CONTROL OF BONE RESORPTION BY HEMATOPOIETIC TISSUE

The Induction and Reversal of Congenital Osteopetrosis in Mice through Use of Bone Marrow and Splenic Transplants*

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Cross-circulation between normal and osteopetrotic littermates has provided a permanent cure for mice (1) and rats (2) with the congenital skeletal disease. Cross-circulation was needed only to initiate recovery. Even when the parabiotic union was interrupted 1 or 2 wk postoperatively recovery of the osteopetrotic mutants progressed to completion in the succeeding weeks or months (3). The effect of a temporary parabiosis was thought to involve the migration from normal to osteopetrotic partner of cells essential to resorption of bone and calcified cartilage (4). In the current investigation the pathogenesis of congenital osteopetrosis and mechanism of recovery was explored further through use of bone marrow and splenic transplants.

The experimental plan used in the transplantation work incorporates criteria derived from Koch's Postulates. Paraphrased to express our present objectives these criteria might be stated as follows: to establish the specificity of defective osteoclasts in the etiology of osteopetrosis, (*a*) these must be demonstrated in all cases of the disease, (*b*) infusions of isolated precursors of defective osteoclasts must produce osteopetrosis in normal littermates, and conversely, (*c*) infusions of isolated precursors of competent osteoclasts must reverse the disease in osteopetrotic mutants. Results of various investigations help to satisfy the first criterion. The deficiencies disclosed in osteoclasts of osteopetrotic animals include reduced lysosomal and oxidative enzymatic activities as revealed by quantitative assays on isolated cells (4), abnormal distribution patterns of enzymatic activities as revealed by histochemical assays (5–8), and partial or complete absence of erosion (4) and ruffled (7, 8) borders.

In attempting to fulfill the second and third criteria as stated above cell infusions prepared from spleens of osteopetrotic mice were administered to irradiated normal littermates and conversely, cell infusions prepared from normal spleens or bone marrow were administered to irradiated osteopetrotic littermates. The transplants decisively altered skeletal remodeling by arresting resorptive activities in all of the normal mice that had received osteopetrotic spleen cells and by restoring resorptive activities in osteopetrotic mice that had received normal mouse spleen or bone marrow cells.

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Materials and Methods

Mice. Microphthalmic mice were obtained from our colony which though not syngeneic is highly inbred. The mice were housed in a constant temperature environment and provided daily with McCollum's diet I (9) as a dry powder and wet mash.

Radiation. Whole-body irradiation was administered from a cobalt 60 source at a dosage of 600 rads for microphthalmic mice and at a dosage of 900 rads for normal mice. These dosage levels are lethal for each of the respective recipients.

Preparation of Transplants. The whole spleen was placed on a depression slide containing 0.2 ml of Hanks' balanced salt solution (HBSS)[1] and thoroughly minced with scissors. The minced spleen was drawn into a tuberculin syringe and injected onto the depression slide through a no. 26 gauge needle. The latter step was repeated using a no. 27 gauge needle, then again using a no. 30 gauge needle. After three passages through the latter the cells were ready for cell count determinations as well as for intravenous injection.

The bone marrow transplants were prepared from the combined marrow plugs expelled from the femora and tibiae. After the addition of 0.2 ml of HBSS the cells of the marrow were dispersed by injection through hypodermic needles of no. 27 and no. 30 gauges. The HBSS, glassware, and instruments used in preparation and administration of the transplants were autoclaved.

Administration of transplants. The splenic transplants (25–50 million nucleated cells) and the marrow transplants (15–35 million cells) were administered intravenously in a vol of 0.05–0.10 ml to anesthetized recipients within 2 h after radiation. As precautions against hemorrhage anticoagulants were not used and the segment of transverse facial vein used for injection was ligated before removal of the needle (no. 30 gauge, stainless steel). The transplants were administered to mice at 20–25 days of age. Donors were usually of the same sex as recipients.

Antibiotics. Tetracycline (1.0 mg/cc) was added to the drinking water for the first 2 wk, postradiation.

Roentgenography. All mice were X-rayed at monthly intervals throughout the observation period. Exposures were made at twofold magnification using a tube with a focal spot diameter of about 0.3 mm.

Bone Biopsy. At 2, 4, or 6 wk after irradiation, a tibia was biopsied and prepared for histologic study.

Autopsy. All animals were killed 3 mo after onset. The skull, the major long bones, and the lymphatic organs were prepared for histologic study.

Histology. Bones were prepared for general histologic study by a sequence that included: fixation in Bouin's fluid, demineralization in formic acid and citrate, embedding in Paraplast, and staining with hematoxylin and eosin. For cytologic study the preparation sequence included glutaraldehyde fixation, demineralization in buffered ethylene-diamintetraacetic acid, postfixation in osmium tetroxide, embedding in epon, and staining with toluidine blue. The soft tissues were prepared by the same sequences sans demineralization.

Experimental and Control Groups. 65 mice used in the present investigation were distributed into the following groups with 5–10 mice per group as indicated in parentheses.

I. CURED EXPERIMENTALS. Included irradiated osteopetrotic recipients of normal bone marrow cells (10 mice) or normal spleen cells (10 mice).

II. INDUCED EXPERIMENTAL. Included irradiated normal recipients of osteopetrotic splenic cell infusions (10 mice).

III. IRRADIATION CONTROLS. Included irradiated normal recipients of normal bone marrow cells (five mice) or normal spleen cells (five mice).

IV. NONIRRADIATED OSTEOPETROTIC CONTROLS. Included recipients of normal bone marrow cells (five mice), spleen cells (five mice), and untreated mutants (five mice).

