HEMATOLOGICAL CHARACTERIZATION OF CONGENITAL OSTEOPETROSIS IN *op/op* MOUSE

Possible Mechanism for Abnormal Macrophage Differentiation*

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Osteopetrosis is a disease of bone resorption that may be inherited with many separate genes in various mammalian species (1, 2). There are four distinct mutations in the mouse that can individually result in this disorder: osteopetrotic (op) (3), microopthalmic (mi) (4), gray-lethal (gl) (5), and osteosclerotic (oc) (6). A major advance in our understanding of osteopetrosis was provided by the experiments of Walker (7–9), demonstrating the ability of normal hemopoietic cells to cure this disorder in *mi/mi* and *gl/gl* mice. Additionally, it was shown that spleen cells from these mutants transfer the disease into irradiated normal littermate recipients (8, 9). The response of other murine osteopetrotic mutants in transplantation assays remains unknown. At the cellular level, osteopetrosis is a disease of a functionally and/or quantitatively abnormal osteoclast (1, 10). The osteoclast is a multinuclear cell that is most probably formed through a fusion of cells of the monocyte-macrophage lineage (11). Therefore, the osteoclast belongs to the progeny of the hemopoietic stem cell (HSC)[1] (12, 13), and this explains the positive response of *mi/mi* and *gl/gl* mice to transplants of hemopoietic cells. This cure of osteopetrosis by hemopoietic grafts is a more general phenomenon and was recently observed also in juvenile osteopetrosis in man (14, 15) and in two rat mutants: *ia/ia* (16) and *op/op* (17). Studies (18) in *op* rat additionally linked osteopetrosis with abnormalities in the T lymphocyte system. On the other hand, the osteopetrosis in *tl/tl* rat failed to respond to the bone marrow transplant (19), suggesting that the primary lesion in this mutant resides beside the hemopoietic tissue, presumably in the hemopoietic microenvironment (HM). The HM is made by stromal tissue of bones and spleen, histogenetically different from the hemopoietic system and largely derived from cells with fibroblast-like morphology (20). The classic example of the disorder of HM is anemia in Sl/Sl^d mice, which is

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[1] *Abbreviations used in this paper:* CFU-S, spleen colony-forming unit; CSA, colony-stimulating activity; FCS, fetal calf serum; HM, hemopoietic microenvironment; HSC, hemopoietic stem cell; MACCM, marrow-adherent colony-conditioned media.

neither cured with transplants of normal hemopoietic cells nor transferred with hemopoietic cells of defective animals (21, 22). The potent interrelationship between osteopetrosis, the hemopoietic system, and HM prompted us to define the hematological competence of one of the osteopetrotic mutants, the *op/op* mouse. Furthermore, cells of monocyte-macrophage lineage, to which the osteoclast belongs, and fibroblastoid cells that compose the HM were analyzed during their in vitro growth. The results of these investigations constitute the present report.

Materials and Methods

Animals. Male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were used between 3 and 6 wk of age. Osteopetrotic mice and breeding pairs for these mice were also obtained from The Jackson Laboratory. The osteopetrotic breeding pairs are (B6C3)F_1 mice (C57BL/6J × C3H/FeB/J)F_1, which maintain the op gene through breeding procedures involving the transplantation of ovaries from *op/op* mice into the (B6C3)F_1 female (3). There are three genotypes that result in the offspring of these breeding pairs, which are F_2 mice. They are *op/op*, +/*op*, and +/+ genotypically, and the latter two are phenotypically identical. We refer further to them as to the +/? mice. The mutant *op/op* mice are distinguished from the +/? mice by the absence of incisors and a characteristic skull deformation (3). The *op/op* mice used for these studies were 3–4 wk of age. The fact that young animals were used in these studies should be considered in the interpretation of these data, as should the poor nutritional status of these mice caused by their lack of incisors. The diets of these mice were, however, supplemented with a wet mesh chow.

Culture Media. RPMI 1640 (M. A. Bioproducts, Walkersville, MD) and Dulbecco's modified minimal essential media (DME, Flow Laboratories, Rockville, MD) were supplemented with L-glutamine (200 mM, Flow Laboratories), Hepes buffer (25 mM, Calbiochem-Behring Corp., Amerian Hoechst Corp., San Diego, CA), and gentamycin (0.05 mg/ml, Schering Corp., Kenilworth, NJ). When indicated, heat-inactivated fetal calf serum (FCS) (Flow Laboratories; batch 29101224) and/or horse serum (HS) (Flow Laboratories; batch 2911046) were used.

