Effects of Microbial Flora on Levels of Colony Stimulating Factor in Serums of Irradiated CFW Mice[1] (37551)

CHIENKUO F. CHANG AND MORRIS POLLARD

Lobund Laboratory, University of Notre Dame, Notre Dame, Indiana 46556

Colony stimulating factor (CSF), a glycoprotein produced by most murine tissues (1), is required for the *in vitro* development of colonies of granulocytes and/or macrophages from precursor cells in the bone marrow (2). Morley *et al.* (3) reported that the CSF activity in the serum of mice increased after radiation. They observed that the level of serum CSF activity was inversely related to the peripheral neutrophil count and suggested that the production of CSF was related to the degree of neutropenia. Recently, Morley *et al.* (4), found that there was no elevated serum CSF level in X-irradiated germfree mice; so they postulated that the bacterial flora of the intestine may play a role in the increase of serum CSF after irradiation. This is in accordance with the report by Metcalf, Foster and Pollard (5) that serum levels of CSF were lower in germfree than in conventional Swiss mice. In order to clarify the role of microbial flora on serum CSF level, we determined serum CSF levels after irradiation in germfree, in specifically monoassociated, and in conventional mice.

Materials and Methods. Male CFW mice, 6–8 wk of age, were used for the germfree and conventional experiments. The germfree mice were housed in Trexler and Reynold's plastic isolators (6). They were free of horizontally transmitted bacteria, fungi, and macroparasites (7), but they were infected congenitally with leukemia virus, as in all other strains of mice (8). The conventional mice had an undefined, but pathogen-free, microbial flora. One group of germfree mice was associated with gram-positive *Streptococcus faecalis* 547, and another group was monoassociated with gram-negative *Escherichia coli* 7. The latter organism has a high potential for endotoxin production. The organisms were each propagated in nutrient broth for 24 hr, and the culture was sealed aseptically in glass ampoules. The surface of the ampoule was sterilized with 2% peracetic acid, and it was introduced into the isolator through a double-entry lock which was sterilized with 2% peracetic acid. A cotton swab, moistened with the bacterial culture, was used to infect the oral cavity of each animal to insure uniform contamination. The excess broth culture was then added to the drinking water. One day later, the mice were exposed to whole-body X-irradiation. The X-ray source was a 260 kVp clinical therapy X-ray machine operated at 245 kV and 15 mA with filtration of 1.0 mm Al and 0.25 mm Cu (HVL = 1.05 mm Cu) at a rate of approximately 38 R/min as measured in air with a Victoreen condenser R-meter. Mice were given a dose of 850 R, and at various intervals after irradiation, they were bled and killed. The blood was stored at room temperature until clot retraction allowed collection of serum. Serums were dialyzed (3 days with 3 changes of water at 4°); centrifuged (27,000*g* for 30 min at 4°); passed through a 0.45 μm Millipore filter, and the filtrate was stored at −20° until assayed.

Bone marrow cells were prepared by flushing out the femurs of mature CFW mice with Eagle's MEM solution and a syringe and needle. Approximately 10^7 of the pooled cells were injected into the tail vein of each mouse following exposure to 850 R X-rays. The irradiated control mice were not trans-

[1] Supported in part by grants from the Little Red Door, Marion County Cancer Society, Inc., Indianapolis, IN, and NIH Grant RR 00294.

fused with bone marrow cells.

The bone marrow cell culture technique has been described in detail by Metcalf and Foster (9). In brief, bone marrow plugs were collected from pooled femurs of three 2 mo old conventional CFW mice. Single cell suspensions were prepared by pipetting the plugs repeatedly in 3 ml of modified Eagle's medium, containing 10% fetal calf serum and 10% trypticase soy broth. 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% Difco bactoagar in water. This mixture was held at 37°, and bone marrow cells were added to this mixture to give a final concentration of 10^5 cells/ml. Mouse serum to be tested was pipetted (0.1 ml) into sterile 35 mm plastic petri dishes. One milliliter of the bone marrow suspension in agar medium was then added to each plate. The serum was mixed thoroughly with the agar-cell medium which was then allowed to gel at room temperature for 20 min. The cultures were incubated at 37° in a humidified incubator with a continuous flow of 5% CO_2 in air. After 7 days of incubation, the plates were examined at 40 × magnification, and clusters of cells containing more than 50 cells were scored as colonies.