V. NONIRRADIATED NORMAL CONTROLS. Included recipients of osteopetrotic splenic transplants (five mice) as well as untreated normals (five mice).

Results

Skeletal development was permanently altered in all 30 mice used in the adoptive transfer experiments. The changes were apparent initially 2 wk after

[1] *Abbreviations used in this paper:* GVH, graft-vs.-host; HBSS, Hanks' balanced salt solution; OAF, osteoclast-activating factor.

onset and progressed for several weeks so that by the end of the experimental period the recipients had acquired the skeletal characteristics of their respective donors. These changes will be described under the headings which identify each experimental group.

Group I. The normal marrow and splenic cell infusions were equally effective in restoring matrix-resorbing activities to osteopetrotic mice. Micrographs of a mutant that received a bone marrow transplant illustrate bone histology (Fig. 1 *a*), roentgenography (Fig. 1 *b*), and spleen histology (Fig. 1 *c*) of the cured experimentals. The skeletal recovery affected by the transplant is readily appreciated by comparing the tibia of an experimental mouse (Fig. 1 *a*) with that of an osteopetrotic control (Fig. 5 *b*). The abnormal accumulation of calcified cartilage and bone has been replaced completely by bone marrow (Fig. 1 *a*). The massive, club-shaped appearance of the distal femur and proximal tibia characteristics of the untreated osteopetrotic organism (Figs. 5 *a* and 5 *b*) is not seen in the experimental. Instead, the ends of the long bones have become flared (Figs. 1 *a* and 1 *b*) like those of the normal control (Figs. 6 *a* and 6 *b*). The cortex which is poorly developed in the osteopetrotic controls has become a well-defined, uniformly thick, cylinder of compact bone (Fig. 1 *a*).

The earliest sign of recovery was the heavy infiltration of mononuclear leukocytes along the vascular channels of the proximal metaphysis of the tibia, biopsied 2 wk after onset. Moderately large (15–25 μm), nongranular basophilic cells with deeply indented, euchromatin-filled nuclei were especially numerous. Mitotic figures were of frequent occurrence among these cells. 2 wk after onset the osteoclasts were less numerous and smaller than normal. By 1 mo after onset, the osteoclasts were of normal size and number. The increase in the various myeloid elements, especially megakaryocytes, was very conspicuous during the third and fourth weeks of the experimental period.

Removal of the abnormally extensive diaphyseal spongiosa and expansion of the medullary cavities continued gradually for several weeks and by the end of the experimental period the long bones of the mutant recipients closely resembled those of their normal donors.

The incisors which grow continually in rodents were affected beneficially by the resortation of bone resorption in the osteopetrotic mice. In the absence of treatment, enormous odontomata developed under the inductive influence of the fragmented and deformed epithelial root sheath of Hertwig. Odontomata formation was minimal or absent in the mutants that received transplants. However, the incisors remained small and failed to erupt even though no bone lay across the pathway of eruption.

The thymic-dependent zones of the lymph nodes and spleen (Fig. 1 *c*) were well-developed. The red pulp of the spleen contained fewer nucleated elements than untreated mutants (Fig. 3 *c*).

Group II. Conspicuous lesions appeared near all active growth centers of the skeleton of the irradiated normal mice which had received splenic cell infusions from osteopetrotic littermates. In Fig. 2 *b* the intensely radiopaque lesions are displayed in the distal femur, proximal ilium, and proximal tibia. The distribution of the lesions is correlated with the location of the epiphyseal plates which were still contributing to growth in length in mice over 5 wk of age. Only one plate per long bone remains active after 5 wk of age. The age of onset also

