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Analysis of Colony-Stimulating Factors and Macrophage Progenitor Cells in Mice Immunized against *Listeria monocytogenes* by Adoptive Transfer

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Experiments were performed to elucidate the role of colony-stimulating factors in host defenses to the intracellular pathogen *Listeria monocytogenes*. Mice were protected against *Listeria* sp. by adoptive transfer of immune spleen cells and were then challenged with listeriae intravenously. Control mice were injected with spleen cells from uninfected mice. Adoptively immunized (immune) mice had significantly fewer listeriae in spleens and livers 2 and 4 days after *Listeria* challenge than did control mice. During acute infection, colony-stimulating activity in serum was increased earlier (10 h) in immune mice than in controls. Concentrations of colony-stimulating activity were equal at 24 h. By 48 h, values were decreased in immune mice, but were elevated in control mice. Similar changes were noted when a specific colony-stimulating factor, macrophage colony-stimulating factor, was measured in serum by using a radioimmunoassay. The changes in serum colony-stimulating activity in mice adoptively immunized with immune spleen cells were eliminated if spleen cells were first treated with anti-Thy-1.2 monoclonal antibodies. The number of macrophage progenitor cells in bone marrow and spleen were also determined as measures of the hemopoietic potential in these organs. The number of macrophage progenitor cells in bone marrow was higher in immune animals than control animals at 1, 2, and 4 days of infection. Similarly, the number of these cells in spleens was higher during the early stages of infection in immune mice. These results indicate that both the regulation of leukocyte production and the transfer of specific cellular immunity by spleen cells are associated, and they therefore suggest that hemopoietic regulatory factors play a role in immune host defenses.

Listeriosis in mice is an extensively studied model of host-parasite interaction. During the early stages of infection, the production of phagocytes in bone marrow and migration of these cells into infected tissues are essential processes for host resistance (2, 8). Immunity to *Listeria monocytogenes* requires both specifically sensitized T lymphocytes and effector phagocytes, primarily granulocytes and macrophages (5). While many details of the immune reaction have been investigated, the specific mechanism whereby T lymphocytes increase resistance is unknown. In particular, it is unclear how T lymphocytes and their products regulate the production and differentiation of phagocytes.

We have approached this problem previously by studying the changes in parameters that affect macrophage-monocyte production in vivo (12, 15). Specifically, in these earlier studies, we measured macrophage colony-stimulating factor (M-CSF) concentrations in serum and determined the number of macrophage precursors in bone marrow and spleen in mice challenged with *Listeria* sp. Our results showed that M-CSF levels in serum of nonimmune mice rose within 24 h of *Listeria* inoculation and remained elevated during infection. In immune mice, M-CSF levels rose earlier than in nonimmune mice but decreased by 48 h after infectious challenge. The number of macrophage precursors in bone marrow was greater in immune mice, whereas the number in spleens was less. The current studies were designed to determine whether the changes described previously in immune mice are associated with cells that are capable of transferring immunity. To answer these questions, mice were immunized by adoptive transfer with spleen cells and then challenged with *Listeria* sp. Serum levels of colony-stimulating factors and the number of macrophage precursors were measured.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice were purchased from Jackson Laboratories, Bar Harbor, Maine, and were used between 8 and 16 weeks of age. Mice were housed in well-ventilated rooms kept between 21 and 22°C. Rodent laboratory chow (no. 5001; Ralston Purina Co.) and water were given ad libitum. During experiments, mice were kept in a separate isolation room.

***L. monocytogenes*.** The *L. monocytogenes* isolate and its preparation were described previously (15). The 50% lethal dose for C57BL/6 mice is approximately 10^5 listeriae intravenously. Mice were inoculated in a lateral tail vein with a suspension of bacteria in 0.9% NaCl. The number of viable *Listeria* organisms in each inoculum was determined by subculturing dilutions on tryptic soy agar.

Quantitation of *L. monocytogenes* in spleens and liver. The number of bacteria in organs was determined by homogenization and culturing on agar as described previously (13).

