Hemoglobin Synthesis in Mice after Bone Marrow Transplantation (34582)

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Mice can be protected from the lethal effects of whole-body irradiation by the transfusion of relatively small numbers of viable bone marrow cells. These cells undergo rapid proliferation in the irradiated host and, with time, fully reconstitute the hematopoietic resources of the recipient. Elegant analyses of this repopulation and the associated stem-cell renewal have been made by McCulloch and Till (1), Curry and Trentin (2), and Kretchmar and Conover (3).

In adult humans, the proliferation of hematopoietic stem cells is frequently associated with the reappearance of fetal hemoglobin (4, 5). Should a similar phenomenon occur in the mouse it would be of great interest since it would provide a model for the control of gene expression which could be subjected to experimental manipulation. The structural analogies between the human and mouse fetal and embryonic hemoglobins have already been demonstrated by Gilman and Smithies (6). The present study was undertaken in the hope of demonstrating the renewed synthesis of fetal hemoglobin(s) in adult mice.

Materials and Methods. Female mice of the C57Bl/6J and SJL/J strains were used throughout these experiments. Both marrow donors and recipients were 10–12 weeks of age. The animals were housed five to a cage, fed commercial mouse pellets, and allowed water *ad libitum*. Donors were killed by cervical dislocation. Bone marrow was flushed out of both femurs and suspended in Pucks saline (7). An aliquot was removed for enumeration, and an appropriate volume was injected intravenously into the recipients. Transplantation was performed within 2 hr of irradiation.

Irradiation was carried out with 210 kVp X-rays with 1.0 mm aluminum and 0.5 mm copper filtration at an exposure rate of 21.5 R/min. The mice were in a Lucite cage on a rotating table 58 cm from the source of the X-rays, and each mouse received a total of 850 R.

The recipients were killed at various times after irradiation, and the hemoglobin synthesis by isolated bone marrow and/or spleen cells was studied. The cells were washed several times with saline G and then suspended at a concentration of 10^7/ml in the medium described by Borsook (8) with either the leucine and/or valine replaced by their tritiated analogues, or the same medium with cold amino acids supplemented with tritiated delta-amino levulinic acid. After 90 min the cells were washed extensively with saline G and lysed with 4 vol of 0.001 *M* $MgCl_2$. Sucrose (1.5 *M*) was added to the lysate to a final concentration of 0.25 *M* and the lysate was then centrifuged at 27,000*g* (15,000 rpm) for 30 min to sediment the stroma. The supernatant fluid was equliibrated with carbon monoxide and stored at —20° until analyzed. Fetal marker hemoglobins were obtained by treating the peripheral blood of 12-day-old mouse embryos of the appropriate strain in a similar fashion using ^{14}C amino acids to label the hemoglobins (mixed amino acids, sp. act. 0.75 mCi/mg, ICN).

The hemoglobins produced were separated by electrophoresis in 6.0 acrylamide gel, using a Tris EDTA borate buffer, pH 8.6 (9). The electrophoresis, in a E-C Apparatus Co. vertical cell, was for 2.5 hr at 275 V and 100 mA, with continuous recirculation of the buffer cooled to 4.0°. At the end of the run the 3-mm thick sheet of gel was cut into rods

60 × 6 × 3 mm. The slices were made parallel to the direction of migration. Each rod was then scanned in a modified Analytrol (Beckman Inst.) densitometer equipped with a 420-mμ filter and sliced into 1.0-mm slices. The radioactivity within each slice was determined by eluting the content of each slice into a quaternary ammonium compound soluble in toluene (NCS Nuclear Chicago) as described previously (10) and then counting in a liquid scintillation spectrometer (Packard Inst. Model 3375).

