Granulocyte Colony Stimulating Factor III. Effect of Alkylating Agent Induced Granulocytopenia[1] (35813)

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Present concepts indicate that hemopoiesis may be governed by the influence of certain humoral agents on immature precursor or stem cell compartments (1). Erythropoiesis is thought to result from the action of erythropoietin on an erythroid committed stem cell compartment (2). Recent data would also suggest that a circulating humoral agent may be involved in the control of thrombopoiesis (3). In addition, considerable attention has been directed to the study of possible humoral factors which may control granulopoiesis. However, owing to changes in granulocyte distribution resulting from the administration of various nonspecific materials such as foreign protein or endotoxin, *in vivo* responses are probably not an accurate reflection of granulopoietic activity (4).

To avoid the problems inherent in detecting granulopoietic factors *in vivo*, the present studies examine the response of an *in vitro* granulopoietic factor to induced granulocytopenia. Recent studies in irradiated mice have indicated variable results with respect to serum levels of this granulopoietic factor. Measurable increases in serum granulocyte colony stimulating factor (CSF) were not observed after 450 R of total body irradiation (5); whereas increased activity was found in animals given 800 R (6). The latter activity varied inversely with peripheral granulocyte counts; peak levels were noted at the nadir of neutropenia. The response of CSF following administration of cytotoxic agents has not been extensively studied. Mice treated with Methotrexate (7) and dogs treated with vinblastine (8) showed slight increases in urine and serum CSF, respectively, but these changes were apparent within 1–2 days of treatment, a time at which one would not anticipate significant granulocytopenia. We have recently shown that the induction of acute granulocytopenia in rats by a potent antineutrophil serum is associated with marked increases in serum CSF within 2–6 hr after injection (9). However, activity of this factor returned to control within 24 hr despite continued granulocytopenia.

The present studies extend the above observations to alkylating agent induced granulocytopenia in the rat. At intervals following treatment with cyclophosphamide, experimental animals were bled and their sera tested for CSF. The results indicate increased CSF activity at the nadir of peripheral neutropenia—a finding consistent with the concept that this material may be a regulator of granulopoiesis.

Materials and Methods. Female Sprague-Dawley rats, weighing 150–180 g, were used as serum donors for all studies. In individual experiments, groups of 50 experimental animals were randomized and given an intraperitoneal injection of either 100 mg/kg of cyclophosphamide or sterile saline solution. Five animals per treatment group were bled at daily intervals for study of total leukocyte counts, white blood cell differentials, and serum CSF activity. Saline control animals were evaluated on both the first and last day of each study. Blood for leukocyte counts was obtained under ether anesthesia by retro-orbital puncture; animals were then exsanguinated by cardiac puncture for serum CSF assays. Leukocyte counts were done using a model B Coulter counter after lysing the red cells with an acetic acid-cetrimide diluent.

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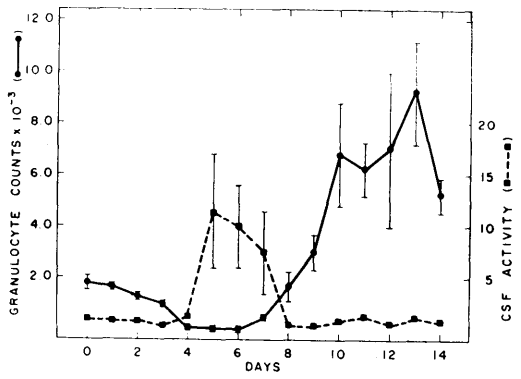

FIG. 1. Peripheral blood granulocyte and serum CSF values at intervals after cyclophosphamide: Points depicted on days 1 through 7 represent the mean ± 1 SE for 10 animals each (2 replicate experiments, 5 animals/day). Days 8 to 14 give the result of a single experiment, 5 animals/point. Serum CSF activity is expressed as the mean number of colonies stimulated by 0.1 ml of test serum. Five culture plates were used for each individual serum assay. CSF activity differed from control observations at the 5% significant level on days 5, 6 and 7 (Kruskal-Wallace H test).

White cell differentials were obtained on Wright-Geimsa stained smears; they were based on evaluation of 200–500 cells.

Serum CSF activity was assayed using minor modifications of the Bradley and Metcalf technique (9, 10). Normal bone marrow cells obtained from CF_{1s} mice were mixed with supplemented McCoy's medium and Bacto agar. Dilutions were such that 1 ml of the final mixture contained 10^5 bone marrow cells in a 0.3% agar suspension. Individual 10 × 35-mm culture plates were prepared by adding 0.1 ml of the rat serum to be tested to 1 ml of the marrow agar suspension; 5 plates were prepared for each test serum. Positive control plates contained an active CSF harvested from the growth of mouse fibroblast cells; negative controls contained only the marrow agar suspension. Following 4-days incubation in a humidified 7.5% CO_2 atmosphere, discrete colonies containing 8 or more cells were enumerated with the aid of a dissecting microscope (20X).

