Bone Marrow Colony-Stimulating Factor Following Ureteral Ligation in Germfree Mice[1] (34658)

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Colonies of granulocytic and mononuclear cells of bone marrow origin can be grown in a semisolid agar medium under the stimulus of a factor, colony-stimulating factor (CSF), found in elevated titers in some sera and urine of humans and mice with leukemia or various hematopoietic and infectious diseases (1–4). *In vitro*, CSF controls both the incidence and the rate of proliferation of colonies of granulocytic and mononuclear cells of bone marrow origin. Continued presence of CSF is necessary for continued growth of these *in vitro* colonies (5). On the basis of the *in vitro* effect of CSF on colonies of granulocytic and mononuclear cells, it appears that CSF may be a leukopoietin. An *in vivo* function of CSF on leukopoiesis, however, is as yet undertermined. This is because of the relatively short *in vivo* half-life of CSF (4) and the last of large pools of a purified preparation of CSF.

In an attempt to produce large amounts of CSF, we have recently shown that unilateral or bilateral ureteral ligation elevates serum CSF for several days (6). In view of this finding, an *in vivo* study of the effects of CSF on the bone marrow colony-forming cells and peripheral blood leukocytes was performed in germfree mice 6 days following ureteral ligation. This study was undertaken in germfree mice for the following reasons: there is less variation in germfree mice, so that the numbers of peripheral blood leukocyte elements are more uniform and there is less variation in the incidence of bone marrow colony-forming cells (7); and serum titers of CSF are uniformly low in germfree mice (8). In addition, it seemed important to evaluate the effect of ureteral ligation on CSF in the absence of any potential for secondary bacterial infection.

Materials and Methods. Mice. Twelve-week-old germfree female Hauschka-Mirand ICR Swiss mice were obtained from the Charles River Breeding Laboratories (North Wilmington, Massachusetts), and were maintained in Trexler-type plastic germfree isolators (9). Inbred 8-week-old conventional DBA/1 males were obtained from the Roswell Park Memorial Institute West Seneca Breeding Colony.

Germfree isolation technique. Strict germfree isolation was maintained throughout the course of the study, with routine cultures of food and feces to detect any inadvertent break in technique (9). The surgical procedures were performed in the germfree isolators. Animals were removed from the germfree isolators immediately before collection of blood and bone marrows for analysis.

Bleeding. White blood cell counts, differential counts, and hematocrits were performed with blood obtained from the tail vein of an unanesthetized mouse. The mouse was anesthetized with ether and killed by bleeding from the axillary vessels, using Pasteur pipettes for blood collection. Clots were allowed to contract for 1 to 2 hr at room temperature, and the sera were stored at −20°.

Bone marrow collection. Using a hypodermic syringe and needle, the entire plug of bone marrow from a single femur was expressed into 2 ml of bone marrow collecting fluid (modified Eagle's medium with 10% fetal calf serum and 10% trypticase soy

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broth). A single cell suspension was prepared by repeated pipetting, and was counted in a hemocytometer chamber.

Assay for bone marrow in vitro *colony-forming cells.* Mouse bone marrow cultures were established, using a technique described extensively elsewhere (4, 10). In brief, double-strength modified Eagle's medium containing 20% fetal calf serum and 20% trypticase soy broth was mixed with an equal volume of 0.6% agar in distilled water. Sufficient bone marrow cells were added to aliquots of the mixture to give final cell concentrations of 5×10^4, 2.5×10^4, and 1.25×10^4 nucleated cells/ml of culture medium. Then 1 ml of each cell suspension was pipetted in a separate 35-mm plastic petri dish containing 0.02 ml of mouse serum from a pool of known colony-stimulating activity (4). Triplicate plates were prepared for each cell concentration.

Plates were allowed to gel at room temperature for 20 min, and were incubated without change of medium in a humidified incubator at 37° with a continuous flow of 5% CO_2 in air. Plates were scored after 7 days of incubation, using an $\times$ 30 dissecting microscope. The criteria for colony scoring have been described previously (1, 2). At these cell dosages, there was a linear relationship between the number of cells cultured and the number of colonies developing for a specified bone marrow. Accordingly, colony counts in plates containing different numbers of bone marrow cells were adjusted, and each count was expressed as the mean number of colonies per 10^6 cultured cells. This latter value was used in determining the number of *in vitro* colony-forming cells per femur.

Assay of serum colony-stimulating factor. Each serum of germfree mice to be tested was pipetted into 35-mm plastic petri dishes at doses of 0.0125, 0.025, and 0.05 ml. A single cell suspension of pooled DBA/1 bone marrow cells from one femur each of three mice was added to the bone marrow culture medium to give a final cell concentration of 50,000 nucleated cells/ml, and the mixture was held at 37°. A 1-ml aliquot was pipetted into each petri dish and mixed thoroughly with the serum. Incubation and scoring were as described for the colony-forming cell assay. As in previously reported work, there was found to be, in general, a linear relationship between serum dose and the number of colonies forming on each plate (1, 2). The data to be described refer only to colony stimulation by 0.025-ml dose levels, since this dose has allowed good discrimination between inactive and active sera, and is not subject to technical problems of surface drying frequently encountered with higher doses of mouse serum (2).

