Stimulation of Granulocytopoiesis by a Diffusible Factor In Vivo

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ABSTRACT A millipore diffusion chamber system was used to cultivate mouse marrow in the abdomens of irradiated and unirradiated host mice for 24 hr. When the irradiated hosts were 72, 96, or 120 hr postirradiation, the number of blasts and promyelocytes in the implanted chambers after cultivation was greater than those in the same marrow cultivated in unirradiated hosts. These data indicate that in vivo, there is stimulation of granulocytopoiesis by a diffusible factor or factors.

INTRODUCTION

The concept that one or more soluble substances may be responsible for the homeostatic control of granulocytopoiesis is supported by recent experimental evidence. The demonstration that hematopoietic colony formation in vitro in agar is enhanced by a factor in mammalian urine or plasma (1-3) suggests that this factor may play a role in the regulation of granulocyte production in vivo. In other studies, DNA synthesis in vitro by granulocyte precursors was enhanced by a plasma factor from leucapheresed animals, as well as retarded by a soluble factor from viable granulocytes (4).

The demonstration that such factors control granulocytopoiesis in vivo has been difficult because such a demonstration requires (a) that the marrow cells affected be accurately quantified and (b) that the test marrow be isolated from cell-mediated stimuli.

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The purpose of this study is to demonstrate an endogenous diffusible granulocytopoietic activity which acts in vivo. A modification of the millipore diffusion chamber of Algire (5) to permit quantitative recovery and morphologic identification of cultivated marrow cells made this demonstration possible.

METHODS

18-21 g male CBA mice (Jackson Laboratories, Bar Harbor, Maine) were used both as marrow donors and as recipients in which millipore chambers containing marrow were implanted for 24-hr cultivation periods.

Blood leukocyte concentration was determined on single samples in triplicate by means of an electronic counter¹ using 0.2 ml vena cava blood anticoagulated with EDTA, 1 mg/ml, and the mean value recorded. 50-200 cell differential counts were done on cover slip smears prepared with a sample spinner² and stained with Wright's stain.

Marrow suspensions for cultivation in millipore chambers were prepared under sterile conditions by flushing the tibias from normal donor mice with Hank's basic salt solution (HBSS)³ in the manner described by Chervenick and co-workers (6). The mean of triplicate electronic counts was recorded and 500 cell differential counts were done on cover slip smears made from the cell suspension (6).

The chambers for marrow cultivation were made by centering 13 mm diameter millipore filters with grid (pore size 0.45 μ) over 11 mm holes punched in Mylar adhesive tape so that the nongridded side adhered to the tape. Then two tape-filter units were joined adhesive side inward with an opening preserved by a toothpick. All chamber surfaces were sterilized in ultraviolet light for 30 min. Hamilton syringes were sterilized with ethylene oxide and used to load the chambers with 0.05 ml of marrow suspension. Care was taken to keep the entry track of the chamber dry, to facilitate sealing. A number of chambers could be loaded with portions of the same suspension, and loaded chambers

¹ Coulter Electronics Industrial Div., Hialeah, Fla.

² Plat model 45T, Plat Engineering Co., Cheltenham, Pa.

³ *Abbreviations used in this paper:* HBSS, Hank's basic salt solution; ³HTdR, tritiated thymidine.

were stored in HBSS until implantation. Using methoxyflurane anesthesia under sterile conditions, chambers were implanted intraperitoneally through a mid-line surgical incision. 2 hr later, 100 U of heparin⁴ was given intracutaneously and 100–150 U every 12 hr thereafter. The recipient mice were handled in groups; one group served as control recipients and one group received 370 rad of total body irradiation 24, 48, 72, 96, or 120 hr before chamber implantation. Chambers were left in the abdomen for 24 hr during which period the mice moved freely in their cages.

The mice were sacrificed by cervical dislocation after the 24 hr cultivation period and the chamber removed for examination of the contents.

RESULTS

In order to count the cells recovered from diffusion chambers, they must be in free suspension. In previous studies with the millipore chamber system, clots formed in the chamber and these were studied by sectioning techniques (5). In the present studies, clotting was prevented by giving heparin, 100 U intradermally, to the host animal 2 hr after chamber implantation and 100–150 U every 12 hr thereafter; coagulation was prevented in all but a rare chamber.

Quantification of chamber contents. After the cultivation period the chambers were retrieved and placed in HBSS. The chambers were inspected for leaks and the exterior cleaned with moist gauze. The tape margin was cut away, and the chamber opened in 0.5 ml of HBSS. The edge of each filter was used to gently abrade the interior surface of the other before both filters were fixed in methanol. Cells collected this way are free of clumping. The cells in the HBSS were counted electronically in triplicate and the mean recorded. The remaining suspension was centrifuged and the cells smeared on a cover slip. A 500 cell differential count was done on each smear. In addition to cells freely floating in the HBSS, other cells were adherent to the chamber surfaces. To quantify these, filters were examined microscopically and cells in the same focal plane as the grid lines considered to be within the chamber. 10 chambers were loaded with $0.75\text{--}1.6 \times 10^6$ marrow cells, cultivated intraperitoneally for 36 hr, and the freely floating cells enumerated. Then, the filter surfaces were evaluated by counting the cells in 10 fields representing 1.2% of the total filter area and the total cells adherent calculated. The number of adherent cells was always <10% of those free floating, and the number of mitotable granulocytes always <4% of those free floating. Thus, in all experiments, the filters are compared with those from the above experiment and if the adherent cells do not exceed those on the reference filters, the freely floating cells are accepted as representative of the culture.