Hematological Measurements. Mice were bled by retroorbital puncture to obtain peripheral blood. The hematocrit and absolute leukocyte count were determined. Differential leukocyte counts were performed on Wright's stained smears. The peritoneal cavity of the mice was lavaged with serum-free medium, the cells obtained were enumerated, and their differential was determined from Wright's stained smears. The cellularity of the spleen and thymus was determined from counts of single cell suspensions from these organs. The marrow cavity of the femurs was flushed out with serum-free media and the cells obtained were enumerated.

Exogenous Spleen Colony-forming Unit (CFU-S) Assay for Hemopoietic Stem Cells. The CFU-S assay was performed as modified and described previously (23). Cell suspensions from the bone marrow, spleen, or liver from either individual test animals or C57BL/6 mice were injected intravenously into groups of four lethally irradiated (750 rad) C57BL/6J recipients. Although the F_2 donor cells were injected into irradiated C57BL/6J mice, no differences between the syngeneic control and the experimental groups were detected suggesting that histocompatibility antigen differences did not affect the CFU-S measurement in this situation. 8 d after the cell transfer, the mice were killed, their spleens were removed and placed in Tellysniczky's fixative. The surface colonies were then enumerated and examined histologically as described previously (23).

Adherent Cell Cultures from Marrow and Spleen. 5×10^6 spleen or bone marrow cells were cultured in 10 ml of DME supplemented with 10% FCS and 10% HS in 25-cm^2 tissue culture flasks (3013; Falcon Labware, Oxnard, CA) as previously described (24). The cells were cultured in triplicate in humidified air, 37°C, 5% CO_2 for 14 d. The culture supernatants were removed and saved for later use as a source of marrow-adherent colony-conditioned media (MACCM). The cells in flasks were air dried, stained with Wright's stain, and macroscopically visible colonies were enumerated. Cultures were then evaluated microscopically. The colonies com-

posed of both fibroblastoid cells and macrophages were apparent as previously described (24). The macrophages observed to surround individual fibroblastoid cells were enumerated. Normally, colonies were made of fibroblastoid cells and were overgrown by macrophages that also grew in intercolony spaces (24).

In Vitro Monocyte-Macrophage Colony Formation. The ability of spleen or marrow cells to form colonies of monocytes and macrophages was determined using an in vitro liquid culture system modified from Goud et al. (25, 26) and described earlier (24). Briefly, 0.5 to 5 × 10^5 spleen cells/ml or 5 × 10^4 bone marrow cells/ml were cultured in 0.24 ml DME, supplemented with 20% HS and 20% MACCM obtained from normal C57BL/6J bone marrow-adherent cell cultures described above. This MACCM served as the source of colony-stimulating activity (CSA). The cultures in slide chambers (Lab-Tek, Naperville, IL) were incubated at 37°C in humidified 5% CO_2 atmosphere for 4 d. The culture supernatants were then discarded, and the cells were air-dried and stained with Wright's stain. The colonies of >30 macrophages and monocytes were scored and had similar cellular composition as those reported by Goud et al. (25, 26), who used embryonal fibroblast conditioned medium as source of CSA.

In Vivo Stimulation of Peritoneal Cells. Mice were injected intraperitoneally with 0.5 ml of 3% Brewers thioglycollate broth (Difco Laboratories, Detroit, MI). 5 d later, the mice were killed and the peritoneal cells recovered by lavage were enumerated. Subsequently, differential cell counts were performed on Wright's stained smears.

Reconstitution of Irradiated Mice. (B6C3)F_2-+/? recipient mice received 750 rad of X irradiation 24 h before cell transfer. 5 × 10^6 spleen cells from (B6C3)F_2-*op/op* or +/? mice were injected intravenously. The recipient mice were usually killed 10–15 d later, and the numbers of cells in the peripheral blood, spleen, femur, and peritoneal cavity were determined.

In Vivo Effects of MACCM. Groups of *op/op* mice daily for 4 d received intraperitoneal injections of 0.5 ml of MACCM of normal C57BL/6J mice or unconditioned DME supplemented with 10% FCS and 10% HS. On day 7 mice were killed and number of peritoneal lavage cells and their differential were determined.