Results. Sera from conventional mice were tested for CSF at varying intervals after exposure to 850 R X-rays. As indicated in Fig. 1, the CSF level increased abruptly after an interval of 3 days, and reached a peak at 8 days. At 9 days, all of the mice were dead of bacteremia. In groups of mice that were transfused with bone marrow cells after X-irradiation, there was a small increase in CSF level, and this level returned to normal (0–5 colonies/plate) at 9 days after irradiation. Germfree CFW mice did not produce increased levels of CSF after administration of 850 R X-rays. As indicated in Table I, gnotobiotic mice, monoassociated with *S. faecalis* developed a CSF pattern following X-irradiations, comparable to that of X-ray

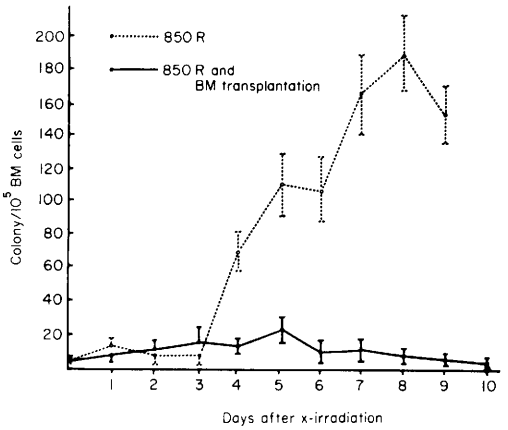

FIG. 1. Serum CSF levels in CFW mice at intervals after 850 R X-irradiation (whole-body) with and without subsequent bone marrow transplantation. The values represent numbers of colonies developing from 1 × 10^5 mouse bone marrow cells ± standard deviations of the means.

TABLE I. Comparison of CSF Activity in Sera of Germfree and of Monocontaminated CFW Mice Irradiated with 850 R.[a]

	Germfree	*E. coli* monocontaminated	*S. faecalis* monocontaminated
Control	0.25 ± 0.5	0	0.35 ± 0.5
Day after irradiation			
1	0	0	0
2	0	0	0
3	0	0.25 ± 0.5	0.5 ± 0.57
4	0.3 ± 0.3	30.3 ± 3.05	0.5 ± 1.0
5	0	30.6 ± 3.05	0.75 ± 0.90
6	0	53.0 ± 3	0.75 ± 0.90
7	0.25 ± 0.25	50.3 ± 5.25	0.25 ± 0.50
8	0	—	0.25 ± 0.50

[a] Results are expressed as the mean ± standard deviation of the number of colonies observed/10^5 marrow cells.

TABLE II. CSF Levels in Germfree and Conventional CFW Mice 3 hr After Injection of 5 μg of *Salmonella typhosa* Endotoxin.

	Control	Endotoxin
Conventional mice	2.6 ± 2.5	166.7 ± 17.3[a]
Germfree	0.25 ± 0.5	143.5 ± 12.9

[a] Mean ± standard deviation.

treated germfree mice: there was very little detectable CSF activity in the serum over the entire observation period. However, sera from irradiated germfree mice which had been monoassociated with *E. coli* showed elevated CSF levels at 4 days after X-irradiation. The increase in serum CSF levels were not as high as in irradiated conventional mice. Germfree mice have lower levels of serum CSF, but following intraperitoneal injection of endotoxin, the serum levels of CSF increased rapidly, with levels comparable to those demonstrated in conventional mice (Table II).

Discussion. These experiments show that gram-negative bacteria are involved in the increased CSF levels after whole-body X-irradiation. Hall (10) has attempted to detect CSF levels in mice after administration of 450 R X-rays. She found a temporary increase in the first 24 hr, but could not detect a definite increase thereafter. Morley *et al.* (3) found that CSF levels in serum from irradiated mice were related in both time and magnitude to the neutropenia which developed following irradiation. They suggested that neutropenia results either in an increased rate of production of CSF, or in a decreased rate of destruction of CSF. These two possibilities are not mutually exclusive. However, they could not detect increased CSF level in irradiated germfree mice (4) and germfree mice like conventional animals, developed postirradiation neutropenia (11). This suggested that the bacterial flora of the gut would have a role in the level of serum CSF following X-irradiation. In this report we could not detect increased serum CSF levels in X-irradiated germfree mice which had been contaminated with gram-positive *S. faecalis*. In contrast to this, X-irradiated germfree mice which had been monocontaminated with gram-negative *E. coli* showed increased CSF levels in serum comparable to levels in their conventional counterparts. These results indicate that gram-negative bacteria play a role in serum CSF levels following irradiation, and this may be due to the production of endotoxin by such microorganisms. This is supported by (a) the time of appearance of serum CSF after irradiation which approximates the time at which one begins to detect endotoxinemia (12), and (b) by the production of high levels of CSF in both germfree and conventional mice following injections of endotoxin. However, addition of endotoxin to normal serums did not lead to the development of CSF activity (13).

