

## Prophylaxis or Treatment of Experimental Brucellosis with Interleukin-1

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Received 21 August 1990/Accepted 1 February 1991

**Intravenously injected recombinant human interleukin-1 alpha (IL-1 $\alpha$ ) given to mice 4 h before infection with *Brucella abortus* 19 depressed the growth of bacteria in the spleen and liver. However, the same dose (10<sup>5</sup> U) or a 10-fold higher dose was not able to decrease numbers of bacteria when given to chronically infected mice. IL-1 injected into normal mice induced a dramatic increase 2 h later in colony-stimulating activity in serum, measured by bone marrow proliferation, and in colony-stimulating factor 1, measured by radioimmunoassay. Colony-stimulating factor levels declined but remained higher than normal for at least 12 h. The early peak stimulation was not observed in chronically infected mice, but the more prolonged elevation was. As a result of IL-1 treatment, the number of colony-forming cells, especially in the spleen, was increased in normal and acutely or chronically infected mice. Myeloperoxidase staining of newly formed monocytes and polymorphonuclear cells in the spleen revealed an increase in the number of these cells in normal and acutely infected mice as a result of IL-1 treatment, but there was no increase in the already high numbers in chronically infected mice. The relationship between these observations and the basis of chronic infection are discussed.**

Injection of mice with the attenuated bovine vaccine *Brucella abortus* 19 results in a chronic infection, which was one of the early models of cell-mediated immunity (9). The bacteria survive within macrophages of the spleen and, to a lesser extent, the liver and bone marrow. The level of infection is controlled by activation of those cells by lymphokines produced by T lymphocytes. After the initial decline in bacterial numbers coinciding with the onset of cell-mediated immunity, there persists for some time a steady infection of about 10<sup>6</sup> bacteria per spleen (3). Both before and after the onset of cell-mediated immunity, macrophages and neutrophils are essential effector cells.

The maturation of these phagocytic cells is dependent on colony-stimulating factors (CSF), a group of cell growth factors that regulate the proliferation and differentiation of hemopoietic precursor cells into macrophages, neutrophils, and other end cells (10). These precursor cells are known as colony-forming cells (CFC) because of their ability to form colonies in semisolid agar under the influence of CSF. In mice infected with *B. abortus* 19, CSF in the serum and CFC in the spleen and bone marrow reach a peak 2 to 3 weeks after infection (4). However, CSF and CFC return to normal levels 4 weeks after infection despite the presence of up to 10<sup>6</sup> bacteria per spleen. It is not clear what the mechanism of this is or whether it is the reason for the chronicity of the infection.

Recent reports have shown that interleukin-1 (IL-1) given before injection of mice with any one of a number of pathogenic bacteria enhances resistance to subsequent infection (5, 13). This is related, at least in part, to increased colony-stimulating activity (CSA) in serum and increased numbers of CFC in treated mice (7, 12, 19). We therefore set out to test whether IL-1 given before infection or during chronic infection would enhance the resistance of mice to *B. abortus*. In particular, the effect of IL-1 during chronic infection would tell us whether the mice had become refrac-

tory to further production of CSFs, as has been suggested (14), or, if not, whether enhancing production of macrophages would lead to early recovery from infection. These questions are relevant to the possibilities for therapy of similar infections, such as tuberculosis and leprosy.

### MATERIALS AND METHODS

**Mice and bacterial infection.** CBA/H mice were bred in the Microbiology Animal Breeding Unit, University of Melbourne, Australia, by strict brother-sister mating. The mice were sex matched within experiments and were infected at 6 weeks of age. The vaccine strain of *B. abortus* 19 (Commonwealth Serum Laboratories, Parkville, Australia) was maintained by weekly subculture on horse blood agar (HBA) and renewed from freeze-dried stock after 25 passages. Twenty-four-hour cultures were washed from the plates with sterile saline. The inoculum was standardized turbidometrically to 5  $\times$  10<sup>5</sup> CFU per mouse for chronic infection or 2  $\times$  10<sup>4</sup> CFU per mouse for acute infection and injected intravenously in 0.2 ml. The dose was checked retrospectively by viable counts on HBA plates. At various times after infection, the mice were killed by fluothane anaesthesia, and spleens and livers were removed aseptically and homogenized in 5 ml of sterile distilled water in sealed containers with an Ultra-Turrax homogenizer (Janke and Kunkel, Breisgau, Germany). After appropriate dilution, samples were plated onto HBA, and colonies were counted after 72 h to give a count of viable bacteria.