Irradiation of Mice. Mice were irradiated using a bilateral X-ray source operated at a dose rate of 120 rad/min at 125 kV and 20 mA with 0.5-mm Cu filtration. Irradiated mice were housed in laminar flow isolators and received sterile food, water, and bedding.

Results

Characterization of Hematological Status of Osteopetrotric op/op Mice. The basic hematological profile of *op/op* mice and their normal +/? littermates was evaluated, and the data obtained are summarized in Table I. The animals used in these studies as littermates were age matched; however, osteopetrotic mice always weighed less than their normal littermates. The direct effect of the nutritional status of the *op/op* mice on their hematological profile is not known, but must be considered during evaluation of these data. The results of this survey demonstrated no differences in hematocrits and absolute granulocyte counts between *op/op* and normal mice. Leukocyte counts were lower in osteopetrotic animals, owing mainly to significant lymphopenia. The numbers of cells obtained from the femoral marrow cavities of *op/op* mice were 10-fold less than from their normal littermates. These results are in contrast to other murine mutants with osteopetrosis, in which there is a near complete obliteration of marrow cavities (2). On cytological examination, these marrow cells appeared to have composition more typical of peripheral blood than of marrow. They consisted of only 4% erythroblasts and 6% proliferating granulocytes (myeloblasts, promyelocytes, myelocytes), whereas nonproliferating granulocytes (metamyelocytes, stabs, and segments) accounted for 47% and lymphocytes for 38% of the total. 5% of cells were unclassifiable. The organ cellularity of the spleen and thymus of *op/op* and littermate mice were essentially similar. In other osteopetrotic mutants (1, 2) splenomegaly does

TABLE I

Basic Hematological Parameters of Osteopetrotic (op/op) Mice and Normal Littermates *

Parameter (unit)	Mean ± SE (number of mice tested)	
	+/? (5)	*op/op* (5)
Weight (g)	11.8 ± 0.5	6.02 ± 0.45‡
Hematocrit (%)	46.1 ± 0.7	47.4 ± 1.1
Leukocyte count/μl blood	6600 ± 700	3500 ± 900‡
Granulocyte count/μl blood	1140 ± 100	940 ± 200
Lymphocyte count/μl blood	5400 ± 800	2500 ± 700‡
Cells/femur × 10^6	18.6 ± 0.8	1.8 ± 0.1§
Cells/spleen × 10^6	70.6 ± 5	43.8 ± 9.1‡
Cells/thymus × 10^6	138.5 ± 12	177.5 ± 25
Cells/ml of peritoneal lavage × 10^6‖	1.12 ± 0.07	0.13 ± 0.016§

* Mice were evaluated at 3–4 wk of age.

‡ Difference significant ($P < 0.05$) when compared with normal littermates.

§ Difference highly significant ($P < 0.001$) when compared with normal littermates.

‖ Each mouse was lavaged with 5 ml of serum-free medium.

occur. In the gray-lethal mice and osteopetrotic (*op/op*) rat models of osteopetrosis, thymic atrophy is a characteristic feature (2, 18). The normal resident peritoneal lavage of *op/op* mice yielded significantly fewer cells as compared with littermate mice, and this difference was further analyzed.

Differentiation of Macrophages in Osteopetrotic and Littermate Mice. Comparison of the indices of various stages of macrophage-poiesis in osteopetrotic and normal mice is shown in Table II. The organ distribution of hemopoietic stem cells in *op/op* mice was altered. The CFU-S concentration in *op/op* marrow was considerably smaller than in normal marrow, which must also be considered in view of the overall reduction in marrow cellularity in these mice (Table I). On the other hand, spleen had increased concentration of these cells, and unlike normal mice of this age, *op/op* mice had considerable numbers of stem cells present in the liver. However, the size and differentiation pattern of spleen colonies formed by *op/op* cells did not differ from similar parameters for normal cells (data not shown). In view of the scarcity of peritoneal lavage cells in *op/op* mice, the unexpected result was the demonstration of very good growth of macrophage colonies from osteopetrotic spleen cells in the presence of MACCM. Not only was the formation of these colonies better (15 times) than in the case of normal spleen cells, but it was also better than the formation of these colonies by normal littermate marrow (97 ± 12 colonies/10^5 marrow cells). The photographs of these colonies are shown in Fig. 1. These data also contrast with other observations; i.e., very low absolute monocyte count in the peripheral blood and extremely low macrophage count per ml of peritoneal lavage. On the other hand, it must be mentioned that is was possible to distinguish individual macrophage-like cells in peritoneal lavage of *op/op* mice. Nevertheless, these studies demonstrated that under appropriate conditions, progenitor cells from *op/op* mice are capable of differentiating in vitro into macrophages, although they largely fail to do so in vivo in *op/op* mice.

