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The Cellular Source of Interleukin-6 during *Listeria* Infection

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The cellular source of interleukin-6 (IL-6) during infection of mice with *Listeria monocytogenes* was investigated both in vitro and in vivo. Peritoneal cells taken at intervals from infected mice and cultured in vitro without added stimulus produced high titers of IL-6 peaking 2 days postinfection in a time course similar to that observed in vivo. Adherent cells with the morphology of macrophages were a major source of this IL-6. Spleen cells similarly harvested at intervals and cultured with heat-killed *Listeria* or heat-killed *Brucella* organisms as specific and nonspecific stimuli, respectively, showed two distinct IL-6 responses: (i) an early-phase response up to 5 days after infection when IL-6 production was elicited by either a specific or nonspecific stimulus, and when depletion of T cells had no effect, and (ii) a later response 7 to 10 days after infection when very high levels of IL-6 were produced in response to a specific stimulus. This response was lost when T cells were depleted in vitro or in vivo or in spleen cell cultures from mice with severe combined immunodeficiency. However, studies in vivo failed to show an important role for T cells governing serum IL-6. We conclude that most of IL-6 detected in vivo is produced by nonlymphocytes. Whether IL-6 produced by T lymphocytes in local foci of infection has any role in resolution of that infection is unknown.

Interleukin-6 (IL-6) is a polyfunctional cytokine whose concentration in serum and tissues is greatly increased by infection, both clinical (2, 7) and experimental (10, 13, 21). It is produced by a variety of cells, including T lymphocytes, macrophages, fibroblasts, endothelial cells, and mast cells (28). The known functions of IL-6 include stimulation of B-cell growth and differentiation, activation of IL-2 receptors and IL-2 production by T cells, induction of acute-phase proteins in the liver, and effects on the nervous system (28). It is not clear whether its main function in infection is to protect or to add to the immunopathology, although injection of recombinant IL-6 into mice before infection with *Listeria monocytogenes* enhanced recovery (18).

L. monocytogenes, a gram-positive intracellular bacterium, localizes in the spleen and liver of mice. Efficient hematopoiesis (31) and an early nonspecific inflammatory response (22) are essential to the initial control of the infection, and deficiency in these mechanisms leads to the genetic susceptibility of some strains of mice (26, 30, 31). Acquired immunity to the infection requires activation of T lymphocytes which in turn recruit and activate monocytes and macrophages at the site of infection (15). During the course of infection, IL-6 levels are greatly enhanced in the serum, peritoneal cavity, and spleen (10, 18, 21). In view of the involvement of both T lymphocytes and macrophages in immunity, it was of interest to examine the role of these two cells in production of IL-6 during infection.

MATERIALS AND METHODS

Bacteria. *L. monocytogenes* was maintained by weekly subculture on horse blood agar (HBA) plates and renewed from freeze-dried stock after 25 passages. Actively growing 24-h-old cultures were used for infecting inocula. Heat-killed listeriae (HKL) were prepared from 24-h-old cultures by washing the HBA plates with lipopolysaccharide-free normal saline (Astra Pharmaceuticals Pty, N. Ryde, New South Wales, Australia), centrifuging, and resuspending in fresh saline. After three spins and resuspension, the bacterial concentration was determined turbidometrically, and the suspension was heated to 60°C in a water bath for 1 h. A sample was checked by culture to ensure that there were no surviving bacteria. Heat-killed brucellae (HKB) were similarly prepared from 72-h-old cultures on HBA plates of *Brucella abortus* vaccine strain 19.

Infection of mice. C57BL/10 mice (*Listeria* 50% lethal dose [LD_{50}] = 2×10^5 intravenously), BALB/c mice (*Listeria* $LD_{50} = 5 \times 10^3$ intravenously) and severe combined immunodeficiency (SCID) mice on BALB/c background were pedigree bred in the Microbiology Animal Breeding Unit, University of Melbourne, Parkville, Australia, and maintained under conventional, but disease-free, conditions. Female mice, 6 to 8 weeks of age, were used in all experiments. The mice were infected intravenously with *Listeria* organisms (10^4 for C57BL/10 and 10^3 for BALB/c and SCID mice) suspended in lipopolysaccharide-free saline. The viable dose was checked retrospectively by sampling dilutions onto HBA plates. Mice were killed by an overdose of Fluothane at specified times after infection, and the spleens and livers were homogenized individually in 5 ml of phosphate-buffered saline with an Ultra-Turrax homogenizer (Janke & Kunkel, Breisgau, Germany). Serial 10-fold dilutions were prepared in 96-well microtiter plates, and samples were placed onto HBA plates which were incubated for 24 h at 37°C.