The cell-tight nature of the chamber. The ability of

FIGURE 1 The effect of 370 rad whole body irradiation upon hematologic tissue of CBA mice. The means and ranges for groups of 10 animals are shown.

the chambers to retain their contents and exclude cells from the host was tested. In one study, 10 chambers were filled with 1.5–2.5 million marrow cells. After incubation of the sealed chambers in vitro for 6 hr in HBSS, no cells were found in the incubating medium after concentration by centrifugation. In another study, 13 empty chambers were implanted in mice. 24–36 hr later, the chambers were retrieved and opened into an HBSS wash. The centrifuged wash fluid contained no cells, either by electronic count or smear. Also the interior filter surfaces were examined, and no contamination with host cells was seen. Bartlett and Prehn also studied adhesive tape skeleton millipore chambers of the

⁴ The Upjohn Company, Kalamazoo, Mich.

FIGURE 2 Blasts plus promyelocytes from bone marrow cultivated in chambers for 24 hr in irradiated or unirradiated hosts. Each data point represents the value for one chamber.

same pore size and found them to be cell-tight to host leukocytes and Erlich ascites tumor cells (7).

Demonstration of an in vivo diffusible stimulator of granulocytopoiesis. To demonstrate a diffusible neutropoietic substance, chambers were loaded with normal marrow and placed in host animals. After a 24 hr cultivation period during which any diffusible substances from the host may act upon the normal target marrow, the chamber contents were examined and quantified.

Host animals were divided into two groups. One group received 370 rad X-irradiation with a 250 kv machine at 50 cm. Dosimetry was carried out with a Farmer secondary standard dosimeter.⁵ This is a sublethal dose that results in neutropenia and myelosuppression (Fig. 1). The other group was unirradiated and served as controls.

In these studies, then, both unirradiated and irradiated mice served as hosts for chambers filled with normal

⁵ Farmer Electric Products, Inc., Natick, Mass.

target marrow 0.8–1.6 million cells. Host mice groups were always paired (irradiated and control) so that control and irradiated hosts received chambers with the same number of cells from the same marrow suspension. In this way, the marrow response in an irradiated host is only compared with the response of the *same* marrow in an unirradiated host. A series of 24-hr cultivations were then performed in hosts irradiated 24, 48, 72, 96, or 120 hr before the cultivation period began. After the cultivation period, the chamber contents were quantified and the results in irradiated hosts expressed as per cent of the mean control value. The chamber surfaces were examined, and the number of adherent cells did not differ between the two groups. The number of myelocytes, metamyelocytes, or polymorphonuclear neutrophil leukocytes (PMN) in the chambers harvested from control and irradiated hosts did not differ significantly after 24 hr of cultivation at 24, 48, 72, 96, or 120 hr postirradiation.

FIGURE 3 The marrow cell uptake of ³HTdR after cultivation in chambers for 24 hr. The disintegrations per minute are expressed as per cent of mean control value, with each data point representing the value for the cells from one chamber.

tion. However, the number of blasts plus promyelocytes was greater in chambers cultivated in irradiated hosts 72, 96, and 120 hr after irradiation than in chambers from the corresponding controls (Fig. 2). In similar studies, the uptake of tritiated thymidine ($^3\text{HTdR}$) by chamber contents was measured. After the 24 hr cultivation period within the host, the target marrow from each chamber was incubated in HBSS which contained $^3\text{HTdR}$, 2.5 $\mu\text{Ci/ml}$, for 1 hr. Then the cells were centrifuged, washed three times in HBSS, and the radioactivity measured in a scintillation counter. The results are shown in Fig. 3. The $^3\text{HTdR}$ uptake by marrow samples cultivated in 120-hr postirradiation animals clearly exceeds the uptake of the control group. In studies carried out 72 hr postirradiation, only two values in the irradiated group exceed the control range.

DISCUSSION

Quantitative study of millipore chamber contents appears to be a suitable assay system for the detection of humoral, granulocytopoietic activity. With this system, marrow can be cultivated *in vivo* and then recovered for morphologic and quantitative study. In the present studies, neutropenic irradiated host animals were capable of stimulating the target marrow even though host cells were excluded, indicating that a diffusible neutropoietic substance entered the chambers and promoted the growth of early granulocyte precursors. Interestingly, the stimulation effect was detected only when the host animals had been irradiated 72 or more hr before chamber implantation. This interval coincides well with the time at which marrow cellularity increases in the shielded hindlimbs of irradiated mice, as shown by Morley and Stohlman (8). At the same time after irradiation, a rise in the *in vitro* colony-forming cells in the shielded limb occurs, and a rise in the colony-stimulating activity of the serum is found (9, 10). It is not yet clear how the present *in vivo* studies relate to the *in vitro* demonstration of granulocytic colony-stimulating activity in the agar plate system (2, 3, 11). Whether the phenomenon of colony formation *in vitro* represents the same event as the promotion of blast plus promyelocyte growth in the millipore system *in vivo* is an open question. However, it does appear that the diffusible stimulator in our system acts upon a cell more primitive than the myelocyte, since only the blast plus promyelocyte category

differed when the irradiated and control groups were compared. The nature of the stimulator in the present studies is yet another question. In these studies, we have seen that a diffusible stimulator of granulocyte production does appear and that it is present in sufficient concentration *in vivo* to exert a granulocytopoietic effect upon normal marrow.

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