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Granulocyte-Macrophage Precursor Cell and Colony-Stimulating Factor Responses of Mice Infected with *Salmonella typhimurium*

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The response of granulocyte-macrophage progenitor cells (in vitro colony-forming cells) and of colony-stimulating (CS) factor in serum were studied in mice infected intraperitoneally with 10^3 viable *Salmonella typhimurium*. Increases in the number of colony-forming cells in marrow and spleen and increases in the serum level of CS factor occurred during the infection. There was no evidence to suggest that progressive infection was associated with failure of macrophage production. Medium rich in CS factor increased the bactericidal activity of macrophages in vitro and it was suggested that CS factor could be involved in macrophage activation.

It is known that resistance to experimental *Salmonella typhimurium* infection in mice is largely dependent upon the bactericidal activity of macrophages and that this activity can be enhanced by immunologically nonspecific (1, 4, 5, 8) or specific (7) mechanisms.

Failure to control the number of organisms below lethal levels could be due to several macrophage-associated factors amongst which may be the animal's ability to generate new macrophages or to maintain their bactericidal activity.

Macrophages and granulocytes share a common progenitor cell which proliferates in semi-solid agar medium to form colonies of differentiating granulocytes or macrophages, or both, and this proliferation of colony-forming cells (CFC) requires the presence of a glycoprotein regulator known as colony-stimulating factor (CS factor). The culture system (reviewed by Metcalf and Moore [15]) therefore allows the quantitative study of granulocyte-macrophage precursors and of factors influencing their development.

Studies in the mouse have shown that the number of CFC in bone marrow and spleen increases (sometimes quite markedly in spleen) in response to injection of a variety of antigens and adjuvants (9, 17), tumors (6), and infection with ectromelia virus (T. A. McNeill et al., Immunology, in press). These changes are usually accompanied by an increase in the serum level of CS factor.

The purpose of the present study was to examine CFC and CS factor responses in mice infected with *S. typhimurium* and thereby to determine if a failure of macrophage production at the precursor cell stage was associated with failure to resist the infection.

MATERIALS AND METHODS

Mice. Female Swiss TO mice 5 to 8 weeks of age were purchased from Animal Suppliers Ltd., N. Finchley, London. *S. typhimurium* could not be isolated from uninoculated mice either before or during the experimental periods. Mice of this strain are relatively resistant to *S. typhimurium* (19).

Bacterial suspensions. *S. typhimurium*, NTCC 5710, was provided by J. Dunbar. Bacteria from 18-h cultures in nutrient broth were washed once by centrifugation and suspended in 0.85% NaCl. Viable bacterial counts were performed by the spread plate method on nutrient agar. Stock cultures were periodically examined for purity by culture on McConkey agar and by slide agglutination with specific antisera.

Mouse inoculation and assay of bacteria in spleen and marrow. All mice were inoculated intraperitoneally with 10^3 viable bacteria from fresh cultures. This dose killed 55% of mice within 2 weeks. For each mouse one half of the spleen was teased with needles into 2 ml of saline, the marrow from one femur was flushed into 2 ml of saline and the cells were suspended by pipetting. These cell suspensions were diluted in cold saline for assay of viable counts. No attempt was made to distinguish between free and cell-associated bacteria. Bacterial counts were expressed per milliliter of these suspensions.

CFC assay. The media and methods used to grow granulocyte-macrophage colonies are given in detail elsewhere (10). The double-layer technique in Nunclon 30-mm plastic dishes was used and CS factor

was provided by the incorporation of mouse embryo conditioned medium (2) in the underlayer. The same batch of conditioned medium was used throughout and in a concentration known to give maximum colony stimulation with cells from this strain of mouse.

Cytology. Blood was collected from the axillary vessels and differential white cell counts were made by standard hematological methods. Counts of total cells per femoral shaft were made by suspending the complete contents of a femur in 2 ml of ethylenediaminetetraacetate in phosphate-buffered saline.

Serum CS factor. The number of colonies stimulated from 5×10^4 normal marrow cells by 0.03 ml of each serum was estimated and expressed as a percentage of the number obtained from the same marrow when maximally stimulated by embryo-conditioned medium. This reference standard was necessary since all sera could not be assayed in the same batch of cultures. At least two cultures were used for each serum.

