Legionella pneumophila Induced Tumor Necrosis Factor Production in Permissive versus Nonpermissive Macrophages (43568)

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Abstract. **The ability of an opportunistic intracellular bacterial pathogen, *Legionella pneumophila*, to induce tumor necrosis factor (TNF) in macrophages from susceptible A/J or resistant BDF_1 and BALB/c mice was determined. Cultures of peritoneal elicited macrophages from these mouse strains produced TNF in response to the Legionella. The TNF levels produced by the macrophages stimulated with either heat-killed Legionella vaccine or lipopolysaccharide were similar and dose dependent, although the amount of TNF produced by macrophages from permissive A/J mice was 2- to 4-fold higher than that produced by macrophages from the nonpermissive mice. Similar differences in TNF levels occurred when macrophages from either permissive or nonpermissive mice were infected with viable Legionella. The TNF levels produced by the A/J mouse macrophages increased as a function of time after infection, with a peak of activity on Day 1 or 2, depending upon the initial concentration of the bacteria. Infection of the A/J mouse macrophages with avirulent Legionella resulted in induced levels comparable to those induced by a virulent strain. Although it is widely believed that TNF production by mouse macrophages is related to resistance to infections, the results of this study did not show a relationship between TNF production by macrophages *in vitro* and resistance versus susceptibility of the macrophage donor mouse strain to Legionella infection.** [P.S.E.B.M. 1993, Vol 203]

Tumor necrosis factor (TNF) is now considered an important immunoregulatory cytokine. This factor was first described as a soluble antitumor serum substance induced by lipopolysaccharide (LPS) in mycobacteria-sensitized animals (1). Studies by others concerning cachectin, a wasting factor found in serum of animals with microbial infection, showed that the LPS-induced antitumor factor and the wasting factor were the same (2). In addition to antitumor and detrimental wasting effects, TNF has now been shown to be important in the activation of macrophages and polymorphonuclear leukocytes and can regulate many immune responses by these and other lymphoid cells (3–6).

Previous studies in this laboratory have shown that BDF_1 mice infected with *Legionella pneumophila*, an important opportunistic intracellular pathogen that infects macrophages preferentially, develop significant levels of TNF in bronchial secretions after respiratory infection (4, 5). The BDF_1 mice were susceptible to lethal infection only when given relatively large numbers of Legionella, but A/J mice were susceptible to low numbers of that bacteria (7). Other studies had shown that anti-TNF antibody could protect infected BDF_1 mice from lethal infection by large numbers of Legionella, possibly by activating polymorphonuclear cells (3, 4). Although macrophages from most mouse strains are not susceptible to infection by these bacteria, and their peritoneal macrophages are nonpermissive for Legionella growth *in vivo*, macrophages from A/J mice, which were found in this laboratory to be much more susceptible to Legionella infection *in vivo*, were

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shown to be highly permissive for growth of these bacteria *in vitro* (7). Previous studies had shown that A/J mice have a much lower LD_{50} threshold for infection with these bacteria than the other mouse strains. Thus, it was of interest to determine whether Legionella could stimulate either permissive or nonpermissive macrophages from these mouse strains to produce TNF *in vitro*.

Materials and Methods

Animals. Female A/J, BDF_1, and BALB/c mice, 6 weeks old, were obtained from Jackson Laboratories, Bar Harbor, ME. They were acclimated to the animal facilities at this institution for at least 1 week before use. The mice were fed Purina mouse chow and water *ad libitum*, and kept in groups of six to 10.

Bacteria. *L. pneumophila*, serogroup 1, used for these experiments was isolated initially from a case of legionellosis at Tampa General Hospital, Tampa, FL. The microorganisms were cultured on buffered charcoal yeast extract agar (Becton Dickinson, Cockeysville, MD) as described previously (5). After 36 hr, the bacteria were harvested into pyrogen-free saline and diluted to a concentration of 10^9 organisms/ml. A killed vaccine was prepared by heating the bacteria to 100°C for 30 min. An avirulent isolate from the virulent strain was prepared by repeated batch culture passage on buffered charcoal yeast extract agar and Mueller-Hinton agar.

Lipopolysaccharide. LPS was kindly provided by Dr. K. H. Wong, Centers for Disease Control, Atlanta, GA, and prepared as a hot phenol extract of killed Legionella. *Escherichia coli* LPS, prepared by phenol extraction, was purchased from Sigma Chemical Co (St. Louis, MO).

Macrophage Cultures. Peritoneal elicited macrophages were obtained 4 days after injection of mice with 3 ml of thioglycolate broth (Difco Laboratories, Detroit, MI). The exudate cells were obtained by aspiration with a needle and syringe, washed three times with Hanks' balanced salt solution, and resuspended to a concentration of 10^6 cells/ml with RPMI 1640 medium supplemented with 10% fetal calf serum. Cells (100 μl) were dispensed into 96-well plates (Costar, Cambridge, MA) and adhered for 2 hr. The nonadherent cells were washed and fresh medium was added. The cultures in triplicate were then stimulated with vaccine or LPS for 24 hr (8). For infection, Legionella was added to the macrophage cultures for 30 min, and then supernatants were collected (Day 0) and fresh medium was added. Supernatants were collected and fresh medium was added again at 24 hr (Day 1) and at 48 hr (Day 2). The Legionella were not toxic for the macrophage cultures during the 48 hr of culture *in vitro* when used at the standard infection ratio of 10:1 (10 bacteria to one macrophage) as determined by the conventional trypan blue stain assay.