**IL-1.** Human recombinant IL-1  $\alpha$  was the generous gift of P. J. Lomedico (Hoffman-La Roche, Nutley, N.J.). The lyophilized material was reconstituted to 2  $\times$  10<sup>8</sup> U/ml (D10 assay; protein concentration, 0.68 mg/ml; endotoxin, <0.5 EU/ml) with pyrogen-free saline (Astra, North Ryde, New South Wales, Australia) and stored at 4°C. Previous experiments showed 10<sup>5</sup> U per mouse as the optimal dose. The control groups of mice received pyrogen-free saline.

**Bioassay for CSA.** Mice were bled from the heart under fluothane anaesthesia. For CSA, serum was pooled within

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each experimental group. The CSA assay was based on that developed by Horak et al. (6). Bone marrow cells from C57BL/10 mice were prepared from tibias by flushing the marrow cavity with 1 ml of IMDM (Iscove modified Dulbecco medium [GIBCO, Grand Island, N.Y.] with 60 µg of penicillin per ml and 100 µg of streptomycin per ml) and 10% fetal calf serum (FCS, Flow Laboratories, Stanmore, Australia). Cells were washed once with IMDM-FCS and adjusted to a concentration of  $4 \times 10^5$ /ml in IMDM-FCS. Twofold dilutions of sera were made in triplicate in 0.1 ml of 96-well flat-bottomed tissue culture microtiter plates (Disposable Products Pty., Adelaide, Australia). A further 0.1 ml of cells was added to each well. After 4 days of culture the cells were pulsed with 0.5 µCi of [ $^3$ H]thymidine per well (Amersham International, Amersham, United Kingdom) for 6 h and then harvested with an automatic cell harvester on filter paper (Titretek Microtitration Equipment; Flow Laboratories). The dried papers were placed in scintillation fluid [0.5% 2,5-diphenyloxazole and 0.01% 1,4-bis(5-phenyloxazolyl)benzene in toluene], and incorporated [ $^3$ H]thymidine was measured with a Packard Tricarb liquid scintillation spectrometer model 3320. The counts were expressed as the mean counts per minute  $\pm$  the standard deviation.

**CSF-1 radioimmunoassay.** Highly purified CSF-1 was radioiodinated as described previously for use in a radioimmunoassay (18). For the CSF-1 assay, sera were collected from individual mice. The concentration of CSF-1 in serum samples from individual mice was assayed by a specific radioimmunoassay as described in detail elsewhere (17).

**CFC assay.** Bone marrow cells were prepared as described above. Spleen cells were prepared by teasing the spleen into IMDM-FCS and passing the cells through an 80-mesh stainless steel sieve. The cells were centrifuged at  $800 \times g$  for 7 min, suspended in Tris-buffered 0.83% ammonium chloride to lyse erythrocytes, underlaid with 1 ml of FCS to remove debris, and centrifuged again. Viable cells were counted by eosin exclusion and diluted appropriately in IMDM-FCS. The agar culture system used to assay CFC was similar to that described by Metcalf et al. (11). Spleen and bone marrow cells were assayed for CFC by using triplicate 1-ml cultures containing  $5 \times 10^4$  viable bone marrow cells or  $10^6$  viable spleen cells in 35-mm-diameter petri dishes (Falcon Products, Becton-Dickson Labware, Oxnard, Calif.) in 1 ml of semisolid agar with IMDM-FCS. A source of CSF was supplied by 0.1 ml of a 1/4 dilution of pooled serum from C57BL/10 mice injected 6 h previously with 5 µg of lipopolysaccharide w (*Escherichia coli* O11:B4; Difco Laboratories, Detroit, Mich.). After 6 days of incubation, colonies were counted under a dissecting microscope (magnification,  $\times 200$ ). Only colonies containing more than 50 cells were scored.

