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The Effect of Bacterial Infection on Granulopoiesis[1] (41115)

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Abstract. Colony-stimulating factor (CSF) is a well-established specific promoter of the committed stem cell colony-forming unit in culture (CFU-C) *in vitro* and thought to be a true regulator of granulopoiesis *in vivo* as well. However, the mechanisms involved in the regulation of granulopoiesis are still controversial. Bacterial products, like endotoxin and live bacteria, enhance levels of serum CSF *in vitro* and *in vivo* both in animals and in man. Data from our laboratory in germ-free mice showed evidence that high levels of bacteria stimulate production of granulocytes by activating the CSF–CFU-C system and that inhibition of granulocyte production may be simply due to clearance of bacteria by granulocytes. This study has been undertaken to determine the role of bacterial infection in conventional mice in the regulation of granulopoiesis *in vivo*. C57/bl 6J mice were injected intraperitoneally (ip) with 1×10^4 *Escherichia coli* (*E. coli*). The animals were evaluated for the following parameters: serum CSF, bone marrow CFU-C, the absolute number of granulocytes in the peripheral blood, and the number of bacteria in peripheral blood and abdominal cavity. Groups of five mice were investigated at multiple time points after injection. The results show peak levels of bacteria both in peripheral blood and abdominal cavity at the 1-hr time point. There is a steady and marked rise in serum CSF reaching a first peak 8 hr, a second peak 96 hr after the injection. The number of CFU-C is increased at the 48- and the 120-hr time point. A pronounced rise in the absolute number of granulocytes is seen 2 hr after infection, which seems to be independent of the activation of stem cells. These findings demonstrate a temporal relationship between the peak in the number of bacteria, release of mature granulocytes, rise in serum CSF, and subsequent activation of bone marrow CFU-C. Following rises in CFU-C there are no marked elevations in peripheral blood granulocytes.

The regulation of granulopoiesis has gained the interest of both basic and clinical scientists over the past years. The entire mechanism is, however, still poorly understood and controversial. On the basis of currently available evidence it appears that there are positive and negative feed-back arms which allow granulocytes to respond to challenges such as infections, consumption, and blood loss. The major stimulatory arm appears to reside in the so-called colony-stimulating factor(s) (CSF) produced by a variety of cells, particularly peripheral blood monocytes, tissue macrophages, and lymphocytes (1–4). A number of stimuli have been defined which elicit the production and/or release of CSF from these cells. These include bacteria and bacterial products such as endotoxin (5–11). They appear to play a modulating role in the production of CSF at least *in vitro* and perhaps *in vivo*. The negative factors, which would dampen the production of granulocytes are less well understood. A number of materials have been described which inhibit granulocyte colony growth *in vitro* or granulocyte precursor cell division in liquid culture systems. Largely these have been derived from mature neutrophils and include chalones and lactoferrin (12–18). Interferon and prostaglandin are also known to have inhibitory activity (19). The physiologic role of these has not been clearly demonstrated.

It has also been proposed that the negative arm of granulocyte regulation may be simply the inactivation of the microor-

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ganism stimulus by mature granulocytes. Evidence for this has been presented using *in vitro* systems and more recently *in vivo* in germ-free mice. In these studies it has been shown that live microorganisms or endotoxin can stimulate the production of CSF, but that addition of live mature granulocytes can abrogate this effect (5–7).

The following studies were undertaken to further characterize this system and to determine whether or not bacterial infection plays a pivotal role in stimulating the production of CSF with subsequent activation of committed stem cells. These studies have shown that following intraperitoneal infection of mice with *Escherichia coli* (*E. coli*) there is a rise in CSF production and/or release accompanied by a rise in peripheral blood neutrophil levels and colony-forming unit in culture (CFU-C) in the bone marrow. Once bacteria are cleared from the peripheral blood and peritoneal cavity, granulocyte levels return to normal, but CSF and CFU-C continue to be elevated in an oscillatory time-related fashion. These findings have added further evidence to the contention that bacterial infection may play a major role in the regulation of granulopoiesis.

