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Bacterial Stimulation of Serum Colony-Stimulating Activity and Neutrophil Production in Germ-Free Mice (40615)[1]

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The factors regulating granulocyte production are poorly understood. The most likely humoral regulator of granulocytic production and maturation is called colony-stimulating factor (CSF) (1). The ability to stimulate granulocyte colony growth *in vitro* is termed colony-stimulating activity (CSA) (2, 3). The major source of CSA in humans appears to be the monocyte–macrophage system (4–8), although lymphocytes have also been implicated in CSA production (9, 10). The factors which modulate CSA production or release appear to be, in part, the result of stimulation by microorganisms and their products, particularly endotoxin. Previous studies have shown that incubation of monocytes with whole live or killed bacteria, or with endotoxin, enhances or increases CSA production by these cells (11–14). Likewise, it has been shown that injection of endotoxin into humans or mice leads to increased levels of serum CSA (15–17).

The role of mature peripheral blood and tissue neutrophils in regulation of their own production is open to controversy. Some authors have suggested that mature neutrophils may make substances which inhibit new granulocyte production and maturation, particularly when they are present in increased numbers (18–20). Recent studies have suggested that neutrophils may modulate their own production by inactivation of microorganisms and their products, thereby negating their stimulatory effect on CSA production (13, 14). The present study was undertaken to investigate further the role of microorganisms and blood neutrophils in the modulation and regulation of CSA production. Changes in serum CSA levels, bone marrow colony-forming cells (CFU-C), absolute granulocyte counts, and bacterial levels in germ-free mice after introduction of bacteria into their environment were investigated. These studies have shown that a marked rise in CSA production occurred immediately after germ-free mice were moved into a nonsterile environment. This rise in CSA was correlated with a rise in stool bacteria levels and was followed by a rise in the absolute granulocyte count. As the absolute granulocyte count rose, both CSA production and stool bacterial levels decreased. These studies are compatible with the suggestions that CSA production is modulated by the levels of microorganisms and their products and that neutrophils serve as a negative feedback arm in the scheme of granulocyte regulation by inactivation of the stimulatory effect of microorganisms.

Materials and methods. Forty-two-day-old Swiss–Webster axenic mice obtained from Charles Rivers Laboratories were used in these experiments. Animals were maintained in a germ-free state in cages provided by the supplier for 24 hr and then moved to a filtered air environment and fed sterile Purina Mouse Chow. They were given 1×10^8 *Escherichia coli* per animal orally at this time. Five mice were sacrificed for each time point prior to, and after movement into, the filtered air environment and introduction of *E. coli*. At sacrifice, blood was collected by cardiac puncture for white blood cell counts, differential counts, and serum CSA values. Stools were collected for measurement of bacteria levels, and bone marrow was obtained for quantitation of CFU-C.

CSA determinations. Serum CSA was determined by methods previously described

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(21). Serum (0.1 ml) from a pool of five test mice for each time point was plated into triplicate 35-mm plastic petri dishes to which was added a 1-ml mixture of 0.5% McCoy's 5A medium containing 15% fetal calf serum. These were then overlayed with 75,000 nucleated cells derived from the femurs of C57/BL mice suspended in 1 ml of McCoys with 0.3% agar. After gelling, the plates were incubated in a fully humidified incubator with a constant flow of 7.5% CO_2 in air. The control stimulus for colony growth was a standard human urine. After 7 days of incubation, colonies were counted using a stereo microscope. Only aggregates containing 50 or more cells were scored as colonies.

Colony-forming unit determinations. CFU-Cs from the femurs of the experimental mice were measured by isolation of a single femur from each animal and the marrow was blown from the shaft using a syringe containing McCoy's 5A medium. A single cell suspension was prepared, followed by a total cell count. Nucleated cells (75,000) were then plated in a 1-ml aliquot of McCoy's 5A medium containing 0.3% agar and 15% fetal calf serum. The stimulus for colony growth was 0.15 ml of a standard human urine. The plates were incubated as above and counted after 7 days.