**Staining of spleen cells.** Cyto-centrifuge smears were prepared by placing 200 µl of FCS and  $4 \times 10^4$  spleen cells into a cyto-centrifuge chamber and spinning at  $44 \times g$  for 10 min. For differential cell counts, cyto-centrifuge smears were fixed in methanol for 5 min and stained with Diff-Quik (AHS, Australia), dried, and mounted in DPX (BDH Chemical, Australia). Slides were examined under oil immersion at  $\times 800$  magnification. Differential cell counts were determined by counting at least 200 cells. For myeloperoxidase staining, the smears were fixed with formol-ethanol for 1 min and stained with a benzidine dihydrochloride mixture as described by Kaplow (8). The slides were dried, mounted in DPX, and examined under oil immersion at  $\times 800$  magnification. At least 500 cells were counted; any cells containing blue granules were considered positive for myeloperoxidase.

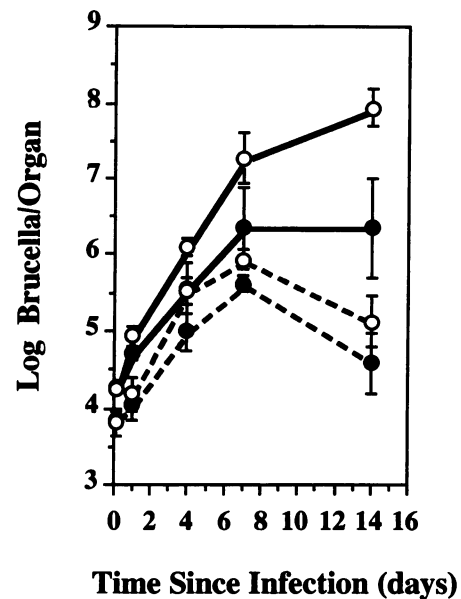


FIG. 1. Effect of IL-1 on bacterial numbers in the spleens (—) and livers (---) of *B. abortus*-infected mice. Mice were infected with  $2 \times 10^4$  *Brucella* cells intravenously 4 h after injection of  $10^5$  U of IL-1 (●) or saline (○). Mice were killed on day 14. Each point represents the mean  $\pm$  standard deviation for five mice.

## RESULTS

**Effects of IL-1 on bacterial numbers.** CBA mice received  $10^5$  U of recombinant IL-1  $\alpha$  intravenously 4 h before infection. A significant reduction in bacterial numbers was observed in spleens and livers of IL-1 $\alpha$ -treated mice compared with those in mice receiving saline only (Fig. 1). Four repeated experiments showed similar results. The IL-1 had no effect on the initial localization measured at 2 h. However, by day 4 after infection, IL-1 treatment had significantly depressed the growth in bacterial numbers, and protection was maintained for the 14 days of observation, when there was a 40-fold difference in bacterial numbers between treated and untreated groups. The effect on bacterial number was more marked in spleens than in livers, in which the infection is more readily resolved even in untreated mice.

Mice that had been infected with *B. abortus* 4 weeks earlier still carried many organisms. When mice were treated with IL-1 as described above or with an even higher dose ( $10^6$  U), bacterial numbers in spleen and liver did not change significantly, averaging around  $3 \times 10^5$ /spleen and  $1 \times 10^3$ /liver regardless of treatment (data not shown). The time of IL-1 administration was in fact found to be critical to the efficacy of IL-1. When mice were injected only 1 day after infection, no depression in bacterial numbers was observed over a 14-day period after infection. Significant protection was observed only when IL-1 was administered before infection.