Bactericidal assay. Macrophage phagocytic and bactericidal activity was assayed in cultures of 10^5 washed peritoneal cells in the cavities of Cooke microtiter plates. Cells from four normal mice were collected in ice-cold medium (Hanks balanced salt solution + 2.5 U of preservative-free heparin per ml), washed, and resuspended in cold Eagle medium supplemented with 10% fetal bovine serum. After 18 h of incubation at 37 C in an atmosphere of 10% CO_2 in air the adherent cells were washed and 3×10^5 opsonized *S. typhimurium* (prepared by treatment with a 0.1 dilution of serum from *S. typhimurium* convalescent mice) in 0.2 ml was added for a further 1 h of incubation at 37 C. Viable counts were made for free and cell-associated bacteria. For the latter, cells were washed three times with cold saline and disrupted by ultrasound at 20 kc/s for 10 s. The number of bacteria phagocytosed per culture was calculated as the difference between the number inoculated and the number free in the medium after 1 h. Bactericidal activity of each culture was the cell-associated viable count expressed as a percentage of the number of bacteria phagocytosed. All media used in these assays were free of antibiotics.

RESULTS

Mortality. Figure 1 shows the percentage mortality per day from a total of 40 infected mice. During the 14 days after inoculation 55% of the mice died and most deaths occurred between the days 5 and 9. All mice surviving at day 14 were found to be carrying *S. typhimurium*.

Course of the infection. Two series of experiments were performed covering days 2 to 8 and 6 to 9, respectively. Groups of eight mice per day were used in each series. Bacterial counts from spleen and marrow, CFC assays from spleen and marrow, and serum CS factor assays were all made from individual mice. In an additional series which covered days 3 to 7 estimates from individual mice were made of spleen and marrow bacterial counts, spleen weight, and the number of circulating granulocytes and monocytes.

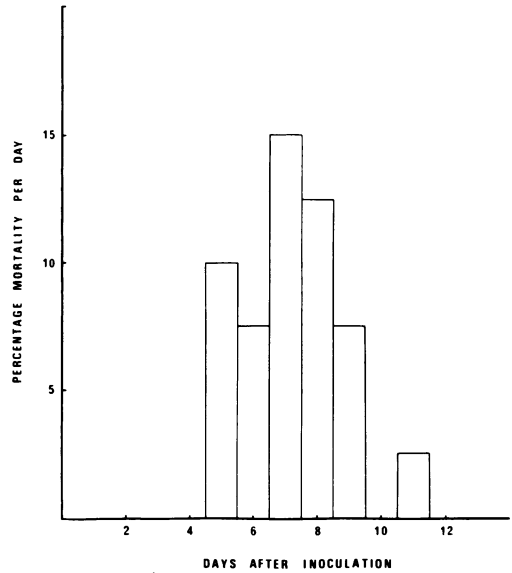

FIG. 1. *Daily mortality after intraperitoneal inoculation of 10^8 S. typhimurium.*

Figures 2 to 6 show the averaged data from all experiments. The level of infection in both marrow and spleen rose to a plateau and then decreased slightly (Fig. 2). There was a rise in serum CS factor levels to over 10 times normal by day 7, followed by a sharp fall (Fig. 3). The numbers of CFC in marrow and spleen were increased throughout the period of observation (Fig. 4) and the numbers of circulating granulocytes and monocytes were maintained at or above resting levels (Fig. 5). Changes in spleen weight (which is a crude index of the total leukocyte count) and the number of cells per femoral marrow (Fig. 6) indicate that gross alterations in leukocyte distribution were also taking place.

Even though these averaged data give an indication of general trends, they do not permit deductions to be made regarding the role or fate of these responses in individual mice since: (i) there was considerable variation from mouse to mouse within each group for all parameters tested, and (ii) from day 5 onwards the data referred only to selected populations of survivors.