Methods and Materials. Eight-week-old C57/b1 6J mice in groups of five from the Jackson Laboratories were used in these experiments. After intraperitoneal (ip) infection with *E. coli* (described below) the mice were sacrificed at the following time points: 1, 2, 4, 8, 24, 48, 72, 96, and 120 hr. After general anesthesia with ether the animals were sacrificed by thoracotomy. Blood was collected by heart puncture for total white blood cell counts, differential counts, serum CSF measurement, and bacterial blood cultures. The bone marrow of one femur was taken for evaluation of CFU-C. The peritoneal cavity was then injected with 1 ml of sterile phosphate-buffered saline (PBS). After careful rinsing of the entire abdominal content, saline was immediately removed and used for the determination of bacterial numbers. CSF and CFU-C are pooled data from five mice per time point. The number of bacteria in the peripheral blood and abdominal cavity is expressed as the mean of the values obtained from two mice at each time point. The white blood cell counts (WBC) and differentials represent mean values from five individual mice per time point.

Bacteriologic studies. The bacteria used in this study were from a strain of *E. coli* K107- derived from human cerebrospinal fluid. This was kindly supplied by Dr. Mary Glode of Children's Hospital, Denver, Colorado. The determination of the lethal dose (LD_{50}) revealed a concentration of 5×10^7 bacteria per mouse after 72 hr according to Reed and Muench (20). Serial dilutions were done from an initial concentration of 1×10^9 bacteria per mouse.

The number of bacteria chosen for ip injection was 1×10^4 bacteria/ml/mouse. The number of bacteria was determined by optical density (OD) (spectronic 20, wave length A540, Bausch & Lomb). This was further established for the above-mentioned strain of *E. coli* by counting the number of bacteria in a Petroff–Hausser and Helber bacteria counter and by the spread-plate method on nutrient agar. The animals were injected 10 min after the OD and chamber counts were done. All injections were done with the bacteria being in the log growth phase.

Bacteriologic cultures. Blood, 0.01 ml, was taken by heart puncture and added to McConkey's culture plates in duplicate. The plates were read after 24 hr of incubation at 37°. The results were expressed as the number of bacteria per milliliter of peripheral blood. The peritoneal wash, 0.1 ml, was taken and added to McConkey's plates in duplicate using undiluted wash, 1:10 and 1:100 dilutions. The plates were read after a 24-hr incubation period and the results were expressed as the number of bacteria per milliliter which represents the number of bacteria per abdomen. Control studies in 10 uninfected mice showed that peripheral blood and peritoneal washings did not contain bacteria.

Measurement of colony stimulating factor. Pooled serum, 0.1 ml, from five mice per time point was pipetted into triplicate 35-mm plastic petri dishes. Because serum at early time points contained *E. coli* the whole blood was spun at 8000 rpm for 5 min in a Beckman microfuge B and the super-

natant was filtered by Swinnex 25 filter units (0.45 μm) to remove microorganisms. One milliliter of a mixture containing McCoy's 5A medium in 0.5% agar was then added. The serum and media were thoroughly mixed by rotation and the plates then were allowed to gel at room temperature. Nucleated bone marrow cells, 7.5×10^4, derived from the femurs of normal 8 to 10-week-old C57/6J mice were suspended in a 1-ml mixture containing McCoy's 5A medium, 15% fetal calf serum (FCS), and 0.3% agar. The bone marrow cells were separated before by means of glass adherence to remove CSF producing cells. This mixture was then placed on top of the underlayer. After gelling, plates were incubated in a fully humidified incubator with a constant flow of 7.5% CO_2 in air. Colony counts were done at 7 days of incubation using a stereo microscope. Aggregates containing 50 or more cells were scored as colonies. Control studies were carried out by using normal C57/b1 6J mice injected ip with PBS. Five control mice were studied at the same time point as were experimental mice.

Colony-forming unit-culture (CFU-C). The marrow was blown from the shaft of a single femur from each animal using a syringe containing McCoy's 5A medium. As indicated, the marrows from 5 mice at each time point were pooled. A single cell suspension was prepared by pipetting and a total cell count was obtained. 7.5×10^4 nucleated cells were then plated in a 1 ml aliquot of McCoy's 5A medium containing 0.3% agar and 15% FCS. The stimulus for colony growth was 0.15 ml of a standard human urine. Plates were incubated as above and scored after 7 days. Results are expressed as the number of CFU-C per femur taking into account the total number of cells found at each time point. Control studies were carried out by using normal C57/b1 6J mice injected ip with PBS. Five control mice were studied at the same time points as were experimental mice.