Operations. All operations were performed in the germfree isolator. Operative and sham-operative groups were anesthetized with 2 ml of pentobarbital injected intraperitoneally. The abdomen was opened through a midline incision, and the left ureter was securely ligated with a 4-0 silk ligature. The incision was closed with clips. The technique for the sham-operative animals was similar, except that instead of ureteral ligation, a small area of the lumbar abdominal wall was suture-ligated. The ureteral ligation and sham operation were performed on alternate mice, using a single set of instruments. At the completion of the study, all animals were autopsied, and all of the mice with ureteral ligations had developed hydronephrosis.

Results. Colony formation. Upon stimulation by active serum CSF, loose globular cell clusters developed in the agar. Between the

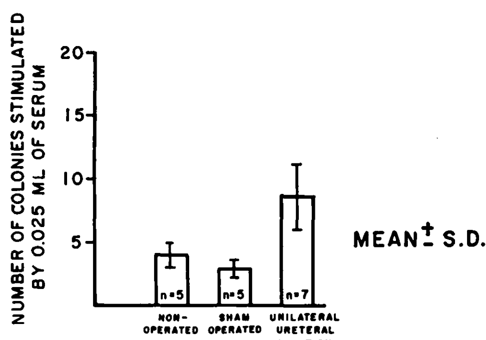

FIG. 1. Germfree mice 6 days after unilateral ureteral ligation. Numbers of *in vitro* granulocytic-mononuclear colonies developing 7 days after stimulation by 0.025 ml of serum collected 6 days after unilateral ureteral ligation, sham operation, or non-operative zero item. Vertical bars are standard deviations.

colonies were scattered single cells and microcolonies containing 2 to 10 cells. Colonies as scored at 7 days usually contained 20 to 500 cells.

Effect of ureteral ligation on serum colony-stimulating factor. When assayed 6 days after surgery, animals with unilateral ureteral ligation had serum CSF activities that were, on the average, four times as high as those of nonoperative controls. Sham-operative animals had levels of serum CSF equivalent to those in normal animals. The difference between the operative groups and the normal and sham groups was significant, p being less than 0.01 (Fig. 1).

Peripheral blood determinations. There was no significant difference among the hematocrits of the three groups of animals. Total leukocyte counts were slightly higher in the ureteral ligation group. There was no significant difference among the counts of the peripheral blood lymphocytes, monocytes, and the eosinophils for the three groups, although both eosinophils and monocytes were somewhat higher in the ureteral ligation group than in the sham and nonoperative groups. The average polymorphonuclear cell count of the animals with ureteral ligation was twice that of the control and sham-operative animals, a difference that is significant, p being less than 0.01 (Fig. 2a, b).

Bone marrow colony-forming cells. There was no detectable difference in the number of colony-forming cells per femur in each of the three groups of mice (Fig. 3). It should be noted that since the absolute number of colonies developing from a specified bone marrow depends upon the strength of the

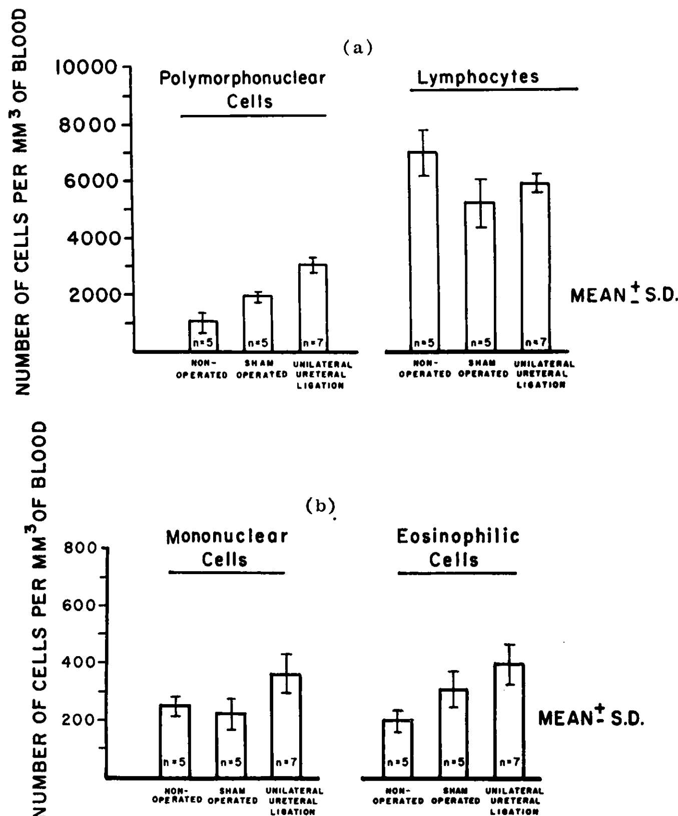

FIG. 2. Germfree mice 6 days after unilateral ureteral ligation. (a) Numbers of polymorphonuclear and lymphocytic cells; and (b) numbers of mononuclear and eosinophilic cells in peripheral blood from germfree mice 6 days after unilateral ureteral ligation, sham operation, or nonoperative zero time. Vertical bars are standard deviations.