In some experiments the recipients were treated with either serum or chorionic gonadotropins. The procedures used in these animals were identical to those described above except that: groups of 10 animals (SJL/J were given either 50 units of serum gonadotropins (Equinex, Ayerst Lab.) or 50 units of chorionic gonadotropins (APL, Ayerst) on alternate days until each group had received a total of four injections. The first dose was given 2 days before irradiation; the second on the day of irradiation and bone marrow transplantation (1.0×10^6 nucleated cells/animal); and the third and fourth injections on Day 2 and Day 4 after irradiation. The hormones were dissolved in saline G and injected intraperitoneally. The animals were killed on Day 6 after irradiation, and the hemoglobins being synthesized in the bone marrows and spleen were analyzed as before.

Details of the individual experiments are included in the results.

Results. Groups of C57Bl/6J mice were irradiated as described and 2 hr later each mouse received the appropriate dose of C57Bl/6J bone marrow. Three cell dosages were tested; 3.6×10^5 cells, 3.6×10^6 cells, and 3.6×10^7 cells. Recipients were killed in groups of four or five animals at 4, 6, 8, and 12 days after irradiation. Spleen cells from individual animals were incubated separately in the labeling medium, while the bone marrow cells from each group were pooled. Figure 1 illustrates the results obtained with the pooled bone marrow of the groups that received 3.6×10^6 nucleated cells and killed 4 and 12 days after irradiation. Figure 2 shows the results obtained after the short-term culture of the bone marrow cells isolated on Day 6 after irradiation from groups of animals which had received 3.6×10^5, 3.6×10^6, or 3.6×10^7 cells per animal. Also shown in these figures are the positions to which marker fetal hemoglobins electrophoresed in the same analysis. When synthetic mixtures of adult and fetal hemoglobin were prepared and co-electrophoresed, the fetal hemoglobins could be consistently identified when any one fetal component made up 10% of the total, and could usually be detected when half that amount was present. No evidence of fetal hemoglobin synthesis

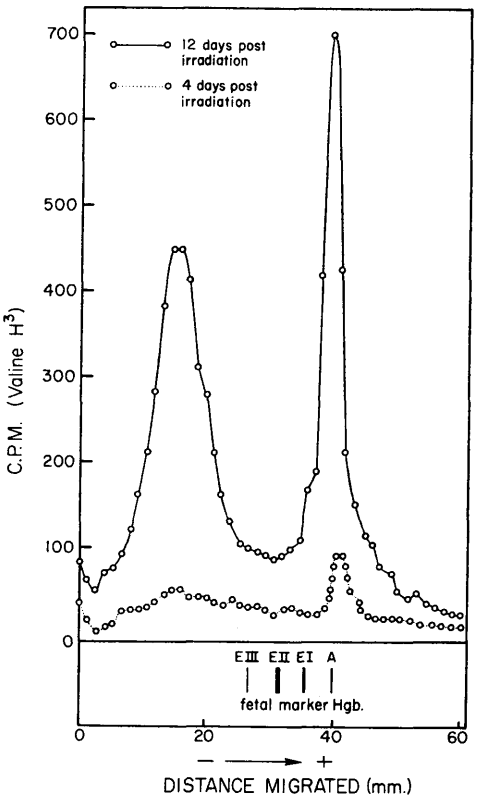

FIG. 1. Electrophoretic separation of the soluble proteins synthesized in short-term cultures of C57Bl/6 bone marrow 4 and 12 days after irradiation with 850 R and protection with 3.6×10^6 viable nucleated cells. Marrow cells obtained from the tibias and femurs of five animals were pooled before culturing in the presence of the Borsook medium (containing 10 mCi/ml valine-2, 3, H^3; sp act 6000 mCi/mmole; Schwarz Bioresearch Inc.). Normal adult mouse hemoglobin was found 41 mm from the origin. The broad peak at 15–19 mm is a non-heme-containing group of proteins.