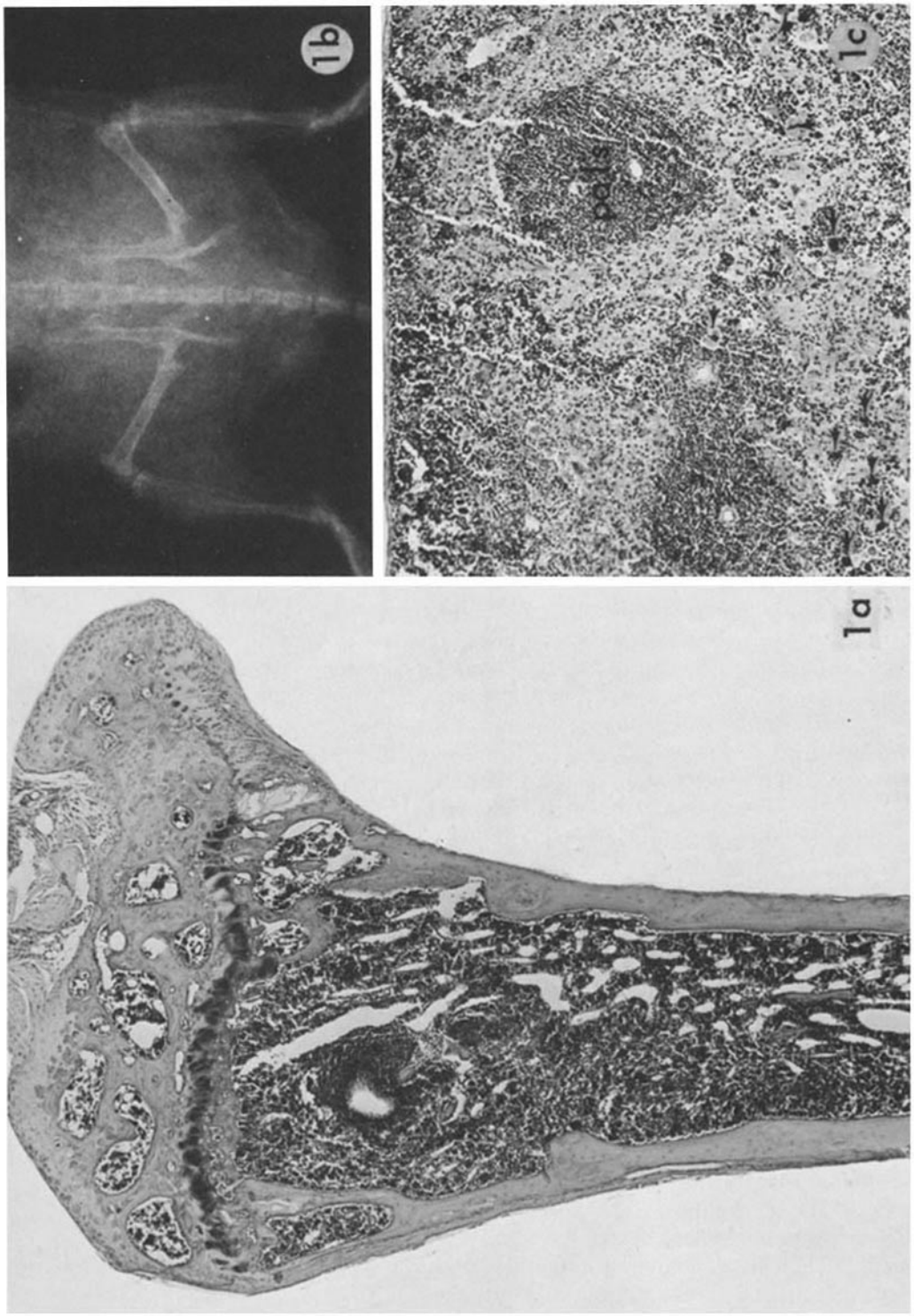


FIG. 1. A cured experimental, 115 days of age. Within 2 h after whole-body irradiation (600 R) this osteopetrotic mouse received 25 million bone marrow cells from a normal littermate at 25 days of age. The proximal tibia (*a*) has a fully expanded medullary cavity occupied by densely nucleated marrow and bounded by the proximal growth plate above and a well-developed cortex laterally (× 48). A radiogram (*b*) of the same animal reveals that the ilium and femur as well as the tibia are of normal appearance (× 2). The spleen (*c*) has well-developed periarterial lymphatic sheaths (*pals*) a thymic-dependent zone. The paler stained red pulp which surrounds the *pals* has many dilated sinuses filled with red blood cells. As compared with the untreated mutants the spleen of the cured mutant posesses sharply reduced numbers of erythropoietic and granulopoietic cells. The small arrow heads in the lower half of this illustration identify megakaryocytes. (× 97).