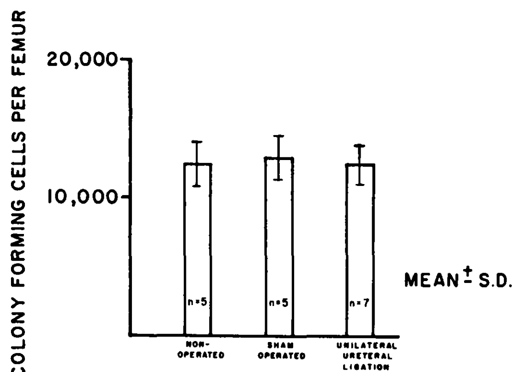

FIG. 3. Germfree mice 6 days after unilateral ureteral ligation. Numbers of *in vitro* colony-forming cells in the femoral bone marrow of germfree mice 6 days after unilateral ureteral ligation, sham operation, or nonoperative zero time. Vertical bars are standard deviations.

stimulating serum, the values for colony-forming units per femur are relative, and represent only a portion of the number of colonies that might develop after maximum stimulation.

Discussion. After unilateral ureteral ligation in conventional mice, CSF is significantly elevated at 72 hr, and remains elevated up to at least 1 week (6). The use of germfree animals in the present study rules out the possibility that the elevation of CSF is due to a proliferation of bacteria in the obstructed urinary system. CSF is excreted into the urine of both normal and leukemic humans (11, 12) and the elevation of serum CSF in mice following ureteral ligation is apparently due in large part to decreased renal clearance (6).

Ureteral ligation is also known to cause elevation of an erythropoietic factor (13, 14), erythropoietin. Although erythropoietin levels were not determined in the present studies, the probability that erythropoietin levels were also elevated should be taken into consideration when noting the effect of elevation of CSF on the bone marrow colony-forming cells. Other conditions (*e.g.*, anemia and anoxia) that cause elevation in erythropoietin, but not in CSF (15), cause a decrease in the number of *in vitro* colony-forming cells (16, 17). It has been suggested that the *in vitro* colony-forming cells and the erythropoietin-sensitive cells have a common precursor, and that increased demands on erythropoiesis shunt cells away from the *in vitro* colony-forming pool. In the present study, there was an elevation in CSF, and presumably also an elevation in erythropoietin, but no change in the number of the *in vitro* colony-forming cells. It is possible that there were opposing demands of erythropoiesis (mediated by erythropoietin) and granulopoiesis (mediated by CSF) influencing the *in vitro* colony-forming cell population. In other circumstances, where CSF alone is elevated, an increase in the numbers of *in vitro* colony-forming cells might be expected.

Other studies in mice with leukemia (1, 2) and following viral infection (4) have failed to demonstrate a consistent correlation between peripheral blood granulocytes and the level of CSF. Nevertheless, both infection and leukemia probably have various direct and indirect effects on leukopoiesis which make analysis complex. Germfree animals are advantageous because of the absence of a possibility for bacterial infection, the uniformity of their peripheral blood counts, the normally low levels of CSF, and the uniformity in the numbers of *in vitro* colony-forming cells. In the present study, the elevation in serum CSF titers following ureteral ligation was associated with an elevation in granulocytes in the peripheral blood, suggesting a granulopoietic function of CSF *in vivo*. The possibility that some regulatory mechanism other than CSF is altered by ureteral ligation and causes the granulocytosis cannot, of course, be excluded.

Proof of increased granulopoiesis in these mice must await kinetic studies, as an increased peripheral blood level in a dynamic population might reflect also a decreased destruction or change in the ratio of circulating to marginated cells. The findings in the present study are at least compatible with an *in vivo* granulopoietic function of the CSF which is capable of inducing granulopoiesis *in vitro*.

Until the source of the mononuclear cells in the *in vitro* colonies is settled (whether they are derived from granulocytic cells or

from a second precursor cell incorporated in and induced by the microenvironment of the granulocytic colonies), it seems best to regard CSF tentatively as a "leukopoietin" that may have more than simply granulopoietic effects.

Summary. Six days after unilateral ureteral ligation, serum colony-stimulating factor (CSF) is four times as high as in nonoperative germfree mice. The elevation in serum CSF is associated with a significant increase in peripheral blood granulocytes, but not lymphocytes, monocytes, or eosinophils. This finding supports the view that CSF not only is capable of stimulating proliferation of granulocytes and mononuclear cells *in vitro*, but also promotes granulopoiesis *in vivo*, and can be regarded as a leukopoietin.

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