Hematologic data. Blood obtained by heart puncture was drawn into a 44.7-μl capillary pipet and added to a Unopette container (Unopette Test 5925, Becton and Dickinson Co.) and the total WBC count was determined in an automatic cell counter. Smears were prepared and differential counts were done on 100 cells. The normal range for WBC counts and differentials was determined in 30 mice of the same strain and age group. The normal range for WBC counts was $5.50 \pm 1.49 \times 10^6$ cells/ml, absolute granulocyte counts $0.427 \pm 0.236 \times 10^6$/ml, total lymphocyte counts $5.0 \pm 1.35 \times 10^6$/ml, and total monocyte counts $0.44 \pm 0.07 \times 10^6$/ml.

Results. Figure 1 shows the levels of bacteria in the peripheral blood and abdominal wash at various time points after injection of 1.0×10^4 bacteria/ml into the intraperitoneal cavity. Maximum counts in both blood and peritoneal cavity occurred 1 hr after injection. There was a rapid decrease in the number of bacteria in the peripheral blood after 4 hr with sterile blood cultures occurring 24 hr after injection. Bacterial levels in the peritoneal cavity remained elevated at 4 hr but had fallen rapidly by the 8-hr time point and at 48 hr the first sterile cultures were obtained. A

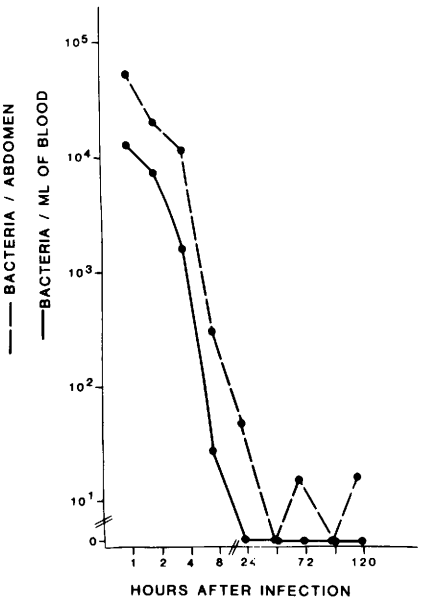

FIG. 1. The ordinate shows the number of *Escherichia coli* K107- on semilogarithmic scale. The abscissa shows the various time points after infection. The solid line represents the bacteria/ml of peripheral blood. The broken line shows the number of bacteria per abdomen.

few bacteria were obtained at later time points (72, 120) but the number of bacteria was small in each case.

Figure 2 shows the changes in serum CSF (panel A) and bone marrow CFU-C levels (panel B) at various time points following bacterial infection. CSF levels rose rapidly reaching a peak of 415 colonies at the 8-hr time point. CSF levels then fell to the normal range at the 72-hr time point with a second peak above the control value at the 96-hr time point. The fall in CSF levels is closely correlated to the fall in bacterial levels in the peripheral blood and peritoneal cavity. CFU-C levels were markedly elevated above the control value at the 48-hr time point. A second rise was seen 120 hr after injection.

Figure 3 shows the changes that occurred in total WBC counts, absolute granulocyte and lymphocyte counts during the various time points studied. Total WBC and granulocyte counts showed an early peak (2900 ± 1600) at 2 hr after which the granulocyte count returned to the normal range and remained there throughout the rest of the study with a moderate elevation at the 96-hr time point. The most striking feature was a marked rise in total WBC count and lymphocyte counts with a peak occurring 96 hr following injection.

Discussion. These studies have shown a temporal correlation between bacterial infection and production and/or release of CSF in mice. The peak of CSF seen in these experiments closely follows the peaks of bacteria in the peripheral blood and abdominal cavity. Furthermore, as bacteria were cleared CSF levels fell. This is similar to the findings noted *in vitro* with human cells where addition of bacteria or endotoxin to monocyte or macrophage cultures leads to the production and/or release of CSF. The mechanisms by which bacteria carry this out are not totally clear. On the basis of *in vitro* experiments from these laboratories (5, 6) and those of others (21) it would appear that the initial recognition of bacteria takes place by a lymphocyte population which then sends a message to the monocyte/macrophage system where CSF is actually produced or released. The

