Colony-Stimulating and Inhibiting Factors in Sera from Normal and Endotoxin-Treated CF_1 Mice[1] (37280)

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Bone marrow cells can be grown successfully *in vitro* by using an agar culture technique (1). Both granulocytic and macrophage colonies are formed in the presence of an appropriate stimulus which may be derived from a variety of sources and is termed colony stimulating factor or CSF. Sera from normal CF_1 mice are either devoid of or have very low levels of CSF (2, 3), but when these mice are irradiated (4) or treated with endotoxin (3), significant levels of CSF can be detected in the serum. A reasonable explanation for this is increased CSF generation. It was found, however, that normal serum contains factors which inhibit the growth of colonies in soft agar (3, 5, 6). From this it might be argued that the increase in CSF was the result of a decrease in inhibition rather than a true increase in CSF production. Support for such a mechanism is provided by the observation of Stanley *et al.* (7), who demonstrated CSF activity after removal of inhibitory material from the previously inactive sera of C_3H or BALB/C mice. The present study was done in order to ascertain whether increases in serum CSF seen in the CF_1 mouse after various perturbations reflected an increase in production of CSF or were due to a decrease in inhibitory factors.

Materials and Methods. Adult female CF_1 mice (Carworth Farms) were used throughout the study. Serum was collected from animals given 50 μg or 5 μg of *S. typhosa* endotoxin (Difco) intraperitoneally 2 hr or 6 hr prior to bleeding. Normal sera was obtained from untreated mice. Sera collected on seven different occasions from control and endotoxin-treated mice were separated and tested for stimulatory and inhibitory factors. Conditioned medium was prepared from mouse embryos by the method of Bradley and Sumner (8) and concentrated by utilizing an Amicon ultrafiltration cell with a PM 10 membrane. Both serum and conditioned media were fractionated on a Sephadex G-150 column at room temperature using a 0.1 *M* phosphate buffer of pH 7.4 as the eluant. Radio-iodinated human serum albumin (^{131}I-RISA) served as a marker. In order to effect a separation of the inhibitory material, some sera were treated with ether and others were dialyzed according to the methods described by Chan *et al.* (6). Rat red cell concentrations were prepared by the method of Bradley *et al.* (9). Rat blood was collected in heparin by cardiac puncture, the buffy coat removed after centrifugation and the erythrocytes washed 5 times in 0.85% saline with added penicillin and streptomycin. Packed cells were then resuspended in an equal volume of saline and the cells used within 1–2 days at a dose level of 0.1 cc per petri dish. All of the material to be tested was sterilized by Millipore filtration through a 0.45 μm membrane and stored at $-20°$.

Colony-stimulating activity was assayed by the single layer soft agar technique of Metcalf and Foster (10) as modified by Rickard

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et al. (2). Two different doses, 0.05 and 0.2 ml of test material, were used for the stimulator assay. The fractions were also tested for their ability to inhibit colony formation by active endotoxin serum. In this case, a dose of 0.15 ml from various fractions was pipetted into each petri dish and mixed with 0.05 ml of an active endotoxin serum and the number of colonies per 10^5 normal marrow cells noted. Three plates were cultured for each dose level in both the stimulatory and inhibitor assays. In four additional experiments a double-layer agar technique (8) was employed. The source of CSF was mixed with Eagle's media and agar so that the final concentration of agar was 0.5%. One milliliter of this mixture was used for the underlayer. The overlayer consisted of 1.0 ml of Eagle's media and agar with a final concentration of agar of 0.3%. The marrow cells to be assayed were mixed with the latter to give the appropriate concentration of cells (usually 1.0×10^5/ml). This technique tends to reduce the influence of inhibitory factors.

The endotoxin concentrations in serum or fractions of serum were measured using the Limulus assay (11).

Results. The sera from endotoxin-treated mice uniformly had significant elevations of CSF whether assayed by the single or double-layer technique. In contrast, normal sera from CF_1 mice had low or undetectable colony-stimulating activity with either assay. The addition of rat red cells, which potentiates CSF activity, to dishes containing normal serum did not result in further colony production. After extraction with ether or dialysis against water, to remove inhibitory activity of the type described by Chan *et al.* (6), normal CF_1 sera did not further stimulate colony growth.

We had previously reported that when using the single-layer technique increasing the concentration of serum from mice that had received endotoxin (endotoxin serum) in the dishes from 0.05 ml to 0.15 ml was associated with a decrease in colony formation. In contrast, an increase in the concentration of medium conditioned by mouse embryo did not inhibit but rather further stimulated colony growth. This suggested that serum from endotoxin-treated mice contained an inhibitor. Further, when the double layer technique was used and the endotoxin serum placed in the underlayer, the inhibitory effect of higher concentrations of serum was reduced. From this we concluded that the inhibitory activity resulted from a large molecule which does not diffuse in significant concentrations from the underlayer to the overlayer whereas CSF does.