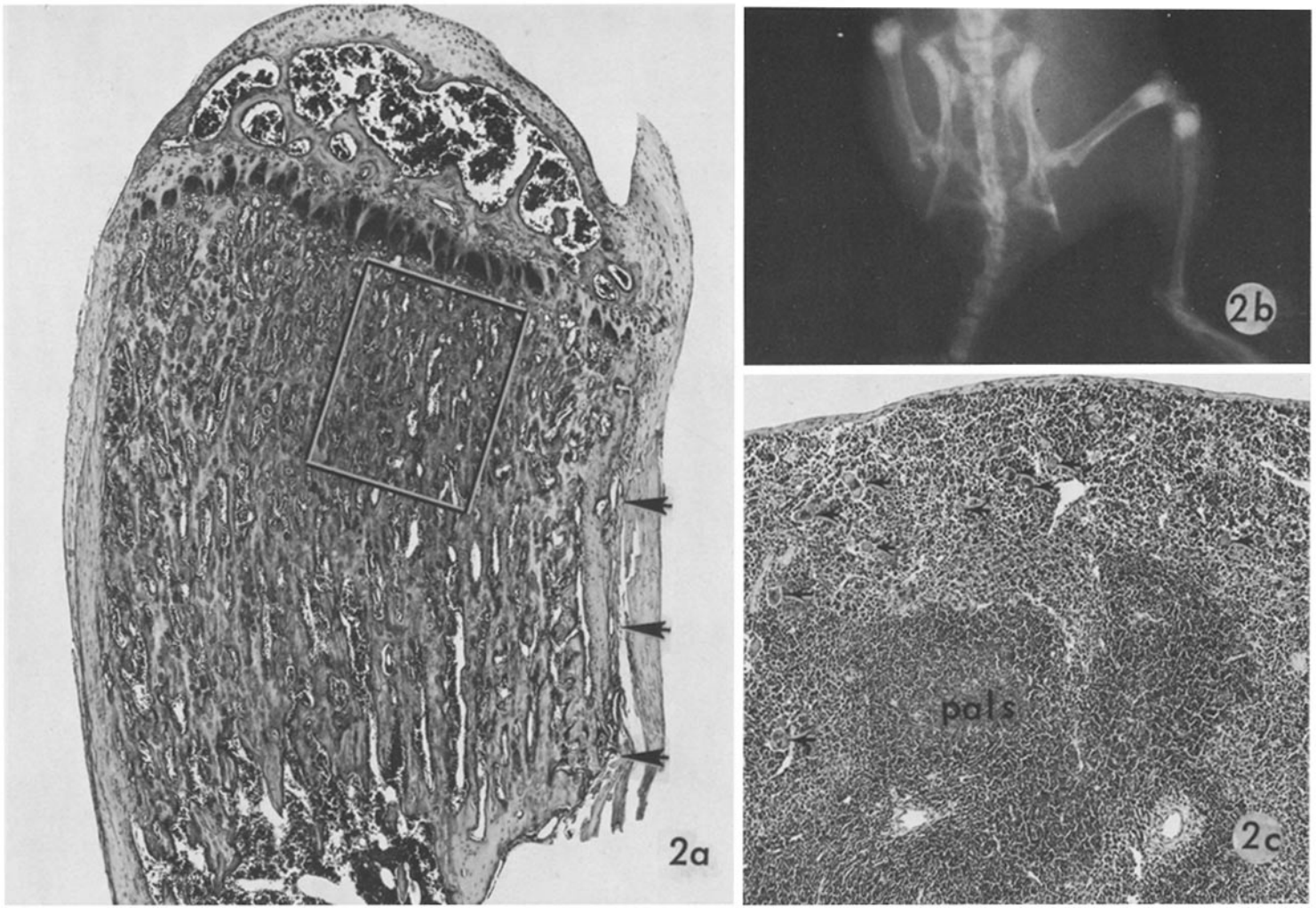


FIG. 2. Induced experimental, 115 days of age. After lethal radiation (900 R) at 20 days of age this genetically normal mouse received 35 million nucleated cells from its osteopetrotic littermate. Bone and calcified cartilage have accumulated in the proximal tibia. (*a*) forming an abnormally dense and extensive spongiosa. The failure of matrix resorption accounts also for the clubbing seen along the right margin of the metaphysis (large arrow heads) (× 48). The abnormal metaphyseal accumulations appear roentgenographically (*b*) as intensely radiopaque masses in the distal femur and proximal tibia and ilium (× 2). In the spleen (*c*) the periarterial lymphatic sheaths (*pals*) are thick and extensive. Megakaryocytes (small arrow heads) are present in concentrations two to threefold greater than normal (× 97).

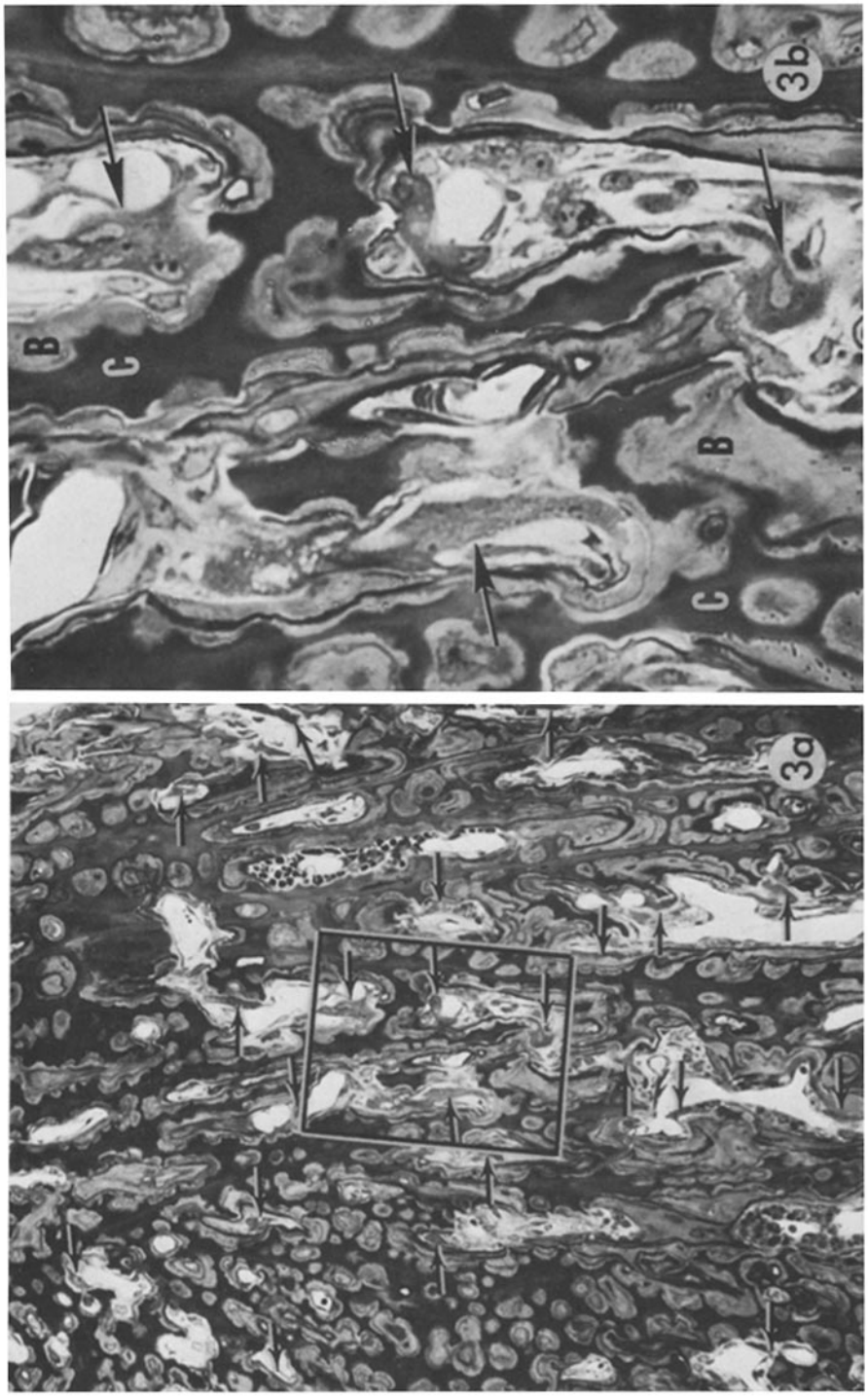
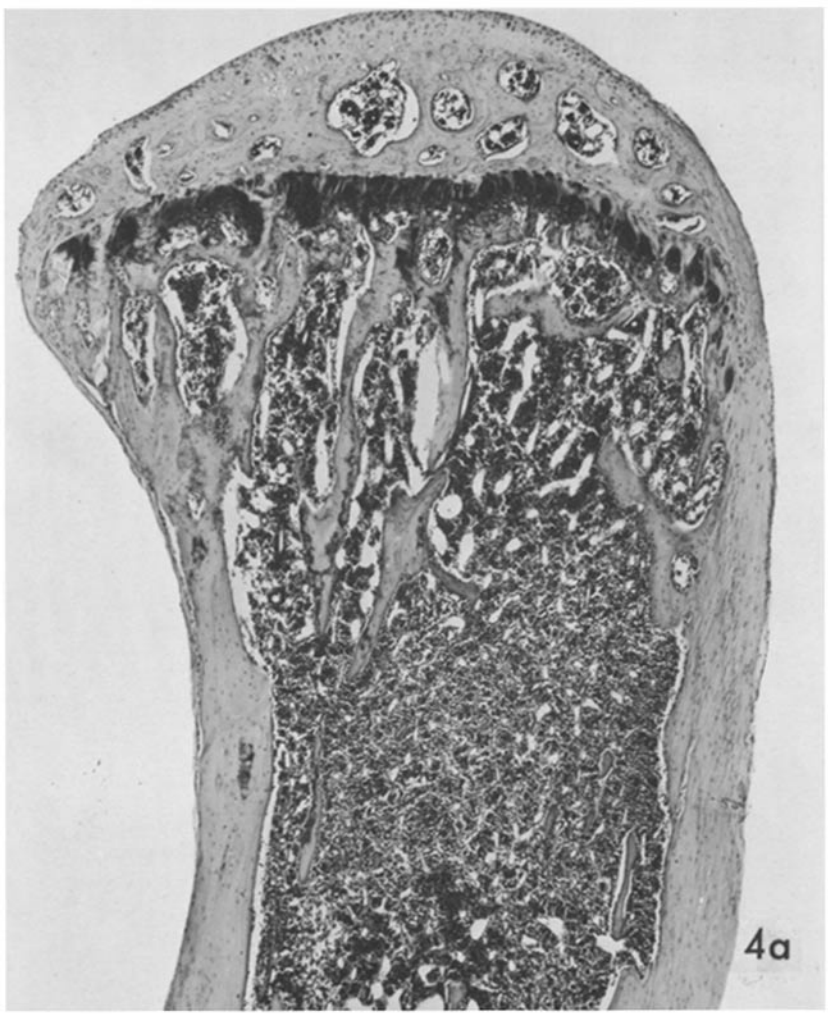
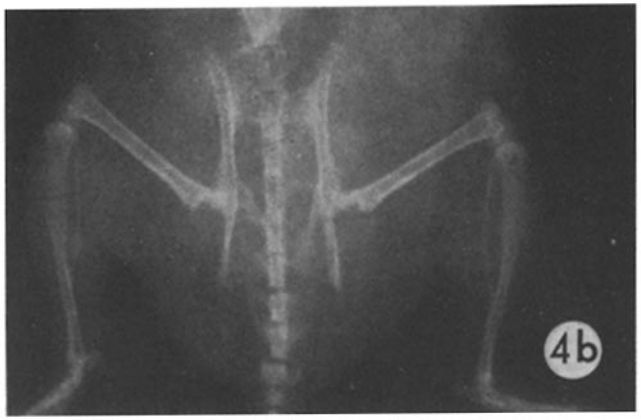