Characterization of Adherent Cell Growth in Liquid Cultures of Marrow and Spleen from

TABLE II

Comparison of Various Stages of Macrophage-Poiesis in Osteopetrotic and Littermate Mice *

Stage of differentiation tested	Index	Mean ± SE (number of mice tested)	
		+/? (7)	*op/op* (5)
Hemopoietic stem cell	Exogenous spleen colony formation:		
	by marrow/10^5 cells	27.1 ± 2.2	6.8 ± 1.3‡
	by spleen/10^6 cells	57.6 ± 5.3	88.7 ± 1.8‡
	by liver/10^6 cells	None	12.1 ± 3.2‡
Monoblast	Monocyte-macrophage colony formation by spleen/10^5 cells	18.7 ± 5.1	270.3 ± 15‡
Monocyte	Monocytes/μl of peripheral blood	730 ± 60	29 ± 10‡
Macrophage	Macrophages/ml of peritoneal lavage × 10^5§	4.2 ± 0.4	0.085 ± 0.01‡

* Mice were evaluated at 3–4 wk of age.
‡ Difference highly significant ($P < 0.001$) when compared with normal littermates.
§ Each mouse was lavaged with 5 ml of serum-free medium.

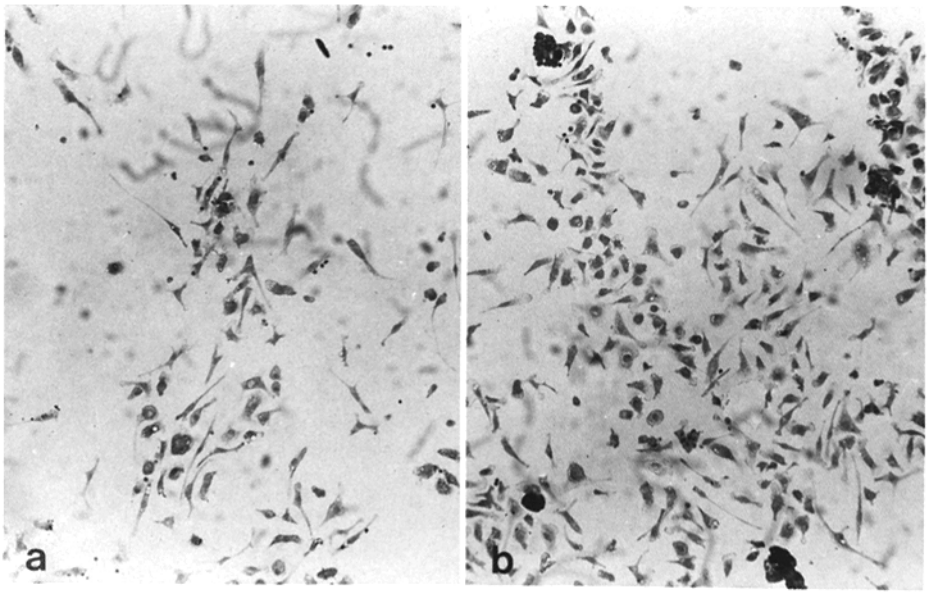

FIG. 1. Formation of macrophages in vitro in the presence of CSA from MACCM in 4-d liquid cultures: (a) macrophage colonies formed by normal +/? spleen cells; (b) macrophage colonies formed by equal numbers of *op/op* spleen cells. Wright's stain, 200 ×.

Osteopetrotic and Normal Mice. Both marrow and spleen cells from *op/op* mice formed lower numbers of adherent macroscopic colonies (Table III) than similar cells from their normal littermates. Moreover, cellular composition of these colonies was essentially altered in *op/op* marrow and spleen cultures. Both marrow and spleen cells from *op/op* mice formed almost pure colonies of fibroblastoid cells and the number of

TABLE III

The Formation of Adherent Cell Colonies by Cells from Hemopoietic Organs of Osteopetrotic and Littermate Mice *

Origin of tested cells	Genotype of cell donor (number of mice tested)	Mean (± SD) number of macroscopic colonies/10^6 cells	Mean (± SD) number of macrophages per fibroblastoid cell in these colonies‡
Marrow	+/? (4)	12.1 ± 1.1	13.3 ± 5
	op/op (4)	1.8 ± 0.3§	1.15 ± 1.4§
Spleen	+/? (6)	7.2 ± 1.3	9.6 ± 4.1
	op/op (6)	5.7 ± 1.3	2.8 ± 1.9§

* Mice evaluated at 3–4 wk of age.