Tissue preparation for IL-6 assay. Under Fluothane anaesthetic, mice were bled for preparation of serum and spleen extracts; extracts were prepared as previously described (18). All samples were stored at −20°C until assay.

Spleen cell cultures. Spleens from groups of five mice were removed aseptically into Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). Cell suspensions were prepared as previously described (5) and treated with Tris-buffered 0.83% ammonium chloride to lyse erythrocytes. They were cultured in 1 ml in 24-well trays (Costar, Cambridge, Mass.) at a concentration of 2×10^6 cells per ml together with 1×10^8 HKL or 1×10^8 HKB or medium alone. The supernatants were collected after 24 h for assay of IL-6, IL-2, and gamma interferon (IFN-γ).

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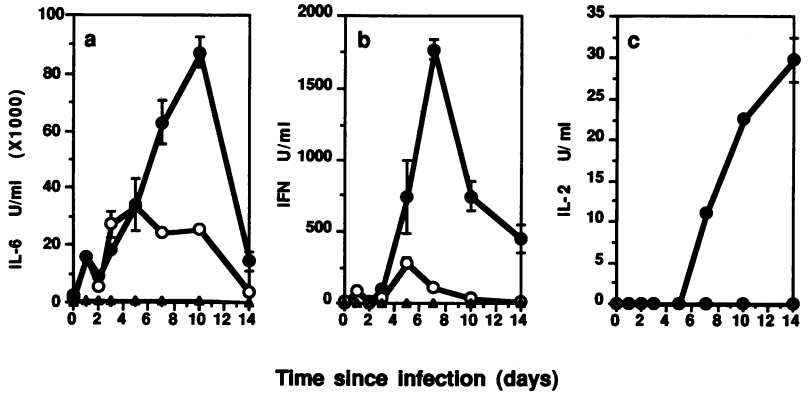

FIG. 1. IL-6 (a), IFN-γ (b), and IL-2 (c) in the supernatants of cultured spleen cells with specific or nonspecific antigen. Spleen cells from infected or uninfected C57BL/10 mice were cultured with HKL (●) or HKB (○) or medium alone (△). Each point represents the arithmetic means of triplicate cultures ± standard deviations. Results are typical of numerous experiments.

Peritoneal cell culture. Resident cells were washed from the peritoneal cavity of groups of five mice with 5 ml of DMEM (lipopolysaccharide free; MultiCel Cytosystems Pty Ltd., Castle Hill, New South Wales, Australia) plus 5% FCS and 10 U of heparin per ml. The cells were centrifuged at 800 × *g* for 7 min through a cushion of FCS and resuspended in fresh medium without heparin. Cell concentration was adjusted to 10^6 cells in 1 ml of DMEM (lipopolysaccharide free)–10% FCS and were cultured in 24-well culture dishes. Culture supernatants were collected after 24 h. To prepare adherent cells, after 2 h of incubation, the wells were washed gently three times with medium to remove nonadherent cells. For control wells, the plates were centrifuged to pellet nonadherent cells and the cell-free supernatant was removed. The adherent cell number was compared by a dye binding method for protein assay (8) and found not to differ significantly between groups.

MAbs. Hybridoma cells secreting monoclonal antibodies (MAbs) directed against L3T4 (GK 1.5, rat immunoglobulin G2b) (3) were grown as ascites in pristane-primed BALB/c mice. MAbs were semipurified by ammonium sulfate precipitation of the ascitic fluid, and the protein content was determined by observing the A_{280}. Hybridoma cells secreting MAb directed against Thy1.2 (30H12, rat immunoglobulin G2b) (17) were cultured in DMEM–10% FCS. When the cells were confluent, the supernatant was collected, centrifuged, and stored at −20°C.

To deplete $CD4^+$ cells in vivo, we injected mice intraperitoneally with 1 mg of anti-L3T4 MAb (in 0.2 ml of pyrogen-free saline) 2 days and 1 day before infection with listeriae. A second dose of antibody was given 4 days after infection. Control mice were similarly injected with anti-influenza virus hemagglutinin (HA) (106) (also a rat immunoglobulin IgG2b) (a gift from M. Anders, Department of Microbiology, The University of Melbourne, Australia).