FIG. 3. (*a*) An area similar in size and location to that enclosed in Fig. 2 *a* enlarged to reveal presence of osteoclasts (arrows). 23 osteoclasts were counted in this area which is less than 0.5 mm^2 (× 175). (*b*) Shows the area enclosed in 3 *a* enlarged to facilitate identification of bone (B), cartilage (C) and osteoclasts (large arrows) (× 400).





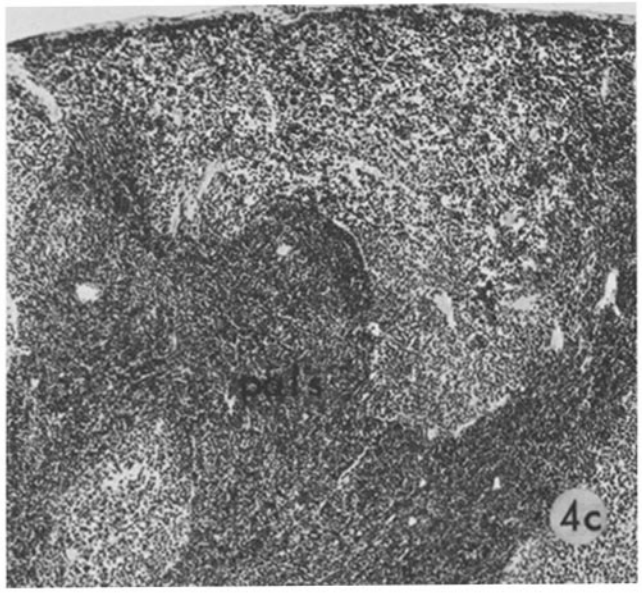


FIG. 4. Radiation control, a normal mouse which after lethal irradiation received a bone marrow transplant from another normal littermate. The photomicrograph of the proximal tibia (*a*) and the radiogram (*b*) closely resemble those of the normal control shown in Fig. 6 *a* and *b*. The periarterial lymphatic sheath (*pals*) of the radiation controls (*c*) is somewhat hypertrophied but the red pulp is normal histologically (× 97).