**Effect of IL-1 on CSA in serum.** We have previously shown that brucella infection results in increased CSA in serum 1 to 3 weeks later but that the level declines by 4 weeks postinfection (4). The effect of IL-1 on CSA in serum was therefore tested in three categories of mice: normal, acutely infected, and chronically (4 weeks) infected. Total serum CSA was measured by the bone marrow proliferation assay, whereas the radioimmunoassay was used to measure specifically

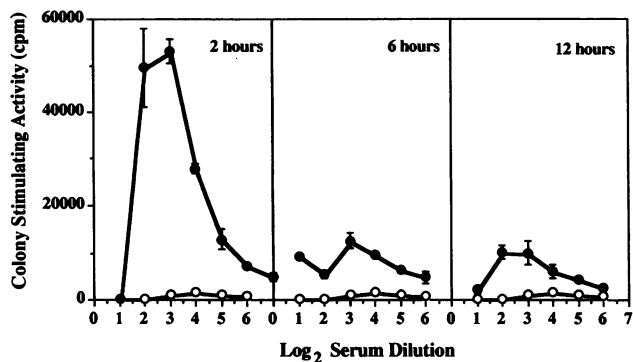


FIG. 2. Effect of IL-1 on CSA in serum of uninfected mice. Mice were bled 2, 6, and 12 h after injection of  $10^5$  U IL-1, and dilutions of serum were tested for CSA. Each point represents the mean  $\pm$  standard deviation for triplicate cultures from IL-1-treated (●) or saline-injected (○) mice.

CSF-1. Experiments were performed three times with similar results.

When IL-1 was injected into normal mice, the CSA in serum increased dramatically 2 h later (Fig. 2). This increase in the CSA in serum gradually subsided but was still measurable at 6 and 12 h. Mice infected 4 h after IL-1 injection showed increased CSA 6 h after IL-1 injection compared with that in infected but untreated mice (Fig. 3). By 12 h after IL-1 injection, the effect of infection alone overtook that of IL-1 pretreatment.

Figure 4 shows the contrasting situation in normal and chronically infected mice injected with IL-1. Whereas at 2 h after injection of normal mice, there was the usual marked increase in CSA, the increase in 28-day-infected mice was modest (5-fold difference in peak counts per minute compared with 45-fold in the normal mice). At 6 h, the CSA levels in IL-1-treated mice were similar in normal and chronically infected mice.

These results were broadly confirmed by specific measurement of CSF-1 with the radioimmunoassay. Levels of CSF-1 in chronically infected mice were actually below the level in normal mice throughout, but the level was markedly

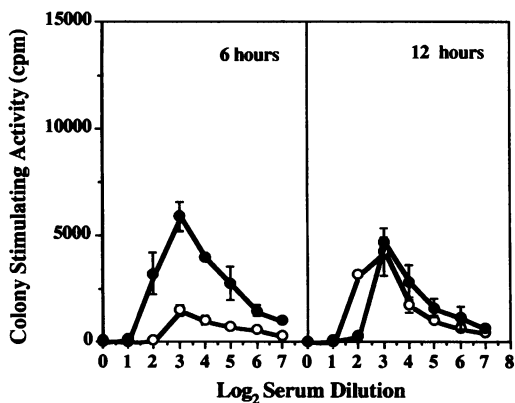


FIG. 3. Effect of IL-1 on serum CSA in acute infection. Mice were injected intravenously with  $10^5$  U of IL-1 (●) or with saline (○) and infected 4 h later with  $2 \times 10^4$  cells of *B. abortus* 19. Serum was taken 6 and 12 h after IL-1 treatment (2 and 8 h after infection). Each point represents the mean  $\pm$  standard deviation for triplicate cultures.