$Thy1.2^+$ cells were depleted in vitro by incubating spleen cells (3×10^7/ml) with a 1:4 dilution (final concentration) of anti-Thy1.2 MAb for 15 min at room temperature and 15 min on ice, mixing every 5 min. After centrifugation (800 × *g* for 7 min), the cells were incubated with a 1:8 dilution of rabbit complement at 37°C for 45 min. An untreated control, a group treated with anti-Thy1.2 MAb alone, and a group treated with complement alone were included.

Cytokine bioassays. IL-6 was assayed by its ability to support proliferation of the hybridoma cell line 7TD1 (29), IL-2 was assayed by its support of proliferation of mouse CTLL cells (6), and IFN-γ was assayed by its inhibition of proliferation of cell line WEHI-279 (24). All assays were performed in triplicate. When appropriate, specificity was checked with neutralizing antibodies.

RESULTS

IL-6 production in vitro. Spleen cells from mice infected for various times were cultured in the presence of HKL or HKB antigen. High concentrations of IL-6 could be detected in the supernatants from spleen cells cultured with HKL, peaking at 10 days postinfection (Fig. 1a). IFN-γ and IL-2 were detected in the supernatants at this time (Fig. 1b and c), suggesting that T cells were activated at 5 to 7 days after *Listeria* infection. Production of IFN-γ and IL-2 was not increased by nonspecific stimulation with HKB. In contrast, HKB induced a level of IL-6 comparable to that induced by a specific stimulus in the early stage of infection. It was only at 7 and 10 days that the specific HKL was far more efficient at inducing IL-6 production, suggesting a role for T cells at these times.

To directly test the role of T cells, we treated spleen cells from 2- or 10-day-infected mice with anti-Thy1.2 MAb and complement. The remaining cells were washed and cultured for 24 h. At 10 days postinfection, T-cell depletion significantly reduced the titer of IL-6 compared with controls (Table 1). Some capacity to produce IL-6 remained after removal of T cells, although IFN-γ production was completely abolished, indicating the success of the T-cell depletion. At 2 days postinfection, T-cell depletion did not affect the ability to produce IL-6, while no IFN-γ was produced at this time.

In contrast, peritoneal cells cultured for 24 h without further stimulus showed a peak in IL-6 production at 2 days postinfection (Fig. 2). It was confirmed that adherent cells were responsible for this production (Table 2). These cells had the microscopic appearance of typical spreading macrophages.

Role of T cells in IL-6 production in vivo. Mice were injected with anti-CD4 MAb or control anti-influenza virus MAb, and groups of five mice were killed at intervals. Interestingly, numbers of bacteria were the same in the two groups of mice (data not shown). There was no significant difference in IL-6 concentrations in serum and spleen extracts between mice treated with anti-CD4 MAb or control MAb (Fig. 3a and b). To check whether T-cell function was

TABLE 1. Effect of in vitro T-cell depletion on IL-6 production[a]

Group	Treatment	Cytokine (U/ml)		
		With HKL		Without HKL (IL-6)
		IL-6	IFN	
Normal	Medium	346 ± 27	<1	<30
	Anti-Thy1.2	359 ± 9	<1	<30
	Complement	293 ± 10	<1	<30
	Anti-Thy1.2 plus complement	311 ± 27	<1	<30
2 days of infection	Medium	3,238 ± 820	<1	126 ± 21
	Anti-Thy1.2	2,997 ± 529	<1	119 ± 10
	Complement	2,645 ± 706	<1	115 ± 12
	Anti-Thy1.2 plus complement	2,075 ± 241	<1	111 ± 10
10 days of infection	Medium	22,671 ± 1,511	313 ± 15	223 ± 58
	Anti-Thy1.2	30,731 ± 12,675	383 ± 55	229 ± 18
	Complement	15,013 ± 1,154	331 ± 19	151 ± 12
	Anti-Thy1.2 plus complement	5,264 ± 726[b]	<1[b]	89 ± 29

[a] Spleen cells, treated as shown, from infected or normal mice were cultured for 24 h with or without HKL. Without HKL in culture, IFN-γ was undetectable. Units of IL-6 or IFN-γ per milliliter ± standard deviations in triplicate cultures are shown. Similar results were obtained in a replicate experiment.
[b] Significantly different ($P < 0.001$ by Student's t test) from treated or untreated controls.

abolished completely by putative CD4 depletion, we cultured spleen cells from each group of mice with or without HKL. At 10 days postinfection, IL-6 and IFN-γ production was markedly suppressed by anti-CD4 MAb treatment compared with treatment with control (Fig. 3c and d), although at up to 4 days postinfection, no significant differences were found between the groups. At both time points, $CD4^+$ T cells made up less than 4% of spleen cells in the depleted mice. These results confirmed that the in vitro IL-6-producing ability of spleen cells depended on the presence of immunized T cells and specific stimuli at later stages of *Listeria* infection.