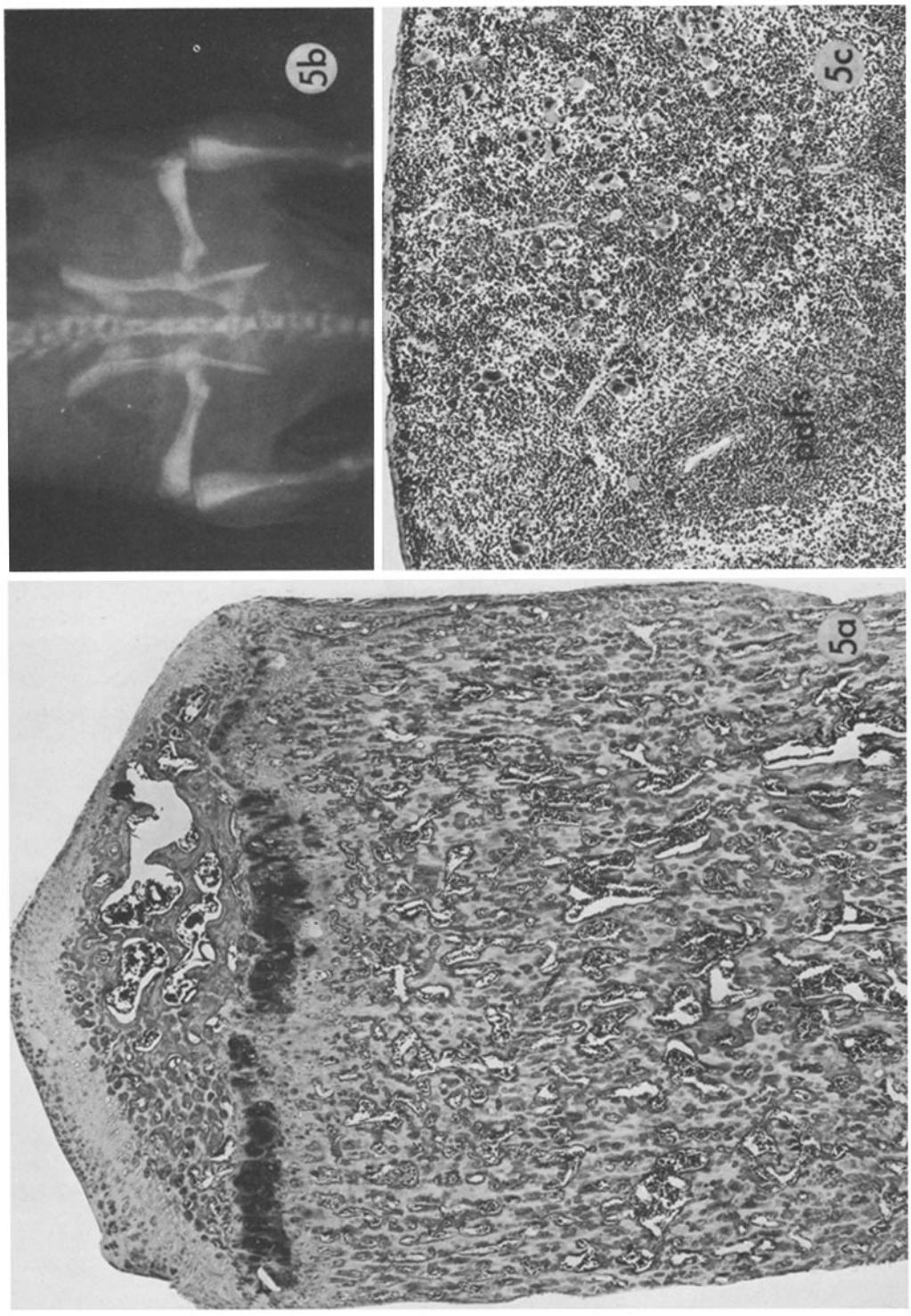


FIG. 5. An untreated, osteopetrotic control, 115 days of age. The proximal tibia (*a*) is abnormally broad, and lacks a cortex as well as a medullary cavity. The interior of the diaphysis is occupied by a dense meshwork of calcified cartilage and bone which is responsible for the intense radiopacity of the skeleton evident in the X-ray (*b*). Both white (*pale*) and red pulp of the spleen (*c*) are well developed. The red pulp has a very high concentration of developing blood cells of red as well as white lineages. The megakaryocyte count (about 50 cells in this field) helps to reveal the intensity of hematopoietic activity in the spleen which in the untreated osteopetrotic organism must compensate for a deficiency of bone marrow. (*a*) × 48; (*b*) × 2; (*c*) × 97.

