INFECTION AND IMMUNITY, Feb. 1995, p. 720–723 Vol. 63, No. 2
0019-9567/95/$04.00+0

Differential Induction of Macrophage-Derived Cytokines by Live and Dead Intracellular Bacteria In Vitro

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Received 3 October 1994/Returned for modification 4 November 1994/Accepted 2 December 1994

Marked differences in the abilities of living and heat-killed *Brucella abortus* and *Listeria monocytogenes* organisms to induce production of tumor necrosis factor alpha by in vitro-cultured macrophages were observed. Interleukin-1 and interleukin-6 appeared to be under different control. The results are discussed in relation to the induction of gamma interferon-producing Th1 cells and acquired cellular resistance to infection by living vaccines but not killed vaccines.

Facultative intracellular bacteria, including *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Brucella abortus*, and *Salmonella* spp., survive within normal resident macrophages and other nonprofessional phagocytes. Gamma interferon (IFN-γ)-producing $CD4^+$ T lymphocytes play an important role in recovery from infection by these organisms (1, 11, 21), while antibody plays at best a minor part (2). Only immunization with live attenuated vaccines induces satisfactory acquired cellular resistance (ACR) (10). In support of this observation, we have found that infection of mice with live *B. abortus* 19 (a vaccine strain) induced a high frequency of typical IFN-γ-producing Th1 cells, while injection of *Brucella* protein extracts induced Th2-like interleukin-4 (IL-4)-producing cells (22a).

There is as yet little understanding of what biases the immune response to these organisms toward the Th1, IFN-γ-producing arm of the T-cell response rather than Th2-like IL-4 producers which favor antibody production or of why this bias requires live organisms. A partial answer was provided by a recent demonstration that IL-12 derived from macrophages stimulated by heat-killed *L. monocytogenes* organisms favored differentiation in vitro of unprimed T cells toward IFN-γ production (8). However, this does not explain why only living bacteria can induce ACR in vivo. Three other macrophage-derived cytokines, tumor necrosis factor alpha (TNF-α), IL-1, and IL-6, have been shown to play a role in the induction of ACR to *Listeria* organisms (3, 5, 6, 9, 12) and other intracellular bacteria (23). We thus chose to quantitate these three following the interaction of macrophages with living and killed *B. abortus* 19, a gram-negative, lipopolysaccharide (LPS)-containing intracellular bacterium, and with gram-positive *L. monocytogenes*.

The methods of bacterial culture, infection of mice, and harvesting and culture of peritoneal cells have previously been described (22). The bacterial strains used were *B. abortus* 19 (a vaccine strain) (Commonwealth Serum Laboratories, Parkville, Australia), *L. monocytogenes* EGD (obtained from R. V. Blanden [Australia National University, Canberra, Australia]), a temperature-sensitive mutant derived from EGD by the method of Morris Hooke et al. (13), and non-hemolytic *L. monocytogenes* DP-L215 (18), the gift of D. A. Portnoy. To compensate for the growth of viable bacteria, various initial numbers of bacteria were added to 10^6 peritoneal macrophages in 2 ml of Dulbecco's modified Eagle's medium (LPS-free; Cytosystems Ltd., Castle Hill, Australia) with 10% fetal calf serum and no antibiotics in 24-well trays (Costar, Cambridge, Mass.). The number of viable bacteria was checked at the end of the 24-h incubation. Cytokines in 24-h supernatants were assayed for the ability to support or inhibit the growth of appropriate cell lines, and specificity was checked by antibody neutralization as previously described (22).

The production of cytokines by peritoneal cells in response to live or heat-killed *Brucella* organisms was tested first. After a 24-h incubation, substantial amounts of TNF-α were detected in the supernatants of cultures with live brucellae (Table 1). TNF-α was not evident after 8 h of culture, but it was detected at 16 h and its amount increased to 24 h, with no further increase thereafter (data not shown). The same population of cells produced little or no TNF-α when stimulated with heat-killed brucellae. Thus, doses of live bacteria, which increased from 10^4 to 10^7 over the incubation period, resulted in the release of 4.4 ng/ml, compared with less than 0.25 ng/ml following stimulation with 10^9 heat-killed brucellae. In contrast, IL-1 was produced in very small amounts (see Table 3 for IL-1 induced by *Listeria* organisms) in response to either live or dead bacteria. IL-6 production was high and did not differ significantly in response to live or dead bacteria. It should be noted that although the relative amounts of IL-1 and IL-6 induced by live and dead brucellae differed marginally from one experiment to another of the four performed, the absence of TNF-α in cultures stimulated by killed bacteria was an invariable finding. At the end of all experiments, cells stimulated with live brucellae were healthy and very actively spreading on surfaces. Cells cultured with dead brucellae were also healthy in appearance but showed little tendency to spread.