‡ Based on evaluation of between 50 and 100 individual fibroblastoid cells.

§ Significance level $P < 0.001$ when compared with normal littermates.

macrophages in these colonies was very low, equal to approximately one per one fibroblastoid cell (Table III, Fig. 2). In normal mice, macrophages were much more numerous than fibroblastoid cells, were overgrowing colonies formed by fibroblastoid cells, and frequently formed monolayer between colonies of fibroblastoid cells. Although in cultures of cells from *op/op* mice macrophages were also scattered between colonies, their frequency was very low. An index was established to quantitate this decrease in the number of macrophages, and this index reflects the mean number of macrophages attached to the individual fibroblastoid cell. As seen in Table III, there was an ~10-fold decrease in the number of macrophages attached to the individual fibroblastoid cell in cultures of *op/op* cells, the difference being more pronounced in the case of marrow cultures than in the case of spleen cultures.

Attempts to Transfer the Macrophage Defect of the op/op Mouse into Normal Mice with Spleen Cells. Osteopetrotic *op/op* mice are among those mutants whose response to hemopoietic transplants is unknown; similarly, the transferability of the defect with spleen cells was untested. Therefore, the ability of spleen cells from *op/op* mice to transfer the macrophage defect into irradiated (750 rad) (B6C3)F_2-+/? mice was investigated (Table IV). Only one mouse, which did not receive any transplant, survived 15 d, and the results of the evaluation of this mouse are shown as reference. Values of all tested parameters in recipients of both *op/op* and +/? spleen cells were obviously different from the values for the unreconstituted mouse. Moreover, recipients of *op/op* spleen cells by all indices were reconstituted better than recipients of +/? spleen cells. Particularly, the number of macrophages in peritoneal cavity of recipients of *op/op* spleen cells was twice this number in recipients of +/? spleen cells. Similar observations were made for mice studied 11 and 13 d postirradiation (data not shown). There were also two recipients of *op/op* spleen cells that survived 4 wk after transplant. Both of them showed full reconstitution including normal number of cells and normal number of macrophages per ml of peritoneal lavage (data not shown). Unfortunately, none of the recipients of +/? spleen cells survived that long. On no occasion were signs of graft-vs.-host disease observed in recipient animals. Therefore, it appeared that *op/op* spleen cells possessed increased reconstitution potential, in agreement with their increased content of stem cells. This observation also concerned the number of