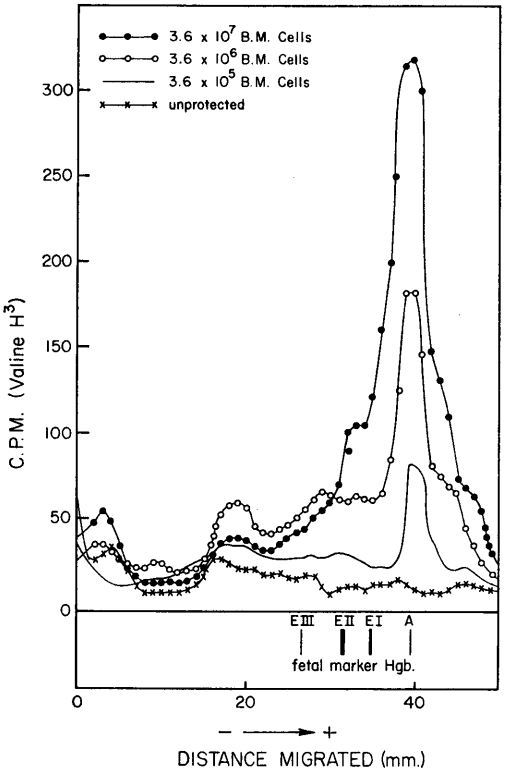

FIG. 2. Electrophoretic separation of the soluble proteins synthesized in short-term culture on Day 6 after irradiation and protection with varying dosages of viable bone marrow cells. Conditions were the same as in Fig. 1.

was seen in this experiment or in any of the 55 other analyses performed with similar material.

Because fetal hemoglobin synthesis normally occurs in an environment rich in gonadotropins, and because there have been reports of the renewed synthesis of fetal hemoglobin in pregnant women and women with tumors of trophoblastic tissue (11), the effects of several gonadotropic agents on hemoglobin synthesis after irradiation were also studied. Groups of animals were treated with either serum or chorionic gonadotropins as described under Materials and Methods. Appropriate controls were processed simultaneously. Representative results obtained with the pooled bone marrow cells are shown in Fig. 3. In 19 additional assays of the hemoglobins produced in the spleens of the treated animals and 9 additional assays on the spleens of the control group only adult hemoglobin was found. Because in many assays a significant amount of electrophoretic "trailing" was found in the region in which one might expect to find the fetal hemoglobins, an additional analysis was performed. The hemolysates obtained from the spleen cells of all of the animals within each group were pooled, concentrated to 0.1 ml with acrylamide pellets (lyphogel, Gelman Inst.), and the electrophoresis was repeated in a 6-mm thick gel in 4.5% acrylamide. The area in question was cut out of the gel slab, macerated with a Dounce-type homogenizer, and its protein content was recovered by elution into the electrophoretic buffer. Seventy per cent of the anticipated counts were recov-

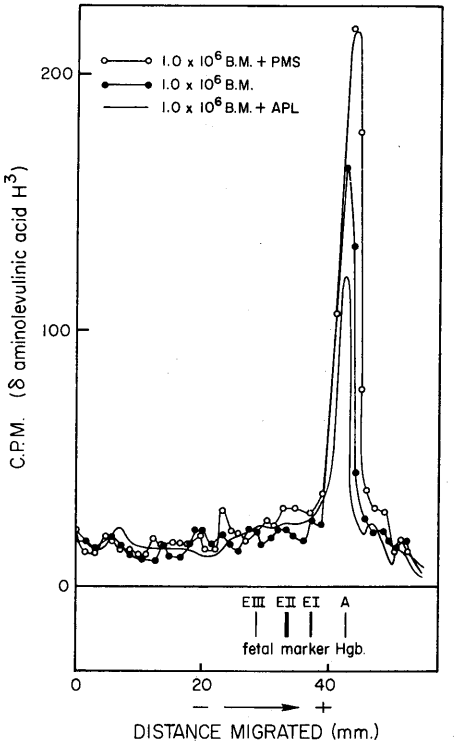

FIG. 3. Electrophoretic separation of heme-containing soluble proteins synthesized in short-term cultures of the bone marrow cells of mice treated with serum gonadotropins (PMS) or chorionic gonadotropins (APL). The cells, obtained on Day 6 after irradiation and protection were incubated in the Borsook medium supplemented with 10 mCi/ml delta/amino levulinic acid-T(G); sp. act 2460 mCi/mmole; Nuclear Chicago.

