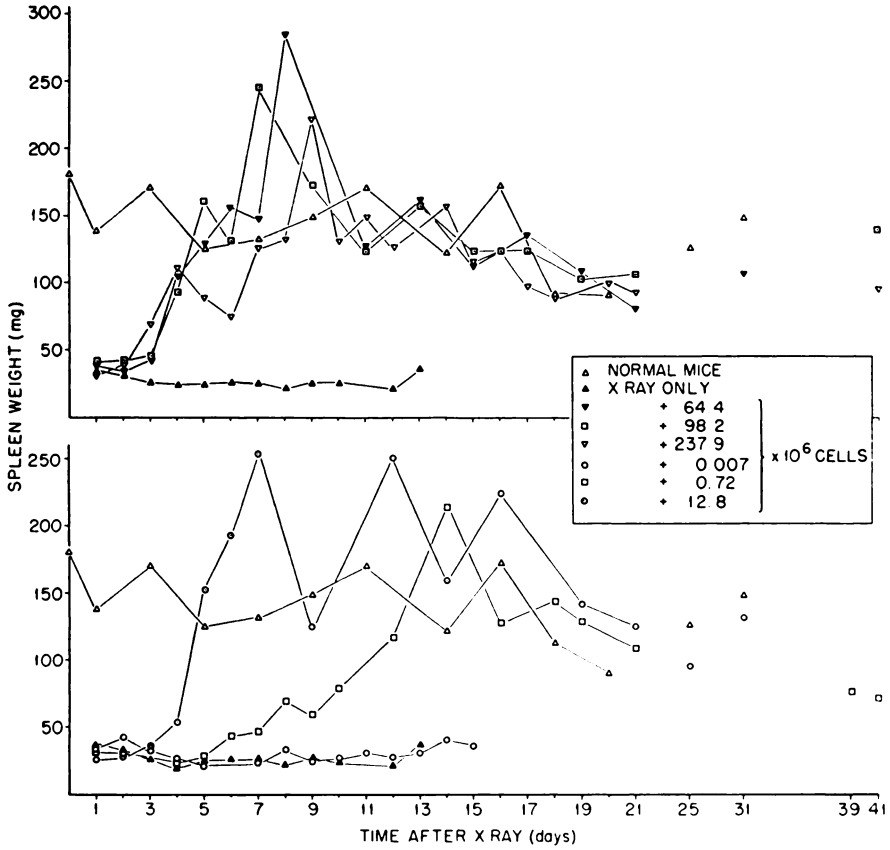

FIG. 5.—Effect of bone marrow dose on spleen weight response of irradiated (900 r) mice. Nucleated marrow cells were given intravenously immediately after irradiation.

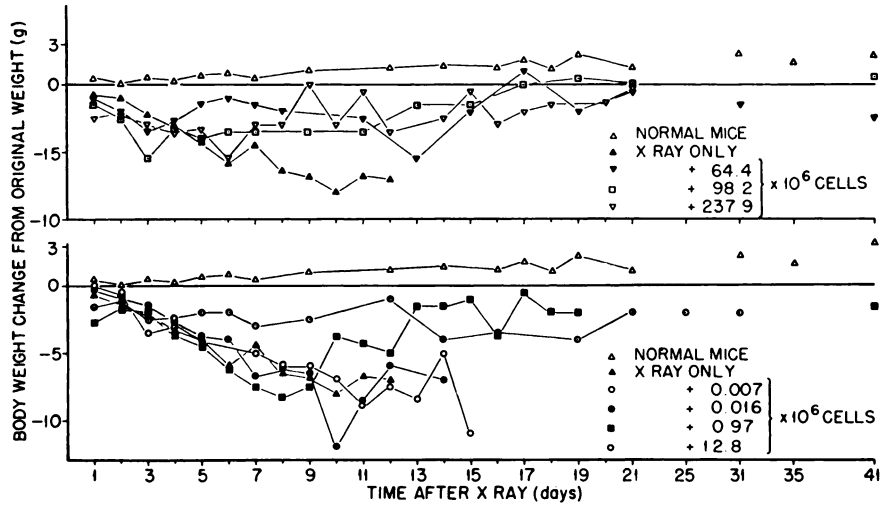

FIG. 6.—Effect of bone marrow dose on body weight of irradiated (900 r) mice. Nucleated marrow cells were given intravenously immediately after irradiation.