Results. The mean results of three experiments are shown in Fig. 1. After a single injection of cyclophosphamide, only modest changes in circulating granulocyte levels were noted for the first 3 days. Counts decreased to 1150/mm^3 on the third day, a value not significantly different from controls of 1800/mm^3. By the fourth day, a further decrease to 250 cells/mm^3 was observed. During this interval virtually no CSF activity could be detected. Generally 0.1 ml of these sera stimulated 0–2 colonies, a value identical to that obtained with sera from saline-treated animals.

Although variation existed among sera from individual animals, a significant increase in CSF was noted by the fifth day after treatment. Serum activity rose from mean values of 0.5 to 1.2 colonies in controls to 11.4 ± 5.8 colonies in cyclophosphamide-treated animals. Heightened activity persisted through the seventh day, a time at which a slight rise in granulocyte levels was evident.

Granulocyte counts returned to normal levels by day 8 (1745 ± 680) with an overshoot to peak levels of 9300 ± 2065 on day 13 of the study. Concurrently, serum CSF activity returned to base line by day 8 and remained in the range of 0.5–1.3 colonies through day 14.

Discussion. Since the introduction of a technique for the clonal growth of bone marrow cells *in vitro* (10, 11), there has been considerable speculation both as to the nature of the cell type involved and to the identity of the factor or material responsible for colony growth. The cell type responsible for *in vitro* colony formation has not yet been morphologically identified. This cell population appears to differ from that responsible for hemopoietic repopulation after irradiation (12) both on the basis of cell replication and kinetic behavior. Approximately 10% of pluripotential stem cells (spleen colony forming units of CFU) are in DNA synthesis as measured by cell kill with either tritiated thymidine or hydroxyurea (13, 14). In contrast, the *in vitro* colony forming cell is an actively replicating cell; approximately 40–50% of the normal population are in DNA synthesis when measured by either of these techniques (15, 16). Further, the growth of this population following irradiation seems to

differ from the growth of CFU paralleling or perhaps preceding expansion of the myeloblast–promyelocyte compartment (17). From these observations, it has been suggested that the *in vitro* technique measures a granulocyte committed stem cell, a concept analogous to the erythroid committed stem cell (2) which is thought to be responsive to erythropoietin.

The material responsible for colony growth, colony stimulating factor (CSF), has been derived from a number of sources including kidney cells (10), embryonic tissue (18), fibroblasts (19), leukocytes (20), and certain sera (21) and urinary extracts (22). Although the diversity of sources might suggest that this material is a nonspecific growth factor, extensive supplementation of the tissue culture media with known nutrients does not lead to appreciable colony formation in the absence of CSF. Therefore, it would appear that CSF may represent a specific granulocyte stimulating factor.

At present, it is uncertain whether CSF may be a true granulopoietin despite its capability of inducing extensive proliferation and maturation of immature granulocyte precursors *in vitro*. Although it may be assumed that a granulopoietin would be released in response to granulocytopenia, only limited data is available regarding CSF levels with induced neutropenia. Studies in which CSF was determined at intervals after irradiation have led to conflicting results. Initial observations in mice indicated no appreciable change in serum CSF following 450 R of total body irradiation (5). More recent studies in which higher doses of irradiation were employed, showed heightened CSF at the nadir of neutropenia; levels of activity bore an inverse relationship to circulating granulocyte levels (6).

Our earlier studies indicated that acute neutrophil depletion in rats, using an antineutrophil serum, was associated with a marked increase in serum colony stimulating factor (9). The present studies extend and confirm these observations. However, cyclophosphamide-treated animals differ from those given antineutrophil serum in that peripheral granulocyte depletion is delayed, as is the CSF response. Moreover, the magnitude of response following administration of this alkylating agent does not approach that seen with acute granulocyte depletion. The latter difference may in theory be a manifestation of CSF turnover (9). Although utilization of CSF has not been demonstrated *in vivo*, consumption of this material appears to parallel the growth of developing granulocytic colonies *in vitro* (23).

The appearance of increased serum CSF after granulocytopenia induced by irradiation, or, as in the present experiments, after cyclophosphamide, suggests that CSF may be responsible for *in vivo* recovery of granulopoiesis. Further findings of a diffusable serum factor 3.5 to 4 days after irradiation, capable of stimulating the growth of myeloid cells implanted in Millipore chambers *in vivo* (24), suggest that certain humoral agents are involved in the recovery of granulopoiesis. The present observations suggest that CSF may be such a granulopoietin. However, it remains to be demonstrated that this factor is active *in vivo* and that it is responsible for control of normal granulopoiesis.

Summary. At intervals following a single injection of cyclophosphamide, groups of rats were tested for the presence of an *in vitro* granulocyte colony stimulating factor (CSF). Increased serum CSF activity was demonstrated on days 5 through 7 after injection, corresponding to a phase of marked peripheral granulocytopenia. The CSF returned to base line levels as peripheral blood granulocytes returned to normal. Increased elaboration of CSF in conjunction with granulocytopenia is in accord with a possible role of this material as a regulator of granulopoiesis.

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