Peritoneal cells from mice whose macrophages had been activated in vivo by *Brucella* infection 6 weeks before were also tested for their response to live or killed brucellae. TNF-α production in these cells was heightened, and while levels in excess of 600 ng were detected in response to live brucellae, TNF-α was still barely detectable in cultures stimulated by killed organisms (Table 2). IL-1 production, although enhanced by macrophage activation, was still low. IL-6 production was high and did not differ significantly in response to live or dead bacteria. The data shown are typical of the three experiments performed.

The production of cytokines by healthy resident peritoneal cells in response to culture with live or heat-killed *Listeria* organisms was tested next. The results showed that killed listeriae were a great deal less efficient than live listeriae at stimulating TNF-α and IL-1 production (Table 3). Among the

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TABLE 1. Cytokine production by peritoneal cells from healthy mice in response to stimulation by live or dead brucellae[a]

Condition	No. of bacteria (CFU/ml)		TNF-α (ng/ml)	IL-1 (U/ml)	IL-6 (10^3 U/ml)
	Initial[b]	Final[c]			
Live	10^4	10^7	4.4 ± 2.2	<1	6 ± 1
	10^5	10^8	8.4 ± 4.1	<1	16 ± 3
	10^6	2×10^9	8.0 ± 1.7	<1	25 ± 8
	10^7	2×10^9	28 ± 12	<1	92 ± 14
Dead	10^6		<0.25	<1	6 ± 1
	10^7		<0.25	2 ± 1	18 ± 3
	10^8		<0.25	6 ± 2	180 ± 60
	10^9		<0.25	35 ± 27	220 ± 61
None			<0.25	<1	3 ± 1

[a] Peritoneal cells from healthy CBA mice (5×10^5 cells per ml) were cultured with the indicated numbers of brucellae for 24 h. Supernatants were harvested, filtered, and assayed for cytokine contents.
[b] Brucellae initially added to cultures.
[c] Number of live brucellae in culture at time of harvesting supernatants.

three strains of live listeriae tested, the two which proliferated at 37°C (either hemolysin positive or negative) stimulated very high concentrations of TNF-α and IL-1, indicating that hemolysin production and the resultant ability to escape the phagosome (18) were not relevant in the in vitro system. Excessive doses of these strains led to overgrowth of cultures, killing cells and inhibiting production of TNF-α and IL-6 or perhaps destroying the cytokines produced. Interestingly, IL-1 production was not inhibited. The temperature-sensitive *Listeria* strain, which did not proliferate significantly at 37°C, stimulated much less cytokine production, although even it was more effective than killed bacteria, particularly for induction of TNF-α and IL-1 production. The differential induction of TNF-α and IL-1 production by live and dead listeriae was replicated in two further experiments. However, IL-6 was always produced in high titers with no consistent difference in response to live or dead bacteria.

It is possible that live bacteria may stimulate cytokine production via a secreted bioactive antigen. Therefore, the requirement for direct contact between cells and bacteria was tested by using a system in which cells and bacteria were separated on either side of a membrane insert (Nunc, Roskilde, Denmark), with a pore size of 0.2 μm, to allow free diffusion of macromolecules but not intact bacteria. The results (Table 4) showed that the separation of cells from bacteria prevented TNF-α production and diminished IL-1 production whenever that cytokine was produced to any extent. There was no consistent effect on IL-6 production. It was also notable that 10 ng of *Escherichia coli* LPS added directly to cultures of peritoneal cells induced high titers of IL-6 (170,000 U/ml) but negligible titers of TNF-α (0.75 ng/ml). Increasing the dose of LPS to 10 μg/ml increased IL-6 production to 360,000 U/ml and TNF-α production to 5 ng/ml.