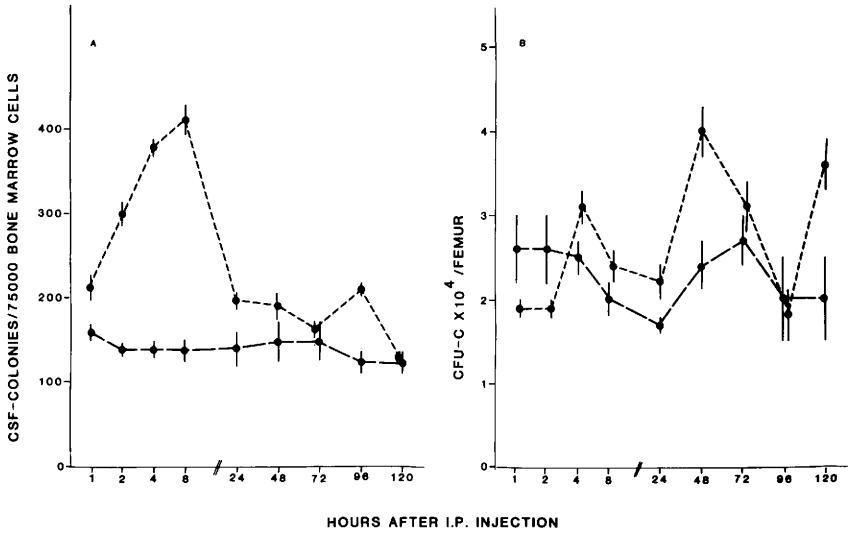

FIG. 2. Panel A. The number of colony-stimulating factor (CSF) colonies per 7.5×10^4 mononuclear bone marrow cells at various time points after infection with *Escherichia coli* are shown as (– – –). The results of control mice, injected with PBS at the same time points, are shown as (———). Panel B. The numbers of colony-forming units in culture (CFU-C) $\times 10^4$ per femur at various time points after injection with *Escherichia coli* are shown as (– – –). The results of control mice are shown as (———). The results are expressed as mean ± SD of pooled observations per each time point. The standard deviation reflects the variability of the technique and not of the experimental data.

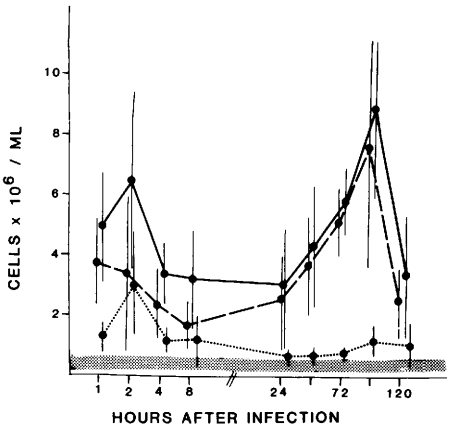

FIG. 3. The ordinate shows the number of white blood cells × 10^6/ml. The solid line represents the total white cell count, the broken line represents the lymphocytes and the dotted line represents the granulocytes. The results are expressed as mean ± SD. The abscissa shows the various time points after infection. The shaded area represents the normal range of the granulocytes.

nature of the mediator between lymphocytes and monocytes and macrophages has not, however, been determined.

There is a correlation between CSF and CFU-C. Following the peak of CSF 8 hr after infection, the peak in CFU-C occurs at the 48-hr time point. The following CFU-C peak at 120 hr after injection is preceded by a CSF peak at the 96-hr time point. No correlation exists between the amount of CSF produced and/or released and the number of CFU-C activated.

The increase in granulocytes 2 hr after infection is explained by action of neutrophil releasing activity (NRA), also termed neutrophilia-inducing factor (NIF), and leukocytosis-inducing factor (LIF). Boggs (22) demonstrated that RNA is not identical with endotoxin and Broxmeyer (23) showed that CSF and LIF were separate entities but both elevated after endotoxin injection. No clear-cut correlations were observed in the present study between peripheral blood neutrophil levels following peaks in CFU-C. This may be a reflection of the fact that peripheral blood neutrophils, when released from the bone marrow, may rapidly egress from the peripheral blood to the site of infection, in this case the abdominal cavity. Recent studies in our laboratory have shown that this, indeed is the case (24). Thus, measurement of peripheral blood neutrophil levels may not reflect accurately the kinetic activity and overall distribution of mature granulocytes. This can be accomplished only by measuring the number of granulocytes actually entering the infected area.

These studies have corroborated and extended previous findings that microorganisms and their products stimulate CSF production and/or release *in vivo* and *in vitro* and may play a pivotal role in activating the committed stem cell CFU-C via CSF.

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