Preparation of spleen cells. Mice were immunized by intravenous inoculation with 10^4 listeriae. Six or 13 days later, they were killed by cervical dislocation, and spleens were removed by sterile technique. The spleens were forced through a wire mesh screen into RPMI 1640 plus 5% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The suspension was passed through a funnel lined with glass wool to remove tissue fragments and then forced through an 18 gauge needle several times to form

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TABLE 1. *L. monocytogenes* in spleens and livers

Organ	Time of count	Organism count in mice injected with cell type[a]:		
		None	Control	Immune
Spleen	Day 2	6.177 ± 0.185	6.578 ± 0.158	2.972 ± 0.235[b]
	Day 4	6.469 ± 0.426	4.584 ± 0.623	1.617 ± 0.702[b]
Liver	Day 2	5.287 ± 0.320	4.494 ± 0.048	3.695 ± 0.157[b]
	Day 4	6.098 ± 0.648	4.360 ± 0.183	2.636 ± 0.660[b]

[a] Mice were injected with 6×10^7 spleen cells from either nonimmune mice (control) or immune mice, or received no cells. Listeriae were then inoculated within 1 h. Spleen and liver counts were performed at 2 and 4 days. Results are expressed as the mean ± 1 standard deviation ($\log_{10}$) of five mice per group. Statistical analysis was by one-way analysis of variance, and grouping was determined by Fisher's least significant difference.

[b] Significantly less than nonimmune cells ($P < 0.01$).

a single-cell suspension. The cells were washed once in the above medium, counted, and brought to an appropriate concentration of viable cells in the same medium as determined by trypan blue exclusion. In some experiments Thy-1.2 antigen-bearing cells were depleted by incubating spleen cells with anti-Thy-1.2 monoclonal antibody (rat immunoglobulin G2b; Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) and rabbit complement (Low Tox-M rabbit complement; Cederlane Laboratories, Ltd., Hornby, Ontario, Canada). Spleen cells (10^7/ml) were incubated with antibody (1.25 μg/ml) for 30 min at 4°C; cells were washed, and complement (1:20 dilution) was added for 30 min at 37°C. The cells were washed, and the depletion was repeated once. The efficacy of the cytotoxicity procedure was confirmed by labeling cells with anti-Thy-1.2 antibody and then labeling with a fluoresceinated goat anti-rat immunoglobulin G $F(ab)_2$ antibody (Cooper Biomedical, Malvern, Pa.). Cells were analyzed for fluorescence on an Ortho Spectrum III flow cytometer (Ortho Diagnostic System, Westwood, Mass.). The mean reduction of Thy-1.2 antigen-bearing cells in our laboratory was 71 ± 11% for 11 experiments.

Adoptive transfer of spleen cells and challenge with *L. monocytogenes*. Mice were injected intravenously with 5×10^7 to 10×10^7 viable spleen cells. One hour later the animals were inoculated intravenously with 5×10^4 to 10×10^4 *L. monocytogenes*.

Determination of CSA in serum by a colony-forming assay. Total serum colony-stimulating activity (CSA) was determined by modification of a colony-forming assay (15). Uninfected mice were killed by cervical dislocation. The right femur was removed and flushed with 2.0 ml of McCoy 5A medium, using a 23 gauge needle. Suspensions of bone marrow cells from three mice were combined, aspirated through an 18 gauge needle to break up clumps of cells, and then counted in a hemacytometer. The cells were brought to a final concentration of 10^5 cells per ml in supplemental McCoy 5A medium containing 15% fetal calf serum, amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin (0.25 μg/ml), and 0.3% agar. The bone marrow cell suspensions were then placed in tissue culture dishes (35 by 10 mm), and 6.25 to 150 μl of serum was added. The plates were incubated for 7 days at 37°C in a humidified atmosphere containing 7.5% CO_2. Five plates were used for each experimental condition. After 7 days, the number of colonies, defined as a cluster containing greater than 50 cells, was scored for each plate by using a dissecting microscope. Control plates stimulated with known dilutions of L-cell-conditioned medium as a source of M-CSF were used a reference.

M-CSF level determinations. M-CSF levels in serum were measured by a double-antibody radioimmunoassay technique as described previously (3). Blood was obtained from the retro-orbital venous plexus at various times after inoculation. Serum samples from five mice were pooled, and duplicate 0.1-ml samples were assayed.

Quantitation of CFUm in bone marrow and spleen cells. The number of macrophage progenitor cells (CFUm) was determined in both bone marrow and spleen by a colony-forming assay (15). Briefly, mice were killed by cervical dislocation, and their femurs and spleens were excised. The right femurs were each flushed with 2.0 ml of McCoy 5A medium, using a 23 gauge needle. Suspensions of spleen cells were prepared by forcing spleens through a stainless-steel mesh. The cell suspensions of each organ from three mice were combined, aspirated through an 18 gauge needle to break up clumps of cells, and then counted in a hemacytometer. The cells were brought to a final concentration of 10^5 cells per ml (marrow) or 10^6 cells per ml (spleen) in supplemented McCoy 5A medium containing 15% fetal calf serum, amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin (0.25 μg/ml), and 0.3% agar. Various dilutions of L-cell-conditioned medium as a source of M-CSF were added (11). The cell suspensions were then placed in tissue culture dishes (35 by 10 mm) and incubated for 7 days at 37°C in a humidified atmosphere containing 7.5% CO_2. Five plates were used for each experimental condition. After 7 days, the number of colonies in each plate containing >50 cells was counted using a dissecting microscope. Only monocytes and macrophages are present in these colonies after 7 days of culture when L-cell-conditioned medium is used as a source of CSF (10). The number of CFUm per femur or spleen was calculated from the mean number of CFUm per plate and the total number of cells per organ.