We next attempted to separate CSF from inhibitory activity on a Sephadex column. Representative results from experiments using a single-layer technique are given in Fig. 1. When mouse embryo condition medium was used, peak activity was observed in the region preceding ^{131}I-RISA, suggesting a molecular weight of greater than 70,000. An increase in the concentration of conditioned medium from 0.05 to 0.20 ml produced fur-

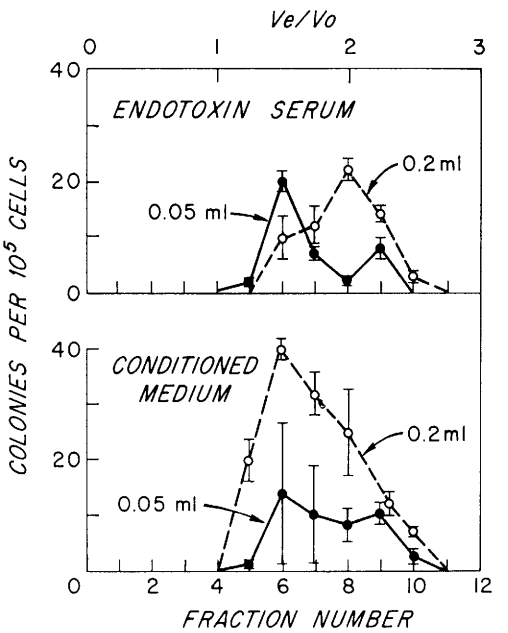

FIG. 1. CSF activity of fractions from endotoxin serum and conditioned medium after separation by Sephadex G-150 chromatography. The fractions were tested at 0.20 and 0.05 ml in each plate. V_e = elution volume; V_o = void volume. Single-layer technique was employed. Endotoxin serum was obtained 2 hr after 50 μg of endotoxin. Fractions from normal serum collected on the same day as the endotoxin serum were without CSF activity at either 0.20 or 0.05 ml per plate.

ther colony growth (Fig. 1). When endotoxin serum, collected 2 hr after 50 μg of IP endotoxin was studied, the results differed from those seen with conditioned medium. In experiments in which 0.05 ml was added to the culture dishes, CSF activity was seen in the same fractions as had been observed with conditioned media (Fig. 1). However, when the concentration of fractionated sera was increased there was no increase in colony formation using the single-layer technique, and, moreover, the curve was shifted to the right (Fig. 1). The above suggested the presence of an inhibitor in those fractions where CSF activity was highest and that the shift in the curve when a 0.2 ml dose level was used represented the downslope of the "true" CSF activity curve. In other words, inhibitors present in fractions 5 and 6 appeared to have obscured the CSF activity in these fractions when a 0.2 ml level was tested. To further explore this possibility the double-layer technique was used to reduce the effect of the inhibitor while at the same time permitting CSF activity to be expressed. In these experiments as may be seen in Fig. 2 there was a dose-related effect on colony growth, 0.2 ml of fractionated endotoxin serum giving a substantially greater effect than 0.05 ml.

In further studies normal mouse serum, serum from endotoxin-treated mice and medium conditioned with mouse embryos were fractionated and the inhibitory effect of the fractions were studied in single-layer cultures in which 0.05 ml of active serum from mice treated 2 hr previously with 5 μg of endotoxin served as the stimulus. To each of the dishes we added 0.15 ml of the fraction under study. As can be seen in Fig. 3 fractions from both normal and endotoxin sera contained potent inhibitors whereas conditioned medium did not.

Endotoxin assay. In order to exclude the possibility of a direct effect of endotoxin on colony formation, the crude sera and the fractions were assayed for endotoxin by the Limulus assay of Levin (11). Serum obtained 2 hr after administration of 50 μg of endotoxin and the first inhibitory fractions (the exclusion fraction) from the same serum were strongly positive for endotoxin. The stimulatory fraction from this same sera, whole mouse sera 2 hr after administration of 5 μg of endotoxin, mouse embryo conditioned media, normal serum, the inhibitory fraction from normal serum, and the fraction from normal serum corresponding to a stimulatory fraction from an endotoxin sera were either very weakly active in the Limulus assay [concentration of endotoxin or endotoxin-like activity less than 0.0001 μg/ml as estimated by comparison with known concentrations of *E. coli* endotoxin (lipopolysaccharide B., *E. coli* 026; B6, Difco Labs., Detroit, Michigan)] or negative. Thus the presence of endotoxin did not seem to be essential for either stimulation or inhibition of colony growth.