TABLE 2. Cytokine production by peritoneal cells from infected mice in response to stimulation by live or dead brucellae[a]

Condition	No. of bacteria (CFU/ml)		TNF-α (ng/ml)	IL-1 (U/ml)	IL-6 (10^3 U/ml)
	Initial[b]	Final[c]			
Live	10^4	4×10^5	6.2 ± 1.5	2 ± 2	91 ± 23
	10^5	8×10^6	130 ± 17	8 ± 4	336 ± 152
	10^6	5×10^7	620 ± 64	40 ± 27	502 ± 2
	10^7	8×10^7	>620	150 ± 49	321 ± 119
Dead	10^6		0.6 ± 0.1	2 ± 2	88 ± 21
	10^7		0.5 ± 0.2	7 ± 3	86 ± 3
	10^8		0.6 ± 0.1	7 ± 4	260 ± 140
	10^9		0.6 ± 0.1	29 ± 14	143 ± 16
None			<0.16	<1	2 ± 0.3

[a] Peritoneal cells from 6-week-*Brucella*-infected CBA mice (5×10^5 cells per ml) were cultured with the indicated numbers of brucellae for 24 h. Supernatants were harvested, filtered, and assayed for cytokine contents.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.

TABLE 3. Cytokine production by peritoneal cells from healthy mice in response to stimulation by *Listeria* organisms[a]

Condition and phenotype	No. of bacteria (CFU/ml)		TNF-α (ng/ml)	IL-1 (U/ml)	IL-6 (10^3 U/ml)
	Initial[b]	Final[c]			
Live, Hly$^+$	10^2	1.6×10^9	69 ± 33	1,300 ± 1,100	440 ± 78
	10^3	2.8×10^9	51 ± 16	7,000 ± 5,000	840 ± 76
	10^4	8.4×10^9	25 ± 6	10,000 ± 580	730 ± 310
	10^5	3.4×10^9	21 ± 10	4,700 ± 780	760 ± 340
Live, Hly$^-$	10^3	4.4×10^9	80 ± 1.0	950 ± 440	4,700 ± 1,400
	10^4	5.2×10^9	27 ± 14	3,300 ± 1,700	2,400 ± 150
	10^5	2.6×10^{10}	<0.25	4,100 ± 1,800	1,300 ± 460
	10^6	7.4×10^9	<0.25	10,000 ± 4,700	590 ± 75
Live, temperature-sensitive	10^5	2×10^4	2.5 ± 1.6	10 ± 2	46 ± 6
	10^6	1.6×10^5	3.1 ± 1.0	16 ± 5	180 ± 32
	10^7	2.2×10^6	3.7 ± 1.1	50 ± 9	440 ± 170
	10^8	4.4×10^8	0.7 ± 0.5	320 ± 21	90 ± 47
Dead, Hly$^+$	10^6		<0.25	<1	73 ± 50
	10^7		<0.25	1.7 ± 1.2	263 ± 2
	10^8		<0.25	6.3 ± 2.1	175 ± 57
	10^9		<0.25	35 ± 27	36 ± 3
None			<0.25	<1	3.5 ± 1.3

[a] Peritoneal cells from healthy CBA mice (5×10^5 cells per ml) were cultured with the indicated numbers of virulent *L. monocytogenes* organisms for 24 h. Supernatants were harvested, filtered, and assayed for cytokine contents.
[b] Listeriae initially added to cultures.
[c] Since live listeriae replicated in cultures, the numbers of listeriae in cultures were determined at time of harvesting supernatants.

The most notable and consistent observation from all of these experiments was the difference in TNF-α induction by live and dead bacteria. Our observations are extended by a report that live but not heat-killed *Leishmania* amastigotes triggered TNF-α production from IFN-γ-activated macrophages (4). In vivo observations also confirm that live, hemolysin-positive *Listeria* organisms induced expression of TNF-α and IL-1 mRNAs, while heat-killed organisms did not (19). In this study of in vivo infection, both live and dead *Salmonella typhimurium* organisms, another gram-negative bacterium, induced TNF-α and IL-1 mRNAs. The contrast with brucellae may reflect TNF-α production by cells other than macrophages or may be the result of difficulties in quantitating mRNA.

TNF-α is a key cytokine in acquired cell-mediated immunity to these intracellular bacteria. An injection of antibody to TNF-α markedly exacerbates infection (5). It is believed that TNF-α activates production of IFN-γ by NK cells, providing early macrophage activation before the activation of T cells. This early IFN-γ may also favor the differentiation of IFN-γ-producing Th1 cells (14). Most importantly, in vitro experiments show that TNF-α acts synergistically with IL-12 and IL-1 to induce optimal proliferation of established Th1 clones (20). TNF-α also promotes proliferation of anti-CD3-stimulated T cells (16) and acts synergistically with IL-1 in upregulating IL-2 receptors on T cells (17).