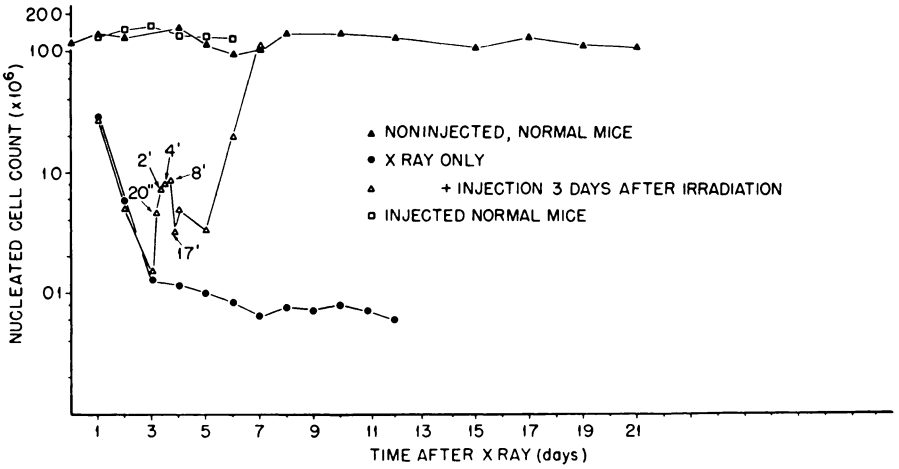

FIG. 7.—Effect of a dose of 237.9 × 10^6 bone marrow cells on the total cell count of the marrow from the right femur of mice irradiated (900 r) 3 days prior to injection.

posture hunched. Some of these animals died after 30 days, which suggests that radiation death could be delayed with minimal doses of bone marrow. Some of the evidence for the latter observations was obtained in additional experiments not reported in this paper.

In a separate experiment to investigate the possibility that the injected bone marrow cells continued to circulate and were responsible for the initial bone marrow cell counts, a massive dose of bone marrow (237.9 × 10^6 cells) was injected into normal mice and into mice that had been irradiated (900 r) 3 days earlier. Figure 7 shows that a sevenfold increase of bone marrow cell count occurred in the irradiated animals within 8 hours after injection but that no increase was detectable in the normal animals. Histologic evidence showed that there were very few nucleated cells in the blood vessels of the femoral bone marrow. These results indicated that, although some circulating nucleated cells were counted, they probably were not responsible for the bone marrow counts on days 1 or 2 after treatment.

Discussion and Summary

The relation between the amount of isologous bone marrow injected into a lethally irradiated mouse and its 30 day survival, its bone marrow response, the histology of the bone marrow in its left femur, its peripheral blood leukocyte count, the weight of its thymus, spleen, and body, and its appearance showed that quicker recovery of these end points occurred with increasing amounts of bone marrow administered.

The bone marrow parameter was the quickest to respond to the varying amounts of bone marrow injected. In addition, no optimum dose of bone marrow was found in these experiments for this end point. This indicates that the bone marrow of an irradiated mouse could be made to show even quicker return to normal by injection of greater amounts of cells.

The peripheral blood leukocyte count and the spleen weight also showed very quick recovery with the massive doses of bone marrow injected. For both end

points, however, an optimum response was reached, which in the leukocytes, was $64.4 \times 10^6 - 237.9 \times 10^6$ cells injected, and in the spleen weight, $12.8 \times 10^6 - 237.9 \times 10^6$. An optimum response for thymus weight was also found with bone marrow doses of $64.4 \times 10^6 - 237.9 \times 10^6$ cells. The cell dose for the optimum response in recovery of body weight was $12.8 \times 10^6 - 237.9 \times 10^6$.