Statistical analysis. Statistical analysis was by Student's *t* test or one-way analysis of variance.

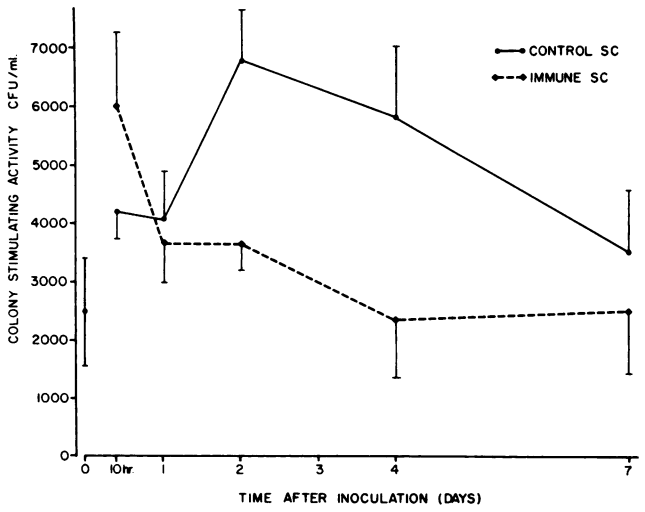

FIG. 1. CSA in serum in mice immunized by adoptive transfer. C57BL/6 mice were injected with 7.4×10^7 spleen cells from mice that had been inoculated 6 days previously with 10^4 listeriae or with saline (control). One hour later 10^5 listeriae were inoculated intravenously. CSA (CFU per milliliter) was measured in serum (pooled from five mice per group) at various times afterwards. Data are expressed as the mean ± 1 standard deviation. The value at time 0 represents the mean of uninfected, uninjected mice.

TABLE 2. CSA in sera and number of *L. monocytogenes* in spleens of mice inoculated with Thy-1.2-depleted spleen cells[a]

Spleen cells	Mean CSA (colonies/ml) ± SEM at (time):			Mean *L. monocytogenes* (log_{10})/ organ ± SEM at 48 h	
	12 h	24 h	48 h	Liver	Spleen
Control	1,040 ± 78	776 ± 182	4,568 ± 471	3.880 ± 0.135	5.262 ± 0.181
Listeria-immune	2,472 ± 215[b]	672 ± 116	464 ± 65[c]	2.323 ± 0.140[c]	1.650 ± 0.173[c]
Listeria-immune + anti-Thy-1.2 + C	1,664 ± 290[b]	560 ± 99	3,664 ± 587	4.086 ± 0.136	5.156 ± 0.140

[a] Mice were injected intravenously with 6×10^7 spleen cells harvested from other mice inoculated 7 days earlier with either saline (control) or 10^4 listeriae. Half of the spleen cells were treated with anti-Thy-1.2 antibodies plus complement. One hour later, mice were inoculated with 5×10^4 listeriae. Serum CSA was determined at 12, 24, and 48 h, and the number of listeriae per spleen was determined at 48 h. Results represent mean ± standard error of 10 samples from two experiments. The data were analyzed by one-way analysis of variance, and grouping was determined by Fisher's least significant difference for each time period.
[b] Values significantly greater than control.
[c] Values significantly less than control.

RESULTS

Transfer of immunity by listeria-immune spleen cells. Experiments were performed to confirm in our model that immunity to listeriae could be transferred with immune spleen cells. Mice were injected intravenously with 6×10^7 spleen cells from either nonimmune (control) or immune mice and 1 h later were challenged with 5×10^4 (0.5 50% lethal dose) listeriae intravenously. Two and 4 days later, the numbers of listeriae in livers and spleens were significantly less in mice that had received immune spleen cells than in mice that had received control cells or no cells ($P < 0.01$) (Table 1).