The effects of live and dead bacteria on IL-1 production

TABLE 4. Requirement for direct contact between cells and bacteria for cytokine production

Organism[a]	Condition	No. of bacteria (CFU/ml)[b]	TNF-α (ng/ml)		IL-1 (U/ml)		IL-6 (10^3 U/ml)	
			−Insert	+Insert	−Insert	+Insert	−Insert	+Insert
Brucellae	Live	10^6	11 ± 1	<0.2	2 ± 1	2 ± 1	88 ± 17	29 ± 7
		10^7	26 ± 2	<0.2	10 ± 5	4 ± 2	102 ± 23	32 ± 12
	Dead	10^8	<0.2	<0.2	10 ± 2	4 ± 1	600 ± 61	29 ± 7
		10^9	<0.2	<0.2	73 ± 38	3 ± 1	1,100 ± 530	97 ± 33
Listeriae	Live	10^2	90 ± 2	<0.2	1,800 ± 960	4 ± 2	251 ± 11	65 ± 21
		10^3	82 ± 2	<0.2	2,200 ± 250	22 ± 7	340 ± 47	138 ± 60
	Dead	10^8	<0.2	<0.2	11 ± 4.4	4 ± 1	170 ± 86	100 ± 4
		10^9	<0.2	<0.2	90 ± 17	22 ± 6.7	154 ± 26	440 ± 110
None			<0.2	<0.2	2 ± 1	11 ± 5	11 ± 4	44 ± 2

[a] Peritoneal cells from healthy CBA mice (5×10^5 cells per ml) were cultured for 24 h with the indicated numbers of *B. abortus* 19 organisms or virulent listeriae, with or without inserts. Supernatants were harvested, filtered, and assayed for cytokines.

[b] Bacteria initially added to culture. Numbers were also determined at time of harvesting supernatants. There was no significant difference in bacterial number between cultures with inserts and those without inserts.

differed for the two organisms tested. Live wild-type listeriae induced about 1,000-fold-more IL-1 than did killed listeriae, while little production of IL-1 was induced by either live or dead brucellae. The overgrowth of listeriae in some cultures had a damaging effect on macrophages when they were examined microscopically, an effect which did not depend on the production of hemolysin. This resulted in lower titers of TNF-α and IL-6 but, if anything, enhanced IL-1 production. This observation is consistent with the report that IL-1 is processed and released during apoptosis and that cell injury is an important stimulus for the release of IL-1 (7). The healthy appearance of *Brucella*-infected cells which failed to produce significant amounts of IL-1 is further support for the concept that IL-1 is the product of dying cells.

IL-1 is well known as a T-cell-activating factor in vitro and has also been shown to enhance T-cell activation in vivo. An injection of recombinant IL-1α before infection with *L. monocytogenes* hastened activation of IFN-γ-producing T cells (3). Recombinant IL-1β injected together with killed *Listeria* organisms also enhanced T-cell activation (9), although it did not lead to IFN-γ production.

We recently demonstrated a role for IL-6 in T-cell activation during experimental *Listeria* infection; an injection of recombinant IL-6 also promoted IFN-γ-producing T cells (12). IL-6 production was always high but rather variable in these experiments. This variation may have been due to extreme sensitivity to the presence of LPS, although every precaution was taken to exclude LPS from these experiments. It is also known that the adherence of macrophages to surfaces can trigger IL-6 production (15). Under these circumstances, it was not possible to demonstrate any consistent difference in the abilities of live and dead bacteria to induce IL-6 production. In addition, cells responded to soluble molecules which passed through 0.2-μm-pore-size membranes by producing IL-6. This was in contrast to the other two cytokines measured. TNF-α was not produced in the absence of direct contact, while IL-1 was produced in very small amounts.

The fact that production of TNF-α and IL-1 varied according to whether macrophages were stimulated by live or dead bacteria suggests that production of other cytokines may also vary. This differential induction of cytokines by live and dead bacteria may well be the key to their differences in ability to induce ACR. Vaccinologists hope that incorporation of the optimal combination of cytokines into a killed vaccine will render it capable of inducing ACR. A better understanding of those cytokines uniquely induced by live organisms will advance this aim.

This work was supported by a project grant from the Australian National Health and Medical Research Council.

We thank Veronica Peros for her cheerful technical assistance.

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