Determination of bacterial levels. After sacrifice, the large intestine of the experimental animal was opened and a single formed fecal sample was removed, weighed, and incubated in a standard amount of trypticase soy broth for 24 hr. The number of bacteria were counted in a hemocytometer and also plated in routine laboratory fashion on a standard agar plate followed by colony counts after 24 and 48 hr.

Results. Figure 1 shows the correlation between serum CSA values, fecal bacterial levels, and absolute granulocyte counts in experimental mice before and after removal from their germ-free environment and introduction of bacteria. Absolute granulocyte counts and serum CSA values were low in the germ-free state when compared with values reported in conventional mice (22, 23). These lower range levels were again attained at Day 7 after the introduction of bacteria as the mice returned to the germ-free state. No bacteria were detected in fecal specimens.

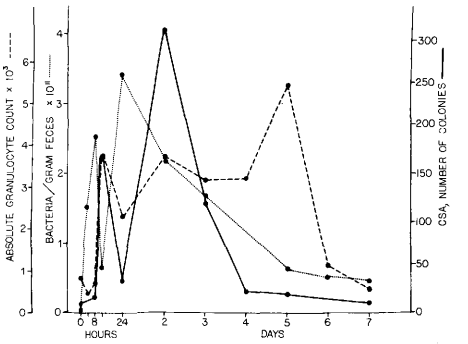

FIG. 1. Changes in serum CSA values (——), fecal bacterial levels (·····), and absolute blood granulocyte counts (– – –) in germ-free mice before and after removal from germ-free environment and oral bacteria introduction. CSA values are the mean colony numbers stimulated of 0.1 ml of pooled sera from five mice. Fecal bacteria levels are expressed as colonies per gram of feces cultured. Time 0 is the point of removal from germ-free environment and introduction to oral bacteria.

Soon after introduction of bacteria and removal from the germ-free environment, there was an initial drop in absolute granulocyte counts at Hour 4 as fecal bacterial levels rose. As fecal bacterial levels continued to rise, so did serum CSA values and absolute granulocyte count. After 2 days, 36 hr following the initial serum CSA rise, there was a second peak in absolute granulocyte counts. The maximum increase in serum CSA values was seen at Hour 48 and this was followed 72 hr later, on Day 5, by a dramatic rise in absolute granulocyte counts. This rise in absolute granulocyte counts was correlated with a concomitant fall in fecal bacterial levels and serum CSA values. The peak of granulocytes was preceded by the commencement of the fall in CSA and in bacteria.

Table I shows the correlation between bone marrow CFU-C levels, serum CSA values, absolute granulocyte counts, and fecal bacterial content in these same animals. It should be noted that the standard deviations reflect the reproducibility of the assay, not variation from animal to animal, since pooled specimens were used. The initial rise in serum CSA values at Hour 12 following introduction of bacteria and removal of these animals from their germ-free environment is correlated with a fall in bone marrow CFU-C levels at Hour 8. This fall in bone marrow CFU-C levels is, in turn, correlated with an

TABLE I. LEVELS OF BACTERIA, GRANULOCYTES, CSA, AND CFU-C BEFORE AND AFTER ADMINISTRATION OF *E. coli* TO GERM-FREE MICE[a]

Time after administration of *E. coli*	No. of bacteria per gram feces ($\times 10^{11} \pm$ SD)	Absolute granulocyte count ($\times 10^3 \pm$ SD)	CSA (No. of Colonies ± SD)	CFU-C per femur ($\times 10^3 \pm$ SD)
Preadministration	0 —	0.81 ± 0.21	7 ± 01.5	10.60 ± 1.70
4 hr	1.68 ± 0.20	0.45 ± 0.13	—	7.09 ± 0.68
8 hr	2.42 ± 0.09	0.69 ± 0.07	15 ± 7	3.36 ± 0.64
12 hr	0.61 ± 0.20	3.62 ± 0.67	168 ± 8	4.05 ± 1.20
24 hr	3.35 ± 0.36	2.36 ± 0.35	31 ± 10	13.03 ± 0.77
2 days	2.10 ± 0.17	3.75 ± 0.72	309 ± 9	17.2 ± 1.40
3 days	1.61 ± 0.15	3.18 ± 0.05	117 ± 1	24.18 ± 0.99
4 days	—	3.23 ± 0.46	19 ± 4	13.81 ± 1.50
5 days	0.48 ± 0.11	5.39 ± 0.08	17 ± 5	18.81 ± 2.01
6 days	0.37 ± 0.02	1.15 ± 0.45	—	14.54 ± 0.12
7 days	0.437 ± 0.04	0.68 ± 0.36	13 ± 5	6.89 ± 0.65