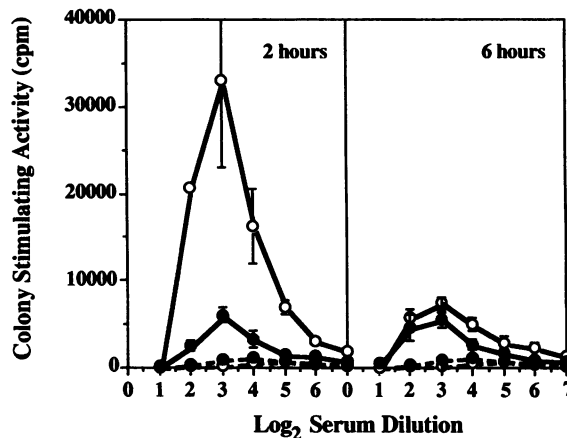


FIG. 4. Effect of IL-1 on serum CSA in normal and chronically infected mice. Mice were infected intravenously with  $5 \times 10^5$  cells of *B. abortus* 19 for 30 days and then injected with  $10^5$  U of IL-1 (●—●) or with saline (○—○). Corresponding normal mice were injected with  $10^5$  U of IL-1 (○—○) or with saline (○—○). Serum samples were taken 2 or 6 h after IL-1 injection, and dilutions were tested for CSA. Each point represents the mean  $\pm$  standard deviation for triplicate cultures.

increased by IL-1 treatment (Table 1). Serum from acutely infected mice also showed an early depression in CSF-1, which was mitigated by IL-1 treatment. Thus, 2 h after infection, untreated mice had only  $450 \pm 212$  U of CSF-1 per ml, compared with  $2,160 \pm 1,486$  U/ml in normal mice. Mice given IL-1 4 h before infection developed  $1,210 \pm 460$  U/ml. By 8 h after infection (12 h after IL-1 injection), untreated mice had  $3,613 \pm 3,327$  U of CSF-1 per ml and treated mice had  $2,733 \pm 1,963$  U/ml.

**Effect of IL-1 on CFC in spleen and bone marrow.** Intravenous injection of IL-1 into normal mice, mice infected 4 h later, or mice carrying a 28-day infection increased the numbers of CFC in the spleen in all three groups as early as 1 day after treatment (Fig. 5). The effect was even greater at 4 days. Although a marked increase in the number of spleen CFC was seen in each of four experiments, the effect on IL-1 bone marrow CFC was variable, occurring in only two of the four experiments, and led to not more than a twofold increase (data not shown).

**Effect of IL-1 on cell composition in spleen.** To quantify the newly formed monocytes and polymorphonuclear cells in the spleen, cells were stained for myeloperoxidase, a bactericidal enzyme found in polymorphs and newly formed

TABLE 1. Effect of IL-1 on CSF-1 measured by radioimmunoassay

Treatment <sup>a</sup>	CSF-1 (U/ml) <sup>b</sup>	
	Normal	Chronic <sup>c</sup>
None	2,160 $\pm$ 1,486	80 $\pm$ 51
IL-1		
2 h	4,775 $\pm$ 2,333	1,483 $\pm$ 678
6 h	2,067 $\pm$ 643	2,050 $\pm$ 636
12 h	2,567 $\pm$ 1,966	1,975 $\pm$ 809

<sup>a</sup> Mice received  $10^5$  U of IL-1 or saline. At the times indicated, mice were bled and sera were prepared from individual animals.

<sup>b</sup> Mean of three to five mice per group.

<sup>c</sup> Mice were infected with  $5 \times 10^5$  *Brucella* cells for 28 days.