ered after 24 hr incubation with constant stirring at 4.0°. The eluate was again concentrated to a small volume and re-electrophoresed. Again no evidence of fetal hemoglobin could be detected. When this concentration procedure was tested with artificial mixtures of adult and embryonic hemoglobins it was found that if as little as 5 of the total original hemoglobin had been fetal it should have been readily detected in the second gel.

In addition to these experiments, studies were made on the hemoglobins synthesized in irradiated animals made plethoric by hypertransfusion before marrow protection, in animals treated with estradiol and/or progesterone, and in animals spontaneously recovering from sublethal doses of X-rays. In no case was any evidence found for the production of any hemoglobin with an electrophoretic mobility different from that of the normal mouse adult hemoglobin.

Discussion. Hemoglobins structurally different from those present in the adult have been found in the fetuses of a variety of species, including man, monkeys, sheep, goats, cattle, and mice (12). The mechanism for the change from one hemoglobin type to another during development has been sought by many investigators, both because it is potentially useful as a model system for the study of differentiation and because in man alterations in the changeover have been found in a variety of pathologic states. Despite the interest aroused by the problem, little is actually known of the mechanism by which the change occurs.

In human fetuses near term, both fetal and adult hemoglobins are present in the circulation, and many cells can be shown to contain both proteins (13). Thus, these hemoglobins must be the products of a single erythroid cell clone. The type of hemoglobin synthesized at any moment seems dependent on genetic (14) and environmental factors (11, 15) as well as the developmental status of the fetus. As noted, rapid erythroid stem cell proliferation is frequently associated with the reappearance of fetal hemoglobin synthesis in humans.

In the experiments described in this communication, extensive stem-cell proliferation occurred. Why mouse fetal hemoglobin was not produced is not clear. Perhaps it should not have been expected since, despite the structural similarities noted by Gilman and Smithies (6), there is no clear evidence for the existence of a distinctive fetal (rather than embryonic) hemoglobin in developing mice. Several workers (16–18) have demonstrated that the nonadult hemoglobins of the mouse embryo are synthesized in cells of extraembryonic origin (*i.e.*, the yolk sac). The hemoglobin synthesized by erythroid cells originating within the developing mouse fetus is indistinguishable from normal adult mouse hemoglobin. It should also be pointed out that, although the reappearance of fetal hemoglobin in adult man is quite common, the human embryonic hemoglobins, Gower I and II (19), have never been found in adults.

In all vertebrates, hemoglobin synthesis begins in yolk-sac cells (20). These cells produce unique hemoglobins during a brief period of embryonic life. There is no evidence that they are ever able to synthesize any other hemoglobin, nor can their product ever be found in adult animals. It seems reasonable to speculate that this embryonic erythroid cell clone is short-lived. It disappears early in development, to be replaced by an independently derived clone of erythroid cells which may, in some species, have the capacity to produce more than one β-like peptide (*i.e.*, β and γ in humans), but which can not synthesize the hemoglobins characteristic of the yolk-sac-derived cells. In such a model, the production of embryonic type hemoglobins in adult animals would be impossible regardless of the intensity of the hematopoietic stimulus or the degree of stem-cell proliferation.

Summary. The hemoglobins synthesized in the spleen and bone marrow of mice protected by the transfusion of isogenic bone marrow from the lethal effect of whole-body irradiation have been studied under a variety of circumstances. Only adult hemoglobin synthesis was observed during the period of ob-

servation. The results are consistent with the evidence that the hemoglobins found in the fetal mouse are of extraembryonic origin and therefore are phylogenetically related to the human "Gower" hemoglobins and not hemoglobin F.

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Received Oct. 24, 1969. P.S.E.B.M., 1970, Vol. 133.