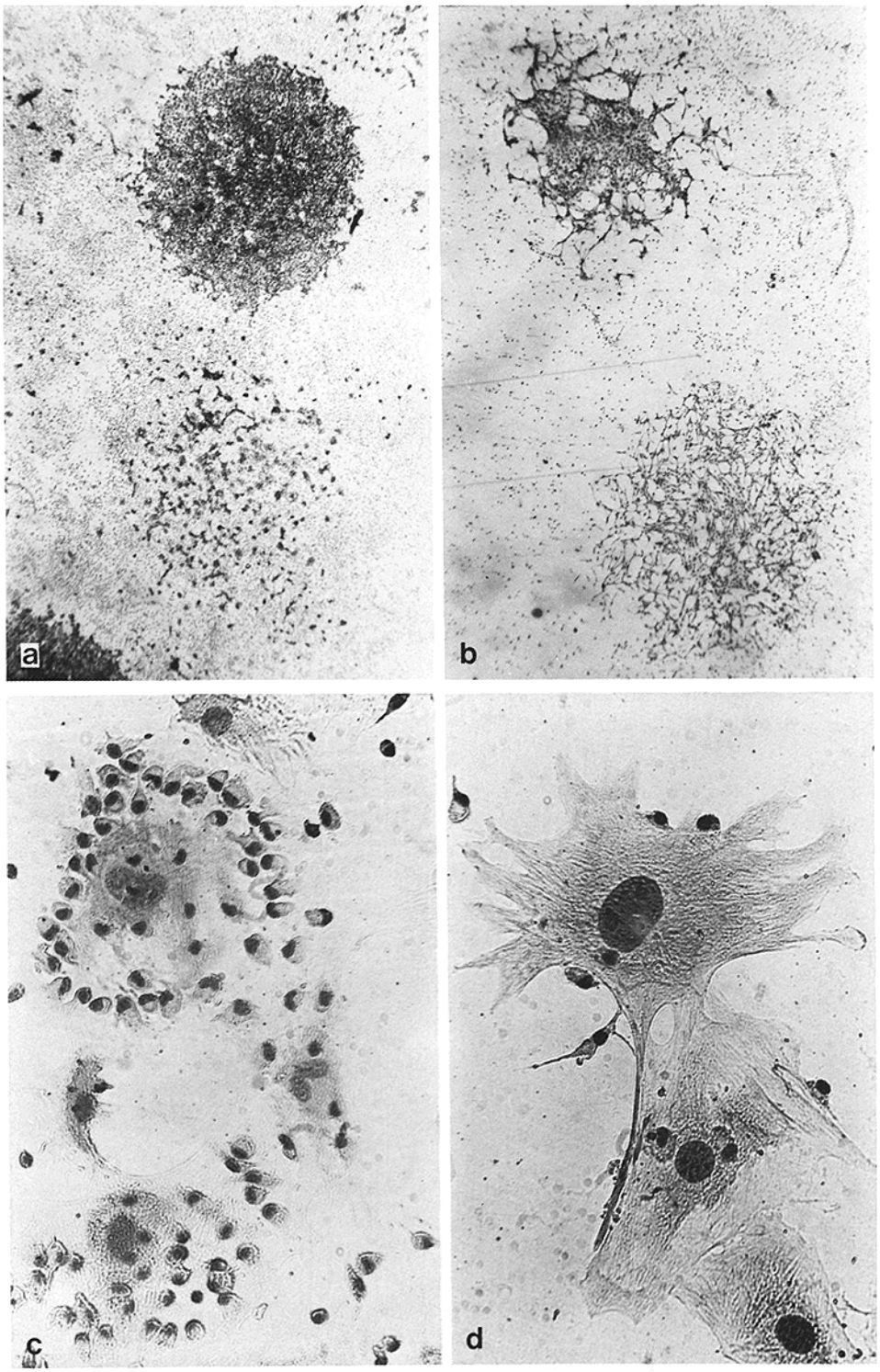

FIG. 2. Adherent colonies formed by normal +/? and *op/op* marrow cells: (a) low-power (15 ×) photograph of representative colonies formed in 14-d liquid culture of +/? marrow; (b) low-power (15 ×) photograph of representative adherent colonies formed in 14-d liquid culture of *op/op* marrow; (c) representative fibroblastoid cells with attached macrophages on peripheries of adherent colony formed by +/? marrow; (d) representative fibroblastoid cells with individual macrophages on peripheries of adherent colony formed by *op/op* marrow. Wright's stain, c and d: magnification 250 ×.

TABLE IV

Postirradiation Reconstitution of (B6C3)F_2 +/? Mice with Spleen Cells of Osteopetrotic and Littermate Mice *

Parameter of reconstitution	Genotype of spleen cell donor: mean ± SD (number of mice tested)		
	None (1)	+/? (5)	*op/op* (7)
Leukocyte count/μl	0.2	0.8 ± 0.3	1.2 ± 0.25‡
Cells/spleen × 10^6	3.0	35 ± 10	56 ± 12§
Cells/femur × 10^6	0.7	2.6 ± 0.6	3.6 ± 0.5‡
Cells/ml of peritoneal lavage × 10^6	0.24	0.5 ± 0.1	0.7 ± 0.15‡
Macrophages/ml of peritoneal lavage × 10^6	0.1	0.2 ± 0.05	0.4 ± 0.06‖

* Recipient mice were irradiated with 750 rad of X rays 24 h before reconstitution and evaluated 15 d after transfer of 5 × 10^6 spleen cells.
‡ Significance level is $P < 0.05$ when compared with recipients of +/? spleen cells.
§ Significance level is $P < 0.01$ when compared with recipients of +/? spleen cells.
‖ Significance level is $P < 0.001$ when compared with recipients of +/? spleen cells.

macrophages in peritoneal lavage. Consequently, the macrophage defect in *op/op* mice appeared to be nontransplantable with *op/op* spleen cells.