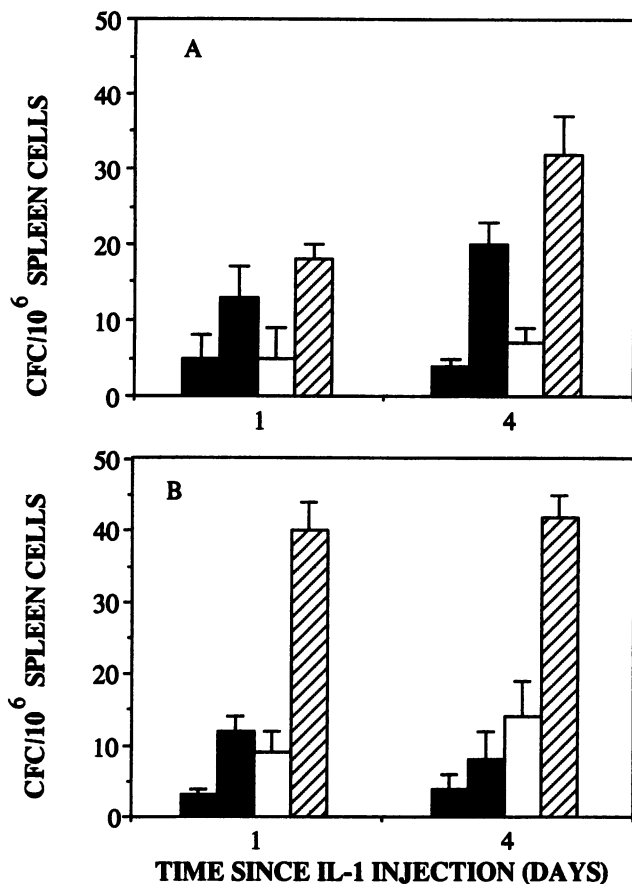


FIG. 5. Effect of IL-1 on spleen CFC in acute (A) or chronic (B) infection. (A) Mice were infected intravenously with  $2 \times 10^4$  *B. abortus* cells 4 h after injection with  $10^5$  U of IL-1 (▨) or with saline (□). At the same time, normal mice were given IL-1 (▨) or saline (■). (B) Mice already infected for 30 days with *B. abortus* 19 were given  $10^5$  U of IL-1 (▨) or saline (□). At the same time, normal mice were given IL-1 (▨) or saline (■). Each point represents the mean and standard deviation for a triplicate determination of CFC from pooled spleen cells of five mice killed 1 or 4 days after injection of IL-1.

monocytes but not in mature macrophages. There were few myeloperoxidase-positive cells in the spleens of normal mice (Table 2). IL-1 treatment resulted 2 days later in an increased percentage of positively staining cells in normal mice and newly infected mice but did not change much that of chronically infected mice, whose spleen already contained a

TABLE 2. Effect of IL-1 on cell composition in spleens of mice 2 days after injection with IL-1 or saline

Mice	% Myeloperoxidase-positive cells		% Polymorphs	
	-IL-1	+IL-1	-IL-1	+IL-1
Normal <sup>a</sup>	1.8	5.0	1.0	4.0
Acutely Infected <sup>b</sup>	1.0	6.6	1.0	2.0
Chronically infected <sup>c</sup>	17.8	17.4	10.0	11.0

<sup>a</sup> Normal controls.

<sup>b</sup> Mice infected 4 h after IL-1 or saline treatment.

<sup>c</sup> Mice infected 30 days before IL-1 or saline treatment.

very high percentage of myeloperoxidase-positive cells. Differential counting in Diff Quik-stained preparations showed that polymorphonuclear cells accounted for only about half the increase in myeloperoxidase-positive cells, which must therefore have included young monocytes.

## DISCUSSION

With the increasing availability of recombinant cytokines, the possibility of their therapeutic use is causing increased interest in their role in infection. The present study was aimed at testing the effect of IL-1 $\alpha$  in particular on chronic infection. If effective, it might provide guidelines for treatment of chronic bacterial infection; if not, it would cast light on the mechanism of chronicity.

IL-1 $\alpha$  clearly increased the resistance of mice to *B. abortus* strain 19 when used prophylactically, i.e.,  $10^5$  U given 4 h before infection. There was no change in the initial localization of the bacteria, but by 7 and 14 days, respectively, there were 10- and 40-fold fewer bacteria in the spleens of treated mice than in those of untreated mice. The timing of the IL-1 injection was quite critical. Delay until 24 h after infection with *Brucella* organisms negated the effect. Why this should be so is unclear, but we observed a similar effect when injecting the interferon inducer poly(A-U), to protect against *Brucella* infection (2), suggesting that quite early events determine the outcome of infection. In contrast to the effect of IL-1 $\alpha$  on acute infection, IL-1 given to mice infected 1 month before and bearing a chronic infection did not lead to a decrease in bacterial numbers. Even a 10-fold higher dose ( $10^6$  U rather than  $10^5$  U) was not effective.