Changes in serum CSA and M-CSF during listerial infection. Our previous studies had shown specific changes in parameters of macrophage production in immune mice that differed from those of nonimmune mice (12, 15). To extend these observations, experiments were designed to determine whether changes in total serum CSA correlated with immunity in mice that had been immunized by adoptive transfer and then challenged with listeriae. The CSA in serum was determined by an in vitro colony growth assay, which is a measure of the total effect of all colony-stimulating factors including M-CSF, granulocyte-macrophage CSF, granulocyte CSF, and multi-CSF (interleukin-3). Mice immunized by adoptive transfer had significantly higher levels of serum CSA 10 h after listerial challenge than mice that had been inoculated with control spleen cells ($P < 0.05$) (Fig. 1). At 24 h, differences were not significant. By 48 and 96 h, immune mice had significantly lower levels than control mice ($P < 0.01$). Levels tended to return to levels of uninfected mice by 7 days. Similar findings were observed in four consecutive experiments. When M-CSF levels were determined by a radioimmunoassay, concentrations were increased 10 h after challenge in immune mice as compared with control mice. At 24 h values were similar, and at 48 h values were lower in immune mice.

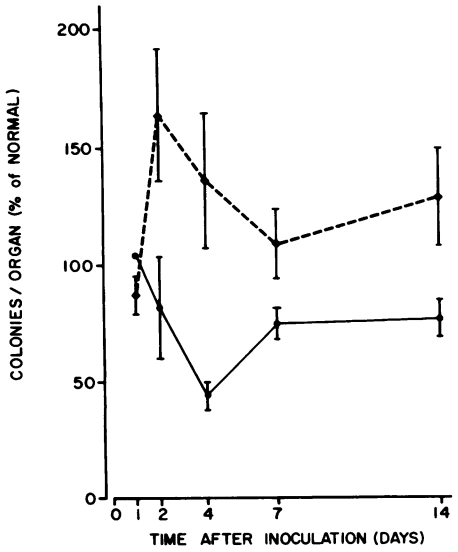

FIG. 2. CFUm in bone marrows of immune mice. Mice received 6.5×10^7 spleen cells from either immune (◆---◆) or control (●——●) mice and then were inoculated with 5×10^4 listeriae. The number of CFUm was measured at various times afterwards.

Effect of depletion of Thy-1.2 antigen-bearing cells on CSA production and on the number of listeriae in spleens and livers. To determine whether T lymphocytes were responsible for the changes in CSA levels documented above, spleen cells were depleted of Thy-1.2 antigen-bearing cells before being used for adoptive transfer. Mice were inoculated with 6×10^7 viable spleen cells and 5×10^4 listeriae in all groups. Serum was obtained 12, 24, and 48 h later, and CSA levels were determined. Mice receiving immune spleen cells had elevated levels compared to controls at 12 h, but significantly lower levels at 24 and 48 h (Table 2). In contrast, mice receiving immune spleen cell populations depleted of Thy-1.2-positive cells had CSA levels modestly elevated at 12 h as compared with mice receiving control spleen cells. Levels at 24 and 48 h were similar to those in controls. The numbers of *Listeria* organisms per organ were lower than controls in mice injected with immune spleen cells, but equivalent to controls in mice injected with Thy 1.2-treated spleen cells at 48 h.

Changes in the number of CFUm in bone marrow. The number of macrophage precursors in bone marrow is one parameter that may reflect the overall capacity of an animal to produce cells of the macrophage lineage. An in vitro colony-forming assay was used to assess changes in CFUm in bone marrow of mice immunized by adoptive transfer and then challenged with listeriae. Results of a representative experiment demonstrated that the numbers of CFUm in marrows of mice given immune spleen cells were higher on days 2, 4, 7, and 14 than those in control mice ($P < 0.01$) (Fig. 2). In other experiments, differences were noted only on days 2 and 4. Numbers of CFUm in control mice fell to a nadir at 4 days of infection and then returned towards normal.