In conventional CFW mice, we found that transplantation of bone marrow cells following 850 R X-irradiation, reduced the serum CSF level which was found in untreated irradiated mice. This could be due to absorption or inactivation of CSF by bone marrow cells (14), or to the restoration of immunocompetence which would reduce the extent of endotoxinemia. The decrease in bone marrow cell populations has been found to be similar in both germfree and conventional animals: by Day 4 after exposure to 700 R, the bone marrow was almost aplastic (11). If the absorption of CSF by bone marrow cells could explain the reduction of CSF in the group which was transplanted with bone marrow cells, then irradiated germfree mice should have elevated levels of CSF in their serums. Since this was not the case, the best explanation is that transplantation of bone marrow cells restored the immunocompetence of the mice, which protected them from endotoxinemia and the resulting associated increase in CSF levels.

The mechanism by which endotoxin influences the level of serum CSF is not clear. Morley *et al.* (14), based on the relation between granulocytopenia and the appearance of CSF in endotoxin-injected mice, suggested that destruction of granulocytes may release CSF, or that granulocytes may bind and neutralize CSF; and that destruction of granulocytes results in increased levels of serum CSF. Shadduck and Nagabhushanam (15)

have induced granulocytopenia by the injection of antineutrophil serum. They demonstrated significant increases in levels of CSF which corresponded to the phase of rapid neutrophil destruction. But they could not detect increased serum CSF levels in mice with cyclophosphamide-induced granulocytopenia; nor could they detect increased CSF when leukocytes were destroyed *in vitro* by antineutrophil serum or by physical methods. This problem requires further clarification.

Summary. Germfree, specifically monoassociated with bacteria, and conventional CFW mice were evaluated for changes in serum CSF levels following 850 R whole-body X-irradiation. Gram-negative *E. coli*-monoassociated mice showed elevations of CSF levels similar to that in conventional mice. However, CSF levels in mice monoassociated with *S. faecalis* remained unchanged, as in germfree mice. In mice with bone marrow transplantations following X-irradiation, serum CSF levels were maintained at the level of untreated mice, which indicates that CSF levels are regulated by functioning bone marrow cells. The data also indicates that the changes in CSF levels that are associated with gram-negative bacteria may be due to the production of endotoxin, which is involved in levels of serum CSF; which, in turn, may play a role in regulating granulopoiesis.

We express our thanks to Dr. Donald Metcalf for his valuable advice in implementation of this project.

1. Sheridan, J. W., and Stanley, E. R., J. Cell. Physiol. **78**, 451 (1971).

2. Robinson, W. A., Metcalf, D., and Bradley, T. R., J. Cell. Physiol. **69**, 83 (1967).

3. Morley, A. A., Rickard, K. A., Howard, D., and Stohlman, F., Jr., Blood **37**, 14 (1971).

4. Morley, A. A., Quesenberry, P. J., Bealmear, P. M., Stohlman, F., Jr., and Wilson, R., Proc. Soc. Exp. Biol. Med. **140**, 478 (1972).

5. Metcalf, D., Foster, R., and Pollard, M., J. Cell. Physiol. **70**, 131 (1967).

6. Trexler, P. C., and Reynolds, L. I., Appl. Microbiol. **5**, 406 (1957).

7. Wagner, M., Ann. N. Y. Acad. Sci. **78**, 89 (1959).

8. Kajima, M., and Pollard, M., Nature (London) **218**, 188 (1968).

9. Metcalf, D., and Foster, R., J. Nat. Cancer Inst. **39**, 1235 (1967).

10. Hall, B. M., Brit. J. Haematol. **17**, 553 (1969).

11. Heit, H., Fliedner, T. M., Fache, L., and Schnell, A., Radiat. Res. **41**, 163 (1970).

12. Wilson, R., Barry, T. A., and Bealmear, P. M., Radiat. Res. **41**, 89 (1970).

13. Quesenberry, P. J., Morley, A., Stohlman, F., Jr., Rickard, K., Howard, D., and Smith, M., N. Engl. J. Med. **286**, 227 (1972).

14. Morley, A. A., Rickard, K. A., Quesenberry, P. J., Garrity, M., and Stohlman, F., Jr., J. Cell. Physiol. **77**, 301 (1971).

15. Shadduck, R. K., and Nagabhushanam, N. G., Blood **38**, 559 (1971).

Received March 12, 1973. P.S.E.B.M., 1973, Vol. 144.