Assuming that the mice with highest bacterial counts have the worst prognosis, comparisons of the level of infection in individual mice with the number of CFC in marrow and spleen or the level of CS factor in serum should indicate any association between failure of CFC

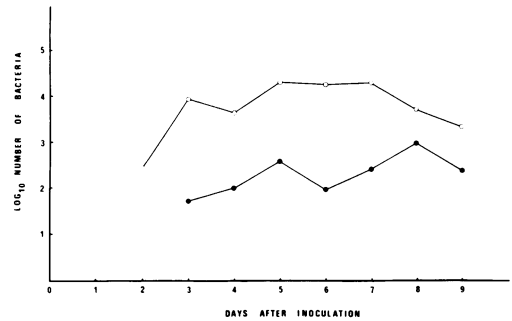

FIG. 2. *Level of infection after intraperitoneal inoculation of 10^3 S. typhimurium. Average of data from groups of 8 to 24 mice. Symbols: O, spleen; ●, marrow.*

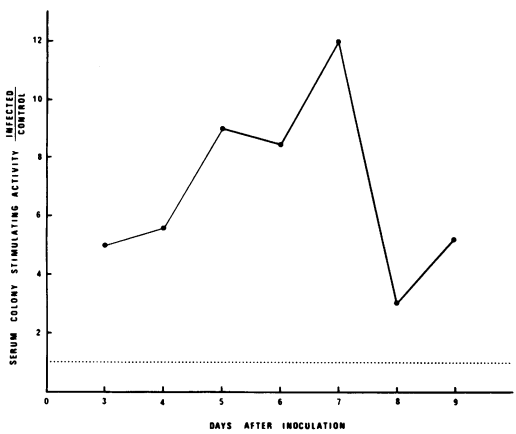

FIG. 3. *Serum colony-stimulating activity after intraperitoneal inoculation of 10^3 S. typhimurium. Expressed as the ratio of percentage CSA in infected sera to percentage CSA in control sera. Average data from groups of 8 to 16 mice.*

production and failure to resist infection. These results show that in most infected mice tissue CFC levels and serum CS factor levels (Fig. 7-9) were higher than in controls and that this increase could therefore be maintained in the presence of a high level of infection.

Effect of conditioned medium on bactericidal activity of macrophages in vitro. CS factor is regarded as a specific regulator involved in the multiplication and differentiation pathways from CFC to mature granulocytes and macrophages. The possibility that it may also be involved in the control of some functions of these mature cells, e.g., bactericidal activity, has not been considered previously. In view of the difficulties involved in equating serum CS factor levels with levels in infected tissues (see Discussion) the data shown in Fig. 3 and 9 do not give any direct information on this question. A more direct approach by determining if embryo-conditioned medium (a rich source of CS

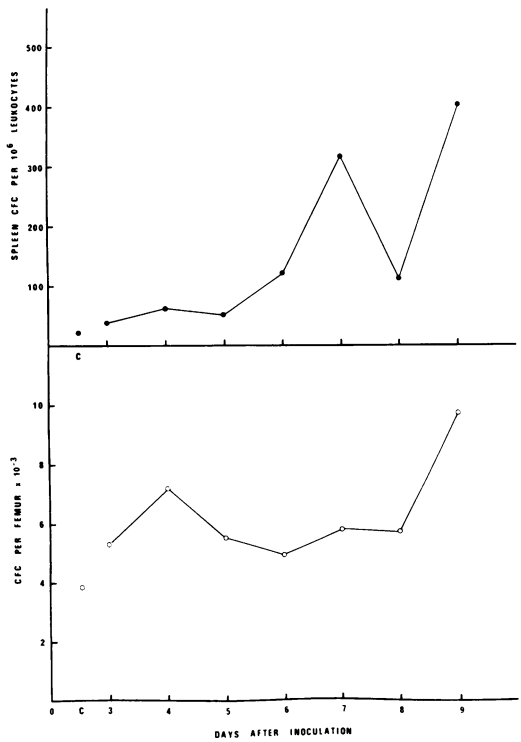

FIG. 4. *CFC levels in marrow and spleen after intraperitoneal inoculation of 10^3 S. typhimurium. Average data from groups of 8 to 15 mice. C indicates level in uninoculated mice.*