Optimum 30 day survival was reached with injection of 64.4×10^6 bone marrow cells in these experiments. However, doses from 12.8×10^6 to 64.4×10^6 cells were not tested. The dose of bone marrow cells that was calculated to give 50 per cent 30-day survival was 0.42×10^6 (0.17×10^6, 1.92×10^6) cells for the males and 1.06×10^6 (0.42×10^6, 2.51×10^6) cells for the females. The calculated dose of bone marrow cells that would give 1 per cent 30-day survival of the males was 0.008×10^6 (0.0008×10^6, 0.0284×10^6) and for the females 0.0102×10^6 (0.001×10^6, 0.040×10^6) cells. These point estimates seem to be unreliable in view of the extremely large 95% confidence intervals. However, in experiments done by other workers at the National Cancer Institute similar to those reported in this paper, M. Schneiderman determined a threshold dose ranging from 0.0026 to 0.0320×10^6 cells by a somewhat different analysis of the data.[7] Jacobson et al. cite a figure of $3–5 \times 10^6$ bone marrow cells from young donor mice as causing 53% 28-day survival in CF No. 1 mice exposed to 900 r.[2] In using bone marrow from older mice, they found that $5–9.9 \times 10^6$ cells caused 54.9% 28-day survival. They also estimate that 50,000 cells are necessary to produce significant recovery of mice after an LD_{99} exposure. This figure is similar to that determined from the results of the present experiments.

The results obtained from mice injected intravenously with a massive dose of bone marrow 3 days after irradiation indicated that the number of cells in the bone marrow of the right femur can be elevated within a few hours after injection. However, this could be observed only in an irradiated animal where the number of cells in the right femoral bone marrow was already low. A similar observation was made by Graevsky.[8] This finding gives further support to the hypothesis that intravenously injected bone marrow transplants to bone marrow sites in the irradiated host.[9]

SUMMARIO IN INTERLINGUA

Le resultatos effectuate in muses per le injection intravenose de doses massive de medulla ossee 3 dies post lor irradiation indicava que le numero de cellulas in le medulla ossee del femore dextere pote elevar se intra alicun horas post le injection. Tamen, iste phenomeno esseva observate solmente in animales irradiate in que le numero de cellulas in le medulla ossee del femore dextere esseva jam basse. Un simile observation esseva facite per Graevsky.[8] Iste constatation corrobora le hypothese que injectiones intravenose de medulla ossee in recipientes irradiate se transplanta a sitos medullar.

REFERENCES

[1] JACOBSON, L. O.: Evidence for a humoral factor or factors concerned in recovery from radiation injury: A review. Cancer Res. *12:* 315, 1952.

[2] ——, MARKS, E. K., AND GASTON, E. O.: Observations on the effect of spleen-shielding and the injection of cell suspensions on survival following irradiation. Radiobiology Symposium, Eds. Z. M. Bacq and P. Alexander. London, Butterworth's Scientific Publications, p. 133, 1954.

[3] FISHLER, M. C., COLE, L. J., BOND, V. P., AND MILNE, W. L.: Therapeutic effect of rat bone marrow injection in rats exposed to lethal whole body X-radiation. Am. J. Physiol. *177:* 236, 1954.

[4] BROWN, M. B., HIRSCH, B. B., NAGAREDA, C. S., HOCHSTETTER, S. K., FARAGHAN, W. G., TOCH, P., AND KAPLAN, H. S.: Some biological aspects of the factor in bone marrow responsible for hematopoietic recovery following systemic irradiation. J. Nat. Cancer Inst. *15:* 949, 1955.

[5] URSO, P., AND CONGDON, C. C.: Quantitative studies on the effect of bone marrow treatment in irradiated mice. Paper given at Radiation Res. Soc., May 1956, Chicago, Ill.

[6] DEWS, P. B., HIGGINS, G. M., AND BERKSON, J.: Error of the determination of the eosinophil count in peritoneal fluid of the rat. Biometrics *10:* 221, 1954.

[7] CONGDON, C. C., AND MCKINLEY, T. W., JR.: Unpublished data.

[8] GRAEVSKY, E. YA.: Investigations of the protection of the animal organism against the harmful action of ionizing radiations. Conf. Acad. Sci. USSR. On the Peaceful Uses of Atomic Energy, July 1-5, 1955, pp. 33-43 (translated into English by Consultants Bureau, 259 West 14th Street, New York City, N. Y.).

[9] CONGDON, C. C.: Experimental treatment of radiation injury. To be published.