Discussion

The hematological characterization of the *op/op* mouse revealed that these animals are deficient in their monocyte and macrophage populations. These mice possess few monocytes in the peripheral blood and few macrophages in their peritoneal cavity. However, the progenitor cells for macrophages and monocytes are present in these mice, as was demonstrated by the in vitro growth of macrophage colonies in the presence of CSA from media conditioned by adherent cells from the bone marrow of normal mice. Bone marrow and spleen cells from *op/op* mice did form adherent cell colonies when cultured in vitro; however, these colonies, when compared with those from normal littermate mice, were composed primarily of fibroblastoid cells, with extremely few macrophages. The macrophage defect in these *op/op* mice could not be transplanted into irradiated mice by *op/op* mouse spleen cells. In addition to these macrophage-related deficiencies, the *op/op* mice showed an altered organ distribution of hemopoietic stem cells with an increased number of these cells being found in the spleen and liver. Furthermore, these *op/op* mice were lymphopenic, yet possessed normal thymus cellularity.

These results strongly suggest that a primary lesion in this particular animal model of osteopetrosis results in the inability of the monocyte-macrophage cell line to fully differentiate. Previous characterization of this osteopetrotic mutant has revealed an absence of functional osteoclasts, the primary cells involved in bone resorption (3). The relationship between macrophages and osteoclasts has been strengthened by the observation that, in vitro, macrophages are capable of effecting osteoclast-like responses to bone resorption stimuli (27, 28). Specifically, the osteopetrosis in the *op/op* mouse would result from a defect in the ability of stromal fibroblastoid cells to produce monocyte-macrophage CSA, resulting in increased numbers of monocyte-macrophage progenitor cells and few mature cells. The lack of these mature cells

would preclude their fusion to form osteoclasts. Therefore, the clinical manifestations of osteopetrosis, resulting from the absence of osteoclasts, are proposed to be the result of the inability of the fibroblastoid cells to promote the maturation of monoblasts to monocytes, macrophages, and osteoclasts presumably because of a lack of production of functional CSA. CSA are a series of glycoproteins that display considerable structural and antigenic heterogeneity, depending on the tissue of origin (29), yet are highly specific with regard to their capacity to stimulate only macrophage or granulocyte maturation (29, 30) from progenitor cells. In the in vitro system used in these studies, the targets of the CSA were already committed cells for macrophage or granulocyte differentiation (25, 26), and it was possible that different CSA glycoproteins were present and responsible for the stimulation of maturation of each of these cell types. In the *op/op* mouse, for example, normal numbers of granulocytes were present. However, macrophage maturation proceeded only to the monoblast stage, suggesting that the *op/op* mouse does not completely lack all types of CSA. On the other hand, it is possible that an epitope of these glycoproteins is required for the full differentiation of each cell lineage and that one of these is absent in *op/op* mice. It is thus possible that it is merely a reflection of a quantitative or structural defect in the glycoprotein essential for this cell lineage. The purification and biochemical characterization of CSA glycoproteins will aid in the study of the different stimuli for macrophage differentiation.

Our data do suggest that the absence of macrophages in the *op/op* mice results from the absence of a biochemical signal for differentiation rather than from a deviation in differentiation. Hemopoietic cells from *op/op* mice can be stimulated to differentiate into macrophages in vitro when provided with a CSA from normal mice. Furthermore, while the injection of a nonspecific stimulant such as thioglycollate failed to induce the appearance of macrophages in vivo, the injection of MACCM as a source of CSA from normal mice into *op/op* mice resulted in the marked appearance of macrophages in the *op/op* mouse peritoneal exudates (unpublished observations).

The lack of the appropriate source of macrophage CSA in *op/op* mice rather than an abnormal monoblast was also suggested by the morphological appearance of fibroblastoid cell colonies from marrow and spleen of *op/op* mice. Fibroblastoid cells that form adherent cell colonies in liquid cultures of bone marrow cells are the precursor cells of bone stromal tissue (20, 31, 32). In vivo, the bone stromal tissue is one of the best sources of CSA (29, 33, 34). In cultures of marrow or spleen cells from normal mice, macrophages adhere directly to fibroblastoid cells. In contrast, the bone marrow cells cultured from *op/op* mice formed fibroblastoid cell colonies, yet these *op/op* cells did not support the attachment of the few macrophages that were present in these cultures to the fibroblastoid cells.