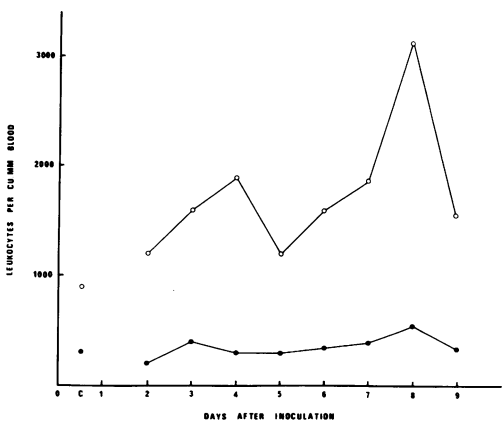

FIG. 5. *Blood leukocyte levels after intraperitoneal inoculation of 10^3 S. typhimurium. Average data from groups of 8 to 24 mice. Symbols: O, spleen; ●, marrow.*

factor) affected bactericidal activity of peritoneal macrophages in vitro was therefore made. Table 1 gives the result of an experiment in which peritoneal cells were preincubated for 18 h with several concentrations of embryo-condi-

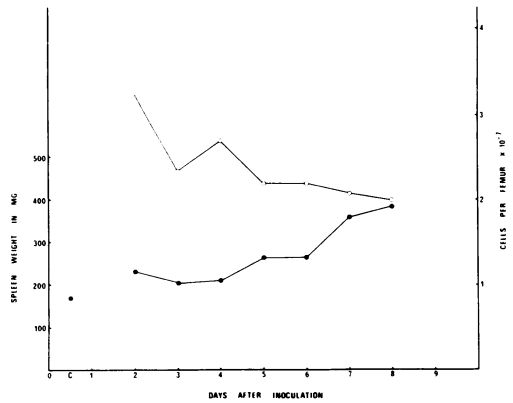

FIG. 6. *Spleen weight and cells per femur after intraperitoneal inoculation of 10^3 S. typhimurium. Average data from groups of 8 to 16 mice. C indicates level in uninoculated mice of (●) spleen weight (○) cells per femur.*

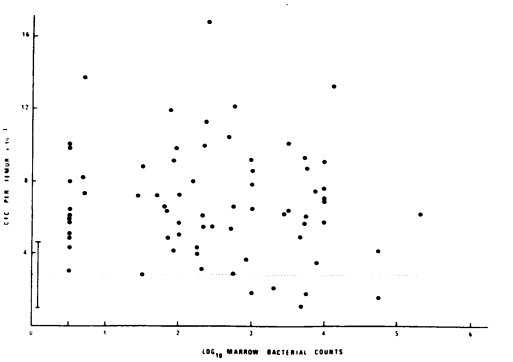

FIG. 7. *Relationship between marrow CFC and marrow infection after intraperitoneal inoculation with 10^3 S. typhimurium. Each point represents one mouse. The mean and standard deviation of CFC per femur in normal mice is shown by the dotted line and bar.*

tioned medium before challenge with *S. typhimurium*. Each value is the mean of 10 replicate cultures and the result shows that conditioned medium had no effect on the number of bacteria phagocytosed but in concentrations greater than 12% did enhance the bactericidal effect.

DISCUSSION

Since macrophages of the reticuloendothelial system provide the principal mechanism of host resistance in *S. typhimurium* infection, the purpose of this investigation was to determine if progressive infection was associated with a failure of macrophage production.

Endotoxin from several enterobacteria is known to cause an increase in the number of CFC in hemopoietic tissues and in the serum

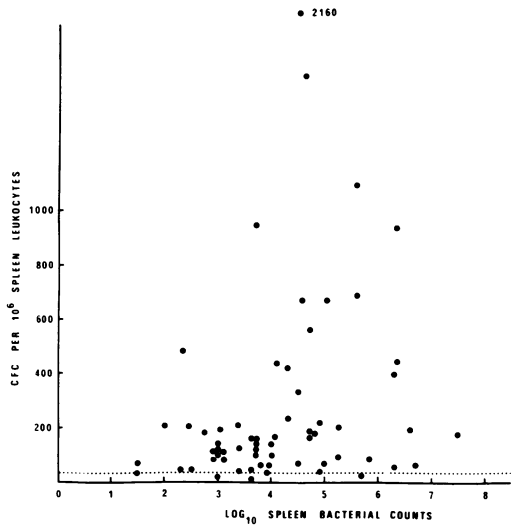

FIG. 8. *Relationship between spleen CFC and spleen infection after intraperitoneal inoculation with 10^3 S. typhimurium. Each point represents one mouse. The mean value for CFC per 10^6 spleen leukocytes in normal mice is shown by the dotted line.*