To further check the role of T cells in IL-6 production in vivo, we infected SCID mice, which lack both T and B lymphocytes, with 10^3 *Listeria* cells per mouse. Bacterial counts (Fig. 4a and b) show that SCID mice were unable to eliminate infection as in control BALB/c mice. Serum IL-6 also remained elevated in SCID mice compared with BALB/c mice (Fig. 4c and d), as did IL-6 in spleen extracts (data not shown). Cultured spleen cells from SCID mice produced high levels of IL-6 under stimulus with either HKL or HKB at both early and later stages of infection (Fig. 4e). Production was only slightly increased by infection. BALB/c mice showed a response similar to that of C57BL/10 mice, in which there was an early nonspecific increase in IL-6 production but the later peak required specific stimulus with HKL (Fig. 4f).

In view of the demonstrated role of T cells in IL-6 production in vitro, the in vivo production of IL-6 in secondary infection was investigated. C57BL/10 mice infected 28 days earlier with 10^4 *Listeria* cells were reinfected with 10^4 *Listeria* cells, and at the same time, control normal mice were given 10^4 *Listeria* cells as a primary infection. Figure 5a shows bacterial counts rising in both primary and secondary infection over the first 16 h. However, by 24 h, secondary infection was all but eliminated, while bacterial numbers in the primary infection continued to increase. IL-6 titers in serum during primary infection peaked with 8,400 U/ml at 48 h, but in secondary infection the peak IL-6 production was only 320 U/ml (Fig. 5b). Nevertheless, at 16 h, before the fall in secondary bacterial numbers, the IL-6 titer was 230 ± 117 U/ml in secondary infection and 35 ± 28 U/ml in primary infection ($P < 0.001$ by Student's t test).

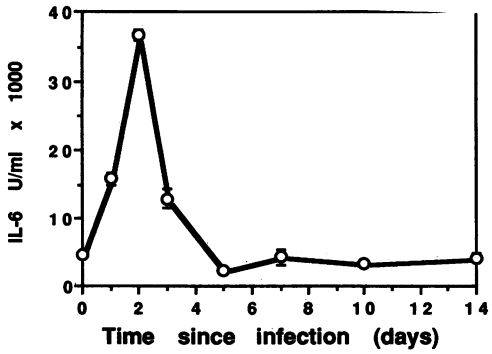

FIG. 2. Production of IL-6 by peritoneal cells. Peritoneal cells (10^6/ml) from C57BL/10 mice infected with 10^4 *L. monocytogenes* cells for the indicated time were cultured for 24 h. Each point represents the arithmetic mean of triplicate cultures ± standard deviation. Results are typical of numerous experiments.

DISCUSSION

The experiments described here show that during infection of mice with *L. monocytogenes*, both T lymphocytes and macrophages are involved in production of IL-6 in vitro.

TABLE 2. IL-6 production by cultured peritoneal cells[a]

Group	U of IL-6/ml	
	Peritoneal cells	Adherent cells
Normal control	4,396 ± 470	2,826 ± 65
2 days of infection	13,472 ± 813	11,638 ± 353
7 days of infection	5,361 ± 440	1,458 ± 40

[a] Total or 2-h-adherent peritoneal cells were prepared as described in the text. Results show units of IL-6 per milliliter ± standard deviations after 24 h in triplicate cultures. Results are typical of three experiments.