The macrophage defect of *op/op* mice could not be transferred into irradiated normal mice by spleen cells. This observation contrasts the osteopetrosis of both *mi/mi* and *gl/gl* mice mutants that are transferable by spleen cells (8, 9). The nontransferability of the *op/op* mouse disease supports an environmental defect rather than a defect intrinsic to cells of hemopoietic lineage. A similar situation may exist in the *tl/tl* rat model of osteopetrosis, which is resistant to cure by hemopoietic cells (19). This rat mutant is also similar to the *op/op* mouse in that the *tl/tl* rat possesses few macrophages and monocytes (W. Wiktor-Jedrzejczak, unpublished observation).

Our hypothesis that fibroblastoid cells from *op/op* mice are defective with regard to

their ability to stimulate macrophage differentiation by the production of a functional CSA is limited by the fact that other cells, such as lymphocytes (35) and macrophages (36) also produce CSA. However, the CSA produced by these cells may not be synthesized and/or functional in the assay systems used in our studies. The ultimate proof of the absence of an active macrophage CSA awaits the purification of CSA.

The abnormal distribution of stem cells and lymphopenia observed in the *op/op* mice are most probably secondary manifestations of osteopetrosis caused by the absence of a functional marrow environment. Indeed, in support of this, a similar abnormal distribution of stem cells was observed in estrone-induced osteopetrosis of mice (37). Similarly, the effects of a poor nutritional status, caused by the absence of incisors in these mice, cannot be disregarded, although earlier studies (2) of all murine osteopetrotic mutants failed to identify any common abnormalities that could be related to starvation of these mice.

In summary, our studies demonstrated a profound absence of mature macrophages in the *op/op* mice. Because these *op/op* mice do possess the progenitor cells for monocytes and macrophages, the defect appears to result from the absence of appropriate stimuli or environment for differentiation. The disease was nontransferable into irradiated mice, further indicating that the lesion resulting in osteopetrosis in *op/op* mice was environmental rather than caused by an abnormal stem cell or progenitor cell. The osteopetrosis of the *op/op* mice is similar to that observed in *tl/tl* rat (unpublished observations), in contrast to the lesions leading to osteopetrosis in *gl/gl* and *mi/mi* mice and *op/op* and *ia/ia* rats. The osteopetroses in these latter models are transferable by hemopoietic cells and corrected by transplantation of normal hemopoietic cells. Furthermore, our data strongly suggest that the environmental lesion in the *op/op* mouse resides in the inability of their hemopoietic stromal fibroblastoid cells to produce monocyte-macrophage CSA. The lesion has been demonstrated to be the result of a single autosomal recessive gene located on chromosome 3 (38). Therefore, this mutant may be useful for the studies of differentiation and functional role of macrophages.

Summary

Compared with normal littermates, the *op/op* mice had very few macrophages in the peritoneal cavity and severely reduced numbers of monocytes in the peripheral blood. Moreover, osteopetrotic animals demonstrated an altered distribution of hemopoietic tissue with a 10-fold decrease in the number of marrow cells. Liver hemopoiesis persisted in 4-wk-old mice as evidenced by the presence of hemopoietic stem cells (HSC). Moreover, the concentration of HSC was decreased in marrow and increased in the spleen of *op/op* mice. In spite of the paucity of cells of monocyte-macrophage lineage in vivo, progenitor cells from hemopoietic tissues of *op/op* mice formed increased numbers of monocyte-macrophage colonies in vitro in the presence of exogenous colony-stimulating activity (CSA). The source of this critical CSA was a medium conditioned by stromal fibroblastoid colonies formed in vitro by normal marrow cells. Therefore, these data suggest that *op/op* mice possess normal monocyte-macrophage-osteoclast progenitor cells but these cells are unable to fully differentiate in the *op/op* mouse microenvironment. In support of this, in cultures of stromal fibroblastoid colonies from *op/op* marrow or spleen, the concomitant growth of macrophages, normally very dense, was drastically reduced. Moreover, transplanta-

tion of *op/op* spleen cells into lethally irradiated littermate recipients resulted in their hemopoietic reconstitution without signs of macrophage defect. Thus, the *op/op* splenic cells do not transfer the disease and are capable of normal differentiation in normal in vivo environment. These observations support the hypothesis that the defect in *op/op* mice is a result of the failure of hemopoietic stromal fibroblastoid cells to release sufficient amounts of CSA necessary for normal differentiation of cells of the monocyte-macrophage lineage.

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