Changes in spleen CFUm. Extramedullary hemopoiesis in organs such as the spleen rises sharply in mice during acute infection. We measured the number of CFUm in spleens as an indication of the number of macrophage precursors in immune and control mice at various times during infection. The number of CFUm was higher in immune versus control

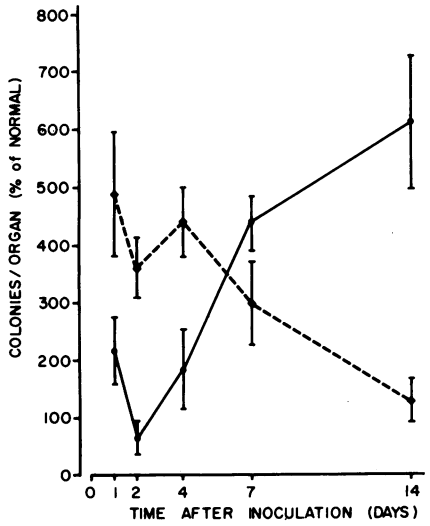

FIG. 3. CFUm in spleens in immune mice. Symbols: ◆---◆, immune spleen cells; ●——●, control spleen cells.

mice on days 1, 2, and 4 after inoculation ($P < 0.01$) (Fig. 3). Control mice had higher numbers 7 and 14 days after infection ($P < 0.05$ and $P < 0.001$, respectively).

DISCUSSION

In previous studies, we showed that immune mice had an accelerated secretion of M-CSF compared to controls in response to challenge with *Listeria* sp. (12). The immune mice also had higher numbers of bone marrow macrophage precursors during infection. In the studies reported here, adoptive transfer techniques allowed us to conclude that spleen cells were responsible for the changes in CSF responses and bone marrow precursors. Specifically, mice immunized by adoptive transfer of spleen cells from immune animals had an earlier increase in CSA and M-CSF concentrations during *Listeria* infection than control mice. This effect could be ascribed to T lymphocytes in the spleen since depletion of Thy-1.2-positive cells ablated the protection and the observed changes in CSA. Similarly, CFUm in bone marrow were increased in adoptively transferred animals as compared with controls. The number of splenic CFUm was increased in immune animals early in infection, a finding not noted in earlier studies, but decreased later in infection. A possible explanation for these differences is that injected spleen cells are distributed differently early in infection compared to lymphocytes in animals actively immunized by infection. Overall, these data demonstrate that both protective immunity and the concomitant changes in parameters of leukocyte production can be transferred by T lymphocytes, suggesting a functional linkage between the two.

The CSA in serum is a measure of the effect of all colony-stimulating factors and their inhibitors and presumably includes M-CSF, granulocyte-macrophage CSF, granulocyte CSF, and interleukin-3. T lymphocytes are known to produce CSFs in response to mitogen or antigen stimulation in vitro (7). The findings in this study are consistent with T-cell production and secretion of CSFs during antigenic stimulation with *Listeria* sp. in vivo. Further experiments from our laboratory indicate that L3T4-positive T lymphocytes are responsible for production of the majority of CSA during immune stimulation in vitro (D. M. Magee and E. J. Wing, manuscript submitted). It should be noted, however, that *Listeria* infection causes elevations in CSF levels in nonimmune mice before T-cell immunity can be detected (15). This indicates that other cell types may be responsible, in part, for CSF secretion during infection. Indeed, many tissues and cell types, including lung, placenta, fibroblasts, and macrophages, have the capacity to produce and secrete CSFs (7, 14).

While the specific functions of CSFs during acute infection are unknown, it seems most likely that a primary role is to stimulate the proliferation and differentiation of leukocyte progenitor cells (7). In vivo, this effect potentially increases the pool of both granulocytes and macrophages available for host defenses. In support of this concept, recent evidence indicates that recombinant human granulocyte-macrophage CSF directly enhances the production of leukocytes in vivo (4). This capacity of CSFs to stimulate leukocyte production may be particularly relevant to immune host defenses, as illustrated by the *Listeria* model. Resistance to *Listeria* sp. depends on the bone marrow production of leukocytes early in infection (2, 8). If bone marrow is irradiated to prevent cell proliferation, the major resistance factor during the first 48 h of infection is eliminated, multiplication of bacteria occurs unchecked, and animals die. Also, studies with genetically resistant and genetically sensitive mouse strains indicate that production of monocytes is critical during early host resistance to this pathogen (6, 9). Therefore, factors that regulate bone marrow and spleen production of leukocytes, such as CSFs, the number of progenitor cells, and tissue stroma, are probably essential for resistance and immunity to this pathogen.

CSFs may also enhance host defenses by stimulating the effector function of mature tissue leukocytes. For example, granulocyte-macrophage CSF and M-CSF increase the secretory capacity of neutrophils and macrophages, respectively (7, 14). CSFs have also been noted to alter the metabolism and morphology of leukocytes and to have significant functional effects that may contribute to inflammatory responses (1, 7). It is tempting to speculate, therefore, that CSFs, which have multiple effects on the leukocyte axis, are produced under the control of T lymphocytes during immune reactions and that immunity itself may depend, in part, on these growth factors.

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