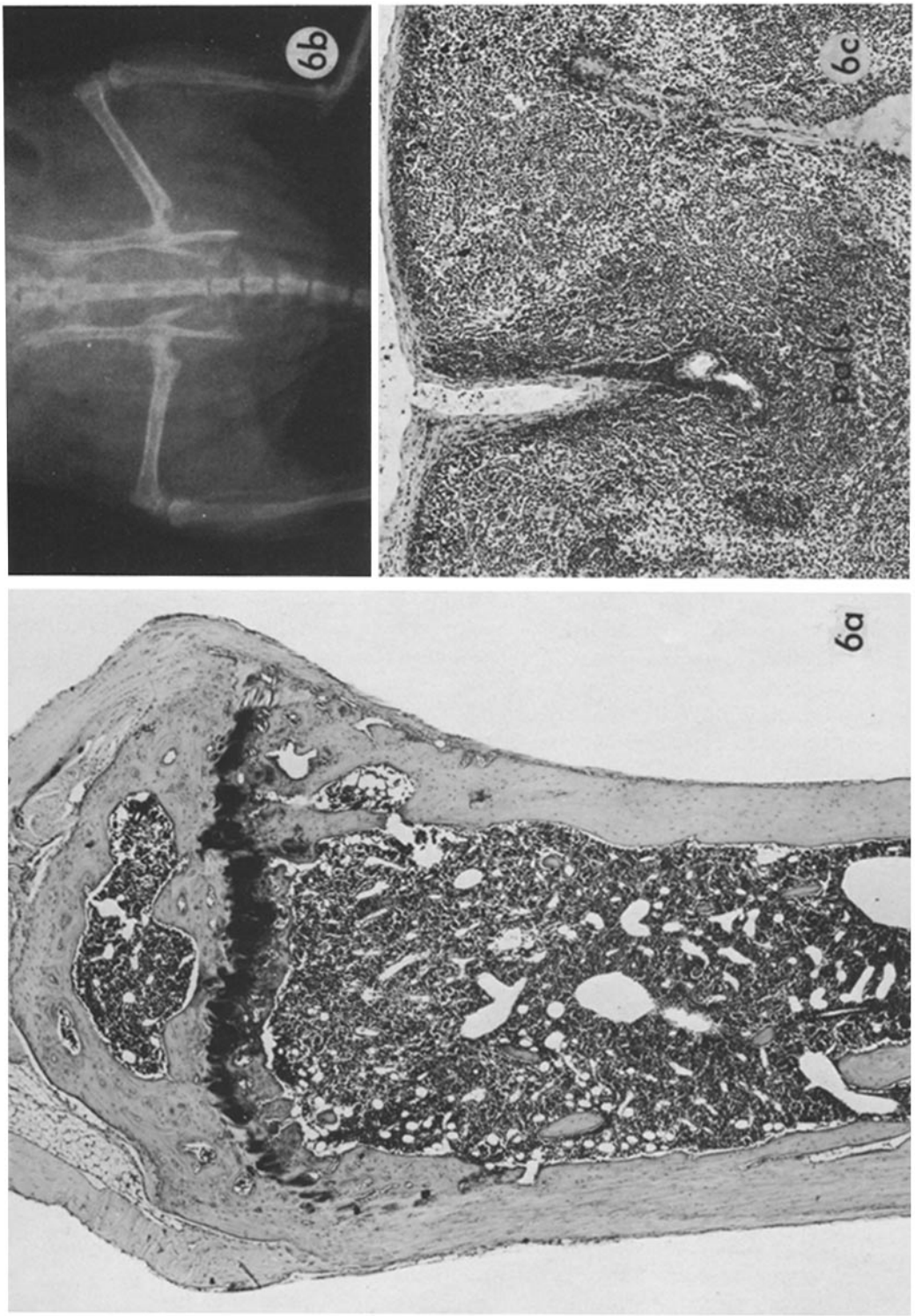


FIG. 6. An untreated normal control, 115 days of age, including the proximal tibia (*a*), radiogram of the hind quarters (*b*), and spleen (*c*). *pals*, periarterial lymphatic sheath. (*a*) × 48; (*b*) × 2; (*c*) × 97.

accounts for the size of the medullary cavities. At 5 wk of age when bone resorption had ceased in mice of group II the medullary cavities were already well-developed. However, no further expansion of the medullary cavities took place though the bones continued to increase in length. This observation may be confirmed by comparing lengths of the tibial diaphyseal components (marrow cavity plus proximal and distal spongiosa) as obtained from the radiograms (mag. × 2) of the normal control (Fig. 6 *b*) and the experimental (Fig. 2 *b*). In the control the tibial diaphysis is about 31 mm in length, including a medullary cavity of 30 mm and spongiosa of 1 mm. In the experimental the diaphysis is about 28 mm in length including a medullary cavity of 23 mm and a spongiosa of 5 mm.

The earliest sign of remodeling failure was observed in the tibial biopsies obtained 2 wk postirradiation, when calcified cartilage had accumulated excessively along the entire extent of the epiphyseo-diaphyseal junction. It appeared that whereas resorption of uncalcified cartilage was maintained at a normal rate within the growth plate, resorption of calcified cartilage and bone matrix had ceased. With continued deposition of bone matrix on the surface of the calcified cartilage trabeculae the vascular channels became abnormally narrow or were obliterated (Figs. 2 *a* and 3 *a*).

Long after resorption of skeletal matrix had ceased, numerous osteoclasts continued to appear along the narrow channels of the metaphyseal spongiosa (Fig. 3 *a*). Osteoclasts of the mice of group II were larger as well as more numerous than normal. Many osteoclasts appeared to possess a marginal or transition zone but there was no sign of a well-differentiated ruffled border (Fig. 3 *b*).

Only a minor degree of malformation of the proliferative zones of the incisors was noted in the experimentals with induced osteopetrosis. However, evidence of obstruction of the salivary ducts by excessively accumulated bone was seen in areas adjacent to the roots of the incisors. Histologically the lymph nodes and spleen (Fig. 2 *c*) appeared normal.

Group III. Irradiated normal controls were used to identify any changes attributable to radiation damage and to help detect influences due to graft-vs.-host reaction (GVH). As illustrated in Fig. 4 *a* and *b* the long bones of the radiation controls closely resembled those of the normal controls (Fig. 6 *a* and *b*). As measured on radiograms magnified twofold (Fig. 4 *b*) the total tibial diaphyseal length was about 31 mm including a medullary cavity 29 mm long and spongiosa, 2 mm in length.