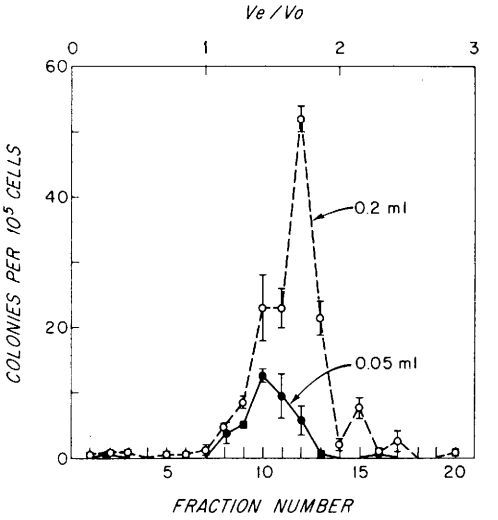

FIG. 2. CSF activity of fractions from endotoxin serum after separation on Sephadex G-150 chromatography tested at 0.20 and 0.05 ml per plate. Double-layer technique was employed. Serum was obtained 2 hr after the administration of endotoxin. At 0.05 ml level the whole serum gave 86 ± 13.9 colonies per 1 × 10^5 cells and at 0.20 ml level 15.7 ± 2.8 colonies per 1 × 10^5 cells. Fractions from normal serum collected on the same day were without CSF activity at either 0.20 or 0.05 ml per plate.

Discussion. The data presented here supports the idea that endotoxin induces CSF production. We could find no evidence that the elevated levels of serum CSF were in fact due to a decrease in a normal inhibitor. Normal CF_1 mice have very low to undetectable serum CSF levels when tested after using

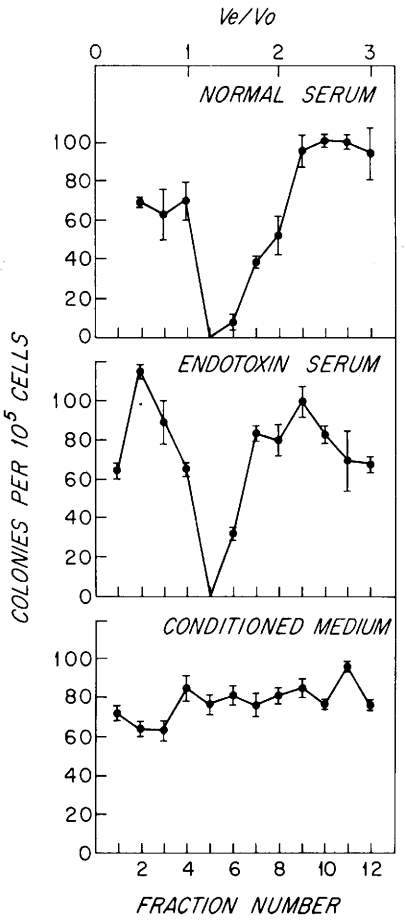

FIG. 3. The inhibitory effect of fractions from normal serum, endotoxin serum, and conditioned medium on growth of soft agar cultures stimulated with 0.05 ml of an active endotoxin serum. The fractions are the same as presented in Fig. 1.

various procedures designed to remove inhibitory factors. Normal serum fractions separated with Sephadex chromatography were without CSF activity, despite the segregation of inhibitory activity to a few fractions. Fractions from endotoxin serum corresponding to fractions from normal serum without inhibitory activity had significant CSF activity. Similarly the use of ether extraction, dialysis against H_2O, or the double-layer agar technique, all procedures which remove inhibitors from serum did not reveal significant CSF activity in normal serum.

We have found, as have others using Sephadex chromatography (12, 13), that CSF activity is present in the prealbumin region, even though CSF may be derived from such diverse sources as human urine (12), medium conditioned by L-cell cultures (13) and in the present study serum from mice treated with endotoxin or medium conditioned by mouse embryos.

The results of the endotoxin assays on crude endotoxin sera and fractions from these sera indicate that the increase in CSF detected in sera after administration of endotoxin does not depend on the presence of endotoxin in such sera.

Both normal and endotoxin sera displayed colony-inhibiting activity. After separation this activity was found in the excluded fraction, which indicates that this factor has a molecular weight in excess of 200,000, the upper limit of the Sephadex gel used in this study. The opaque appearance of the inhibitory fraction and its extractability with ether suggests that we may be dealing with a lipoprotein similar to that described by Chan *et al.* (5, 6).

It is yet to be established whether the inhibitor described herein has a physiologic role in the regulation of granulopoiesis or is merely a nonspecific inhibitor of *in vitro* colony growth. In either event, it seems prudent in the study of physiology of CSF to determine whether any apparent changes in CSF activity might reflect changes in the concentration of an inhibitor rather than true generation of CSF. The results which we have presented herein provide further confirmation of our initial impression that endotoxin generates a true increase in colony-stimulating activity which in turn plays a significant role in the regulation of granulopoiesis.

Summary. Sera from normal and endotoxin-treated CF_1 mice were fractionated by Sephadex chromatography. CSF activity was demonstrated in the prealbumin region in serum obtained from mice that had previously received endotoxin. Identical fractions from mouse embryo conditioned medium also displayed CSF activity. Normal serum had very low or no detectable CSF levels. An inhibitor was demonstrable in the high molecular weight fractions from both normal and endotoxin serums. Its effect is decreased by utiliz-

ing a double-layer technique. These results suggest that there is an increase in CSF levels after endotoxin administration rather than a decrease of serum inhibitory factors. Furthermore, the increase in CSF detected in sera after administration of endotoxin does not depend on the presence of endotoxin in such sera.

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