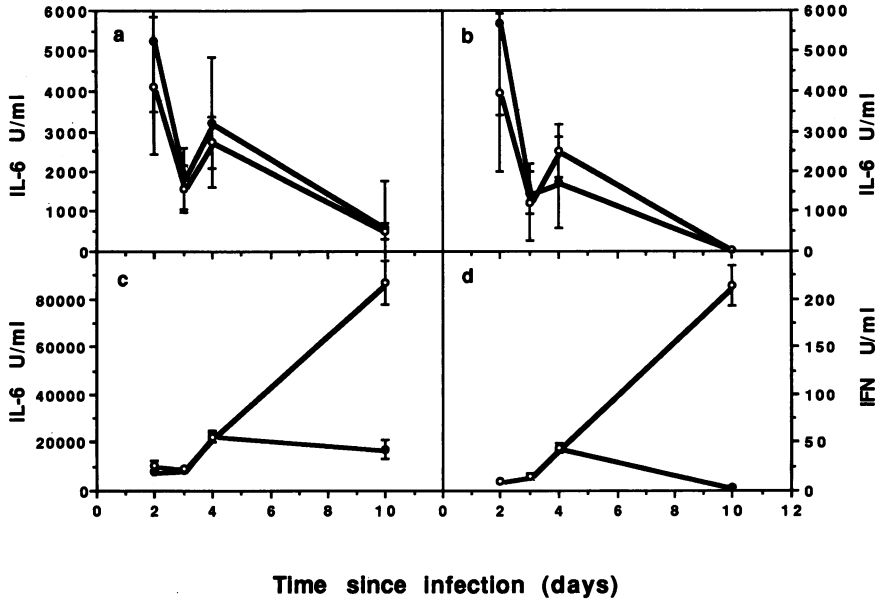

FIG. 3. Effect of depletion of CD4+ T cells on IL-6 production. Mice were treated with GK 1.5 MAb (●) or with control anti-influenza virus HA MAb (○). The upper graphs show in vivo IL-6 levels at various times postinfection: (a) IL-6 in serum; (b) IL-6 in spleen extracts. The lower graphs show the results of spleen cell cultures: (c) IL-6 in supernatants; (d) IFN-γ in supernatants. Each point represents the mean and standard deviation for five mice (a and b) or units per milliliter of triplicate culture ± standard deviation (c and d). Similar results were obtained in a replicate experiment.

However, most IL-6 detected in vivo is produced independently of T cells.

Spleen cell suspensions cultured in vitro for 24 h showed two phases of responsiveness. Cells harvested up to 6 days after infection responded to both specific (HKL) and nonspecific (HKB) stimulus to produce IL-6, while at 7 to 14 days postinfection, there was a heightened specific response in parallel with the specific induction of the typical T-cell-derived lymphokines IFN-γ and IL-2. This later response was abrogated by treatment with anti-Thy1.2 MAb and complement, but the earlier response was not. Note that these experiments do not formally prove that T lymphocytes produce IL-6. Our experiments do, however, establish that T cells control IL-6 production at the time when they are actively producing other lymphokines. Inclusion of antibodies to IFN-γ or tumor necrosis factor α in the spleen cell cultures did not decrease IL-6 production, although the specific lymphokines were themselves neutralized (data not shown), implying that these cytokines were not the true stimulators of IL-6. IL-6 production by freshly isolated T cells has been demonstrated by histochemical staining (12).

This demonstration of a role for T cells is in direct contrast with the results of Huygen et al. (13) using *Mycobacterium bovis* BCG-infected mice. In their case, treatment of spleen cell suspensions with anti-L3T4 antibody and complement to remove CD4+ T cells led to increased IL-6 production in vitro. We found that *Mycobacterium avium*-infected mice