[a] The mice were administered 1×10^8 organisms per animal. At each time point, animals were sacrificed and levels of bacteria, granulocytes, CSA, and CFU-C were assayed. Results (for each time point) are the mean of five animals.

initial rise in absolute granulocyte counts seen at Hour 12. The CFU-C levels continued to rise, but once the maximum rise in serum CSA values had occurred, bone marrow CFU-C levels gradually returned to lower levels over the following 5-day period. The absolute granulocyte counts and serum CSA values also returned to normal levels following the dramatic rise in absolute granulocyte counts on Day 5.

Discussion. There seems little doubt that granulocyte production must be associated with the levels of microorganisms and their products *in vivo*. Any increase in microorganism load over the normal endogenous state leads to a prompt increase in granulocyte production to combat this insult. The mechanism whereby this occurs has previously been unclear. The data presented here, and in previous studies, suggest that this is the result of a stimulatory effect of microorganisms and their products on the factors regulating granulocyte production, maturation, and release (12, 13, 15–17). These data suggest that peripheral blood granulocytes serve a role in regulating their own production by inactivating microorganisms, specifically bacteria, thereby decreasing the production of granulocyte stimulatory factors and, hence, their own production.

Previous studies from this laboratory of patients undergoing bone marrow transplantation have suggested that similar mechanisms may be operative in humans (24). In these studies it was shown that serum CSA values rise dramatically as patients become profoundly granulocytopenic and return to low or normal levels as bone marrow engraftment ensues and the absolute granulocyte counts rise. It was shown in these studies that there was a direct correlation between rises in serum CSA levels and fever curves. In these initial human studies no measurement of bacterial content to correlate with the fever curves and CSA values were carried out. These data might suggest that the microorganism load may have accounted for the rises in temperature and serum CSA values noted in these patients.

Further support for such a mechanism is obtained from the studies of Quesenberry *et al.* (25), who studied the changes in serum CSA values of conventional and germ-free mice rendered neutropenic by whole body irradiation and demonstrated that dramatic rises in serum CSA values occurred in conventional animals with the development of neutropenia, but no such rise was found in germ-free animals, despite similar falls in peripheral blood neutrophil levels. They suggested that the factors necessary to stimulate CSA production were absent in germ-free animals and that peripheral blood neutrophil levels alone were not the major determining or modulating force for CSA production.

Other authors have suggested that mature circulating and tissue neutrophils may produce factors which inhibit their own production and maturation. These factors have been sometimes called chalones (18, 26) but con-

crete evidence for a physiologic role of such materials is, at best, presumptive.

We prefer to view neutrophil regulation from a more physiologic standpoint. In the scheme presented previously (14, 27), we have suggested that the major positive or stimulatory force for neutrophil production is the level of microorganisms and their products and perhaps other antigenic stimuli, and the negative feedback arm inactivation of microorganisms and their products by mature neutrophils. The present data seem in concert with this concept.

Summary. The *in vivo* effect of microorganisms upon granulopoiesis of axenic mice was studied. A marked rise in CSA production occurred immediately after germ-free mice were exposed to microorganisms. This rise in CSA was correlated with a rise in stool bacterial levels and a rise in absolute granulocyte count. As the absolute granulocyte count rose, both CSA production and stool bacterial levels decreased. The peak of granulocytes was reached after the commencement but during the fall of CSA and bacteria levels. These studies are compatible with the suggestion that CSA production is modulated by the levels of microorganisms and their products, and that neutrophils serve as a negative feedback arm in the scheme of granulocyte regulation by inactivation of the stimulatory effect of microorganisms.

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