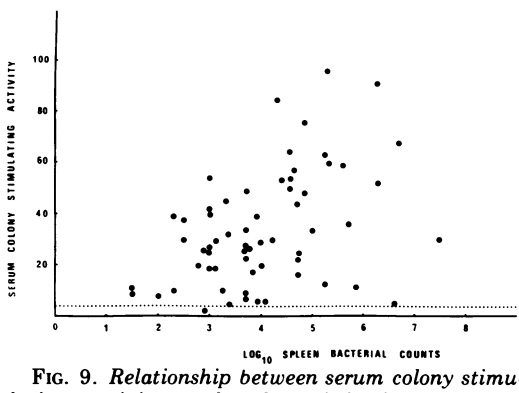

FIG. 9. *Relationship between serum colony stimulating activity and spleen infection after intraperitoneal inoculation with 10^3 S. typhimurium. Each point represents one mouse. The mean value for serum CSA in a group of normal mice is shown by the dotted line.*

TABLE 1. *Effect of mouse embryo-conditioned medium on phagocytosis and killing of S. typhimurium by peritoneal cells in vitro*

Conditioned medium (%)	No. of *S. typhimurium* phagocytosed in 1 h × 10^{-4}	Phagocytosed *S. typhimurium* alive after 1 h (%)
0	4.8 ± 0.2[a]	50 ± 5[a]
3	4.6 ± 0.2	44 ± 5
6	4.0 ± 0.3	46 ± 5
12	4.8 ± 0.2	43 ± 4
25	5.2 ± 0.3	32 ± 4
50	4.2 ± 0.3	19 ± 2

[a] Mean ± S.D. from group of 10 cultures.

and tissue levels of CS factor (3, 9, 13, 18, 20); it seems likely that the increases observed with *S. typhimurium* infection resulted from similar mechanisms. The spleen is a sensitive indicator of changes in CFC number (11) and, like the CFC responses to nonliving antigens and adjuvants (9, 12, 17), the greatest proportional increase over resting levels occurred in spleen. However, it should be pointed out that, since most CFC are present in the marrow, a 100-fold increase in splenic CFC represents the same absolute increase as a 2-fold increase in marrow. Spleen and marrow CFC were increased throughout the infection (Fig. 4) and the data from individual mice showed that these increases could be present despite high bacterial counts (Fig. 7 and 8).

Although it is clearly recognized that peripheral blood cell counts are unreliable as an index of cell production, since marked changes can occur as a result of cellular redistribution (Fig. 6), the maintenance of blood granulocyte and monocyte levels (Fig. 5) suggests that during infection CFC's were able to develop to maturity. Taken together, the results do not give any indication that failure to resist infection was associated with failure of macrophage production.

The results for serum levels of CS factor in this infection are interesting but difficult to interpret. CS factor is regarded as a specific "myelopoietin" largely because of its in vitro effects on granulocyte-macrophage colony growth and apparent lack of effect in several other culture systems (14). Direct evidence for such an action in vivo has been obtained (16) but the magnitude of effect was small in relation to the quantity of CS factor injected.

In the present experiments, serum CS factor levels appeared to fall 7 to 8 days after infection, whereas the number of CFC in marrow and spleen and the number of granulocytes and macrophages in peripheral blood were raised. Analysis of the relationship between spleen CFC and serum CS factor levels in individual mice failed to show any correlation between them ($r = +0.128$, data from 63 mice). These observations would suggest a less than direct relationship between CS factor and CFC responses. However, attempts to confirm or refute a role for CS factor in myelopoiesis on the basis of such data are hazardous since: (i) there is not necessarily a correlation between serum and tissue levels of CS factor, and (ii) the "factor" assayed in the marrow culture system is probably a heterogeneous family of molecules (20).

The possibility that CS factors function other than to regulate myelopoiesis must be regarded as an open question. One alternative role could be that such factors influence the activity of mature phagocytic cells, e.g., be involved in the macrophage "activation" phenomenon described by Mackaness and others (8). The bactericidal activity of normal peritoneal cells was found to increase after exposure to mouse embryo-conditioned medium—a rich source of CS factor (Table 1). Work is in progress to determine the relationship between colony-stimulating and macrophage-activating activities in such media.

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