A major effect reported after injection of IL-1 into normal mice is the increase in the CSA in serum (19). We compared both total CSA (measured by bone marrow assay) and CSF-1 (measured by radioimmunoassay) in normal mice, acutely infected mice, and chronically infected mice. By both assays, there was a sharp increase in CSF 2 h after giving IL-1, with levels declining but remaining above normal for at least 12 h. The relatively large standard deviations in the radioimmunoassay results reflected mouse-to-mouse variation. The fact that the increase in CSF-1 was detected by the radioimmunoassay is important, since the radioimmunoassay, unlike bioassays, is not influenced by synergistic or inhibitory effects. IL-1 can act synergistically with some CSFs in vitro (21), and the apparent rise in total CSA could have been attributed to residual IL-1. Similarly, the increase in CSF-1 could not be due to removal of an inhibitor, which has been reported with a CFC assay (1).

When mice were challenged with *Brucella* organisms 4 h after IL-1 injection, the IL-1-treated mice retained an advantage over untreated mice in terms of total CSA until the infection itself began to stimulate CSA in the serum. Curiously, infection had an initial depressing effect on specific CSF-1, although levels of CSF-1 were higher in the IL-1-pretreated mice. Whether this apparent removal of CSF-1 from circulation could be due to rapid upregulation of macrophage CSF-1 receptors as a result of infection or whether it is due to some other mechanism could only be settled by further experiments. By 24 h, CSF-1 in infected mice is well above normal (3a).

When IL-1 was injected into mice already carrying a chronic (28-day) infection, the dramatic increase in total CSA did not occur at 2 h. The effect on total CSA is unlikely to be due to its removal from circulation, since at 6 and 12 h the levels in normal and chronically infected mice were similar. It may be that chronically infected mice do develop

some tolerance to further stimulation of CSF production, as suggested by Quesenberry (14). As in the early infection, chronically infected mice had only low levels of CSF-1. IL-1 restored the level to normal.

Notwithstanding these variations in CSF responses among the groups of mice, we observed a four- to fivefold increase in the number of granulocyte-macrophage CFC in the spleens of all mice given IL-1, whether they were normal or acutely or chronically infected. In the case of the chronically infected mice, either IL-1 was acting on the CFC to make them more sensitive to CSF, as reported previously (21), or the lesser increase in CSF in these mice was still sufficient to increase their CFC. The effect on bone marrow CFC was slight. It is not unusual for bone marrow CFC to be relatively unaffected by injection of stimulating materials, either CSF (15) or bacterial lipopolysaccharide (16), an otherwise strong stimulator of CFC and CSF, as well as of IL-1. As a result of the increased CFC in our IL-1-treated normal or acutely infected mice, there was an increase in both neutrophils and myeloperoxidase-positive monocytes in the spleen. Both of these cell types are able to phagocytose and kill *Brucella* organisms (20) and presumably contribute to the early control of the infection that we observed. However, the chronically infected mice already had high numbers of neutrophils and monocytes in the spleens, and these were not increased by the treatment, despite increased numbers of CFC.

These results suggest that the basis of chronic infection, at least in this experimental model, is not related to the diminished levels of CSF and CFC that occur after the peak of infection and the onset of specific T cell-mediated immunity (4). Furthermore, they suggest that CSF and the cytokines that stimulate their production may find their application in prophylaxis against infection, rather than in its treatment. This limitation would presumably not apply where there is a definite deficiency in the production or action of the phagocytic defense cells.

#### ACKNOWLEDGMENTS

This work was supported by a grant from the National Health and Medical Research Council of Australia (to C.C.) and by Public Health Service grants CA 32551 and P30-CA1330 from the National Institutes of Health and a grant from the Lucille P. Markey Foundation (to E.R.S.). Y.F.Z. was supported by a Cooperative Research Project Grant from the Australian Department of Science Bilateral Science and Technology Program.

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