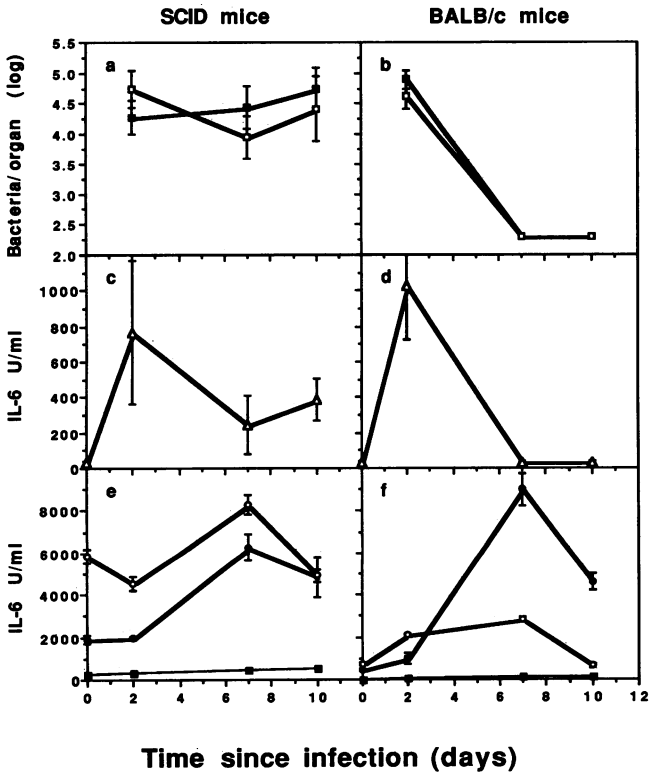

FIG. 4. IL-6 production during *Listeria* infection in SCID (left) and BALB/c (right) mice. Mice were infected with 10^3 *Listeria* cells, and at the times shown, bacterial counts on tissues were made, IL-6 in serum was assayed, and spleen cell cultures were prepared for assay of IL-6 production. (a and b) Bacterial counts on spleen (■) and liver (□); (c and d) IL-6 in serum (△); (e and f) IL-6 in spleen cultures with HKL (●), HKB (○), or medium alone (■). Each point represents the mean and standard deviation for five mice (a, b, c, and d) or units per milliliter of triplicate culture ± standard deviation (e and f). Similar results were obtained in a replicate experiment.

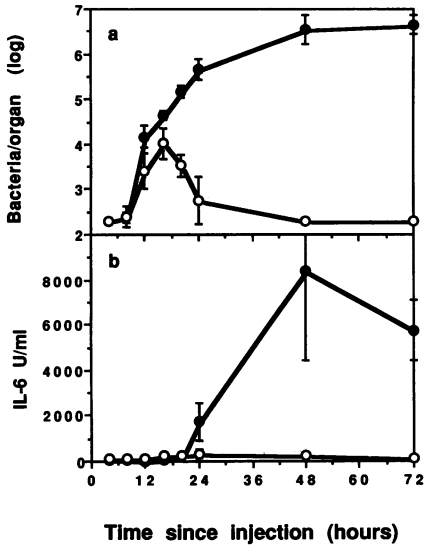

FIG. 5. IL-6 response in primary and secondary infection. C57BL/10 mice infected 28 days earlier with 10^4 *Listeria* cells were reinfected with 10^4 *Listeria* cells as secondary infection (○); at the same time, control normal mice were given 10^4 *Listeria* cells as a primary infection (●). (a) Bacterial numbers in spleen; (b) IL-6 in serum (b). Each point represents the mean and standard deviation for five mice. Similar results were obtained in a replicate experiment.

depleted of $CD4^+$ T cells in vivo remained fully capable of producing IL-6 in vitro (25), suggesting that T cells were not important producers in this infection. On the other hand, $CD4^+$ but not $CD8^+$ T cells taken late in infection of mice with *B. abortus* were able to produce IL-6 in vitro in the presence of specific antigen and antigen-presenting cells (25). It is apparent that generalizations concerning the role of T cells, even among these intracellular bacteria, may not be valid.

In vivo levels of IL-6 were not dependent on T cells. It may be significant that the course of IL-6 production detected in the serum followed more closely the times of IL-6 production by adherent peritoneal cells and the early nonspecific production by spleen cells in vitro than the later T-cell-dependent response of the spleen cells. CD4-depleted mice showed no deficiency in their IL-6 levels in serum or spleen extracts. Interestingly, in agreement with Dunn and North (4), CD4 depletion did not prolong infection (data not shown). SCID mice were unable to eliminate the infection, and IL-6 production in vivo was prolonged in parallel.

Even in the secondary response to infection, we were not convinced of a role for T lymphocytes in vivo. The demonstration of a memory response by T cells under these circumstances is complicated by the rapid removal of the secondarily infecting bacteria, so that there is less antigen to stimulate lymphokine production. Nevertheless, lymphokines must be released to produce this secondary removal. The secondarily infected mice did show a slight advantage in IL-6 production at 16 h when bacterial loads were similar in primary and secondary infection, but the concentration was low and whether it was biologically significant is a moot point. Whether IL-6 produced by T lymphocytes in local foci of infection has any role is unclear.

The function of IL-6 during infection with intracellular bacteria remains unknown. Injection of recombinant IL-6 shortly before infection with *L. monocytogenes* aided recovery from that organism (18). The known effects of IL-6 in activating T lymphocytes (14, 16) and stimulating hematopoiesis (19, 27) have obvious relevance to these infections. The classically described B-cell-stimulating function (11) occupies a more ambiguous position. While antibodies to some other intracellular bacteria are produced, specific antibodies are not produced during infection of mice with *L. monocytogenes* (23). On the other hand, there is a clear polyclonal expansion of B cells during this infection (20). Such an expansion could well be caused by the very high levels of IL-6 seen in mice infected with this organism compared with the levels in other infections (25). Elucidation of the role of IL-6 will be best studied by injection of specific antibody, in analogy with studies of tumor necrosis factor α (9) and IFN-γ (1).

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