Histological study of the tibial biopsies obtained 2 wk after radiation revealed that reconstitution of the bone marrow was virtually complete. Growth plate activity as well as osteogenic and osteolytic activity of endosteum and periosteum remained intact throughout the experimental period as indicated by the tibial growth rate obtained from the radiograms and bone cell distribution patterns evident in the histologic sections. The lymph nodes and spleen (Fig. 4 *c*) were normal histologically.

Groups IV and V. In addition to untreated osteopetrotic mice (Fig. 5 *a*, *b* and *c*) and untreated normal mice (Fig. 6 *a*, *b* and *c*), the control groups included mice which received transplants in the absence of radiation. In all cases,

whether bone marrow or spleen was used as the donor tissue and whether the nonirradiated recipient was normal or mutant, the transplants had no affect on skeletal development. It was assumed that as long as the hosts source of immunologically competent cells remained intact donor cells would be destroyed.

Discussion

The results of the present investigation represent the first evidence in mammals that migratory cells of myeloid tissue control resorption of skeletal matrix. These migratory cells were obtained from the spleen as well as from the bone marrow of normal mice.

Regarding the nature of the mechanism by which the myeloid derivatives control bone and cartilage resorption, three alternatives were considered: GVH, osteoclast-activating factor (OAF), and migratory progenitors of osteoclasts.

GVH Reaction. Osteoporotic-like changes have been observed in young mice which were undergoing GVH reaction (10). It was of interest, therefore, to know whether or not the vigorous resorptive activity observed in the mutants that received normal bone marrow or splenic transplants was a part of a GVH reaction. Although the mice used in the present studies were not syngenic, the stocks had been inbred for over 15 generations. Furthermore, the fact that littermates from our stocks tolerated parabiosis well (1) indicates minimal disparity of histocompatibility antigens (11). Nevertheless, our experimental animals were screened for GVH reaction by the following criteria: (*a*) body and skeletal growth rates, (*b*) osteoporotic changes, and (*c*) hypoplasia of the thymic-dependent zones of the lymph nodes and spleen. By these criteria all experimental and control animals were free of GVH reaction. The strongest evidence against occurrence of GVH reaction was observed in mice of group II, all of which developed osteopetrosis rather than osteoporosis. The general body development and growth rate was normal in all experimental groups. Thymic-dependent zones of the spleen and lymph nodes were consistently well developed. Osteogenic and chondrogenic activities were not permanently affected by either the exposure to radiation or any influence of the donor cells.

Osteoclast Activating Factor. Cultures of human leukocytes release a substance called OAF, which when tested in vitro promotes resorption of bone (12). The possibility exists that OAF plays a role in the recovery of osteopetrotic mice initiated by cross-circulation or myeloid transplants. Unfortunately no published information is available on the existence and properties of OAF in the mouse or the rat. In a pilot study we tried to test the effect of OAF on microphthalmic mice in vivo. The supernate obtained from normal mouse spleen cultures was prepared by the protocol recommended for human leukocytes (13). When given subcutaneously at daily intervals for 3 wk the preparation produced no skeletal effects. Additional investigation currently underway should yield decisive information regarding the possible role of OAF in osteopetrosis.

Migratory Progenitors of Osteoclasts. It has been postulated that control of bone remodeling is mediated by progenitors of osteoclasts which arise in the bone marrow and which migrate via the circulation to sites of active or impending bone and calcified cartilage resorption, including haversian system, fracture

sites, and extraskeletal sites where bone matrix has been implanted (4).

Fischman and Hay (13) described three phases in the life cycle of osteoclasts: a proliferative phase lasting 4 or 5 days spent in the bone marrow (or spleen), a migratory phase lasting 5–13 days when osteoclast precursors (monocytes) migrate via the blood stream to the ossification centers, and a resorptive phase lasting about 10 or 11 days when the cells are multinucleated and actively resorb cartilage and bone. Thus, in the newt it takes at least 10 days to manufacture an osteoclast after which the cell functions for about 10 days. The duration of these stages in the life cycle of the osteoclast could account for the lag times encountered in the parabiotic and transplantation work on osteopetrotic mice. Accordingly, it was learned that a mutant must be connected to his normal littermate by cross-circulation for at least 10 days in order to initiate recovery. Histologic signs of restoration of cartilage and bone resorption in osteopetrotic mice receiving normal marrow or splenic cells were first noted 2 wk after infusion. Signs of failure of cartilage and bone resorption in mice in which osteopetrosis was induced, likewise, were first detected histologically at about 2 wk after onset. These time relationships suggest that mouse osteoclasts, like those of the newt, have a developmental and migratory phase which lasts about 10–14 days and an equally long definitive or functional stage.

Summary

The reciprocal transplantation of hematopoietic tissues was carried out on young, lethally irradiated mice of inbred, microphthalmic stock. The cell infusions prepared from the bone marrow or spleen of a normal littermate fully restored capacity to resorb bone and cartilage in the osteopetrotic recipients. Conversely, cell infusions prepared from the spleen of microphthalmic mice induced osteopetrosis in their irradiated, normal littermates.

It is concluded that resorption of skeletal matrix is controlled by migratory cells, possibly osteoclastic progenitors, derived from the myelogenous tissues. No evidence was obtained to suggest that skeletal changes observed in the experimental animals were mediated by a graft-vs.-host reaction. The earliest skeletal changes in the experimental mice were detected 2 wk after onset which may represent the length or time required to replace the osteoclast population of the mouse.

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