TNF Assay. Cell supernatants, after stimulation with Legionella vaccine or LPS, were tested for TNF activity exactly as described previously (5). In brief, serial dilutions of supernatants in 0.1 ml volumes were added to 96-well plates, to which were added 0.1 ml of WEHI-164 cells labeled with ^{51}Cr. As a standard, murine recombinant TNFα (Genzyme, Boston, MA) was used. After 18 hr of incubation at 37°C, the supernatants from the WEHI cells were collected and counted. TNF units were calculated for each sample based on a TNFα standard. Anti-TNF monoclonal antibody (Genzyme) neutralized activity *in vitro*, but other anticytokine sera such as anti-IL-1 or anti-interferon did not.

Statistical Analysis. *P*-values were calculated with the Student's *t* test.

Results

Legionella antigens induced detectable amounts of TNF in culture supernatants of elicited peritoneal macrophages from all three mouse strains examined (Table I). Induction of TNF by heat-killed vaccine and Legionella LPS was similar and dose dependent for all strains tested. However, the amount of TNF produced by the permissive A/J mouse macrophages was two to four times greater than that produced by the nonpermissive macrophages from the BALB/c or BDF_1 mice. Similar results were obtained with *E. coli* LPS, although the LPS induced greater levels of TNF activity than did Legionella LPS.

Peritoneal elicited macrophages from either permissive A/J or nonpermissive BDF_1 mice were infected

Table I. Production of TNF by Murine Peritoneal Macrophages Stimulated with Legionella Antigens

Stimulator[a]	TNF from macrophages of[b] (units/ml)		
	A/J	BALB/c	BDF_1
None	<10	<10	<10
L. pneumophila vaccine			
10^5	150 ± 30	84 ± 22	<10
10^6	850 ± 110	220 ± 40	110 ± 30
10^7	1110 ± 460	440 ± 160	280 ± 75
L. pneumophila LPS			
0.01 μg	110 ± 25	58 ± 17	24 ± 17
0.1	330 ± 50	130 ± 45	38 ± 14
1.0	860 ± 73	350 ± 80	210 ± 75
E. coli LPS			
0.01 μg	530 ± 169	42 ± 22	26 ± 11
0.1	2200 ± 470	220 ± 28	140 ± 28
1.0	3800 ± 760	640 ± 37	280 ± 37

[a] Peritoneal macrophages (10^5 cells) from A/J, BALB/c, or BDF_1 mice were cultured for 24 hr *in vitro* with indicated stimulator.

[b] TNF activity as units in culture supernatant is expressed as average ± SD for three to five experiments.

in vitro with Legionella, and the induced TNF activity was compared. The level of TNF activity with A/J mouse macrophage cultures increased significantly ($P \leq 0.01$) as a function of time after infection, with the peak of activity on Day 1 or Day 2, depending upon the initial concentration of Legionella (Table II). When an excessive amount of Legionella was used, TNF was produced mainly during the first 24-hr period (Day 1), with little additional TNF evident during the second 24-hr period (Day 2). When less Legionella (1×10^6) was used, the greatest level of TNF activity was evident during the second 24-hr period. In contrast, the nonpermissive BDF_1 mouse macrophage cultures had lower amounts of TNF during infection by Legionella, with most activity occurring during the first day of culture. Permissive A/J mouse macrophages infected with avirulent Legionella had similar levels of TNF activity on Day 1, but very little was produced after Day 1 (Table III).

Discussion

TNF activity was readily induced in both permissive and nonpermissive murine macrophage cultures by Legionella LPS, which is structurally different from LPS of *E. coli* or other gram-negative bacteria. Sonesson *et al.* (9) reported that *L. pneumophila* LPS is a complex structure due to several unusual fatty acids, including branched carbon chains, *guinovosamine*, and glycerol in the lipid A portion (8). Moreover, it has also been reported that *L. pneumophila* LPS is far less toxic in mice and has lower pyrogenic effects in rabbits as compared with enterobacterial LPS (9, 10). Since it is known that TNF is an important cytokine for endotoxic septic activity such as lethality and pyrogenicity (11, 12), it is of interest that Legionella LPS induced TNF, although not to the levels occurring after stimulation with *E. coli* LPS. Therefore, TNF induction by Legionella LPS may be involved more with pathogenicity and less with the detrimental effects generally associated with *E. coli* LPS and sepsis.

Table II. Production of TNF by Peritoneal Macrophages from A/J or BDF_1 Mice During Infection with Legionella Virulent Strain

Mouse strain	No. of Legionella for infection of macrophages	Production of TNF (units/ml) by infected macrophages at[a]		
		Day 0	Day 1	Day 2
A/J	None	<10	<10	<10
	2×10^5	<10	11 ± 10	38 ± 18
	2×10^6	13 ± 7	170 ± 65	860 ± 320
	2×10^7	26 ± 11	910 ± 320	36 ± 20
BDF_1	None	<10	<10	<10
	2×10^5	<10	<10	<10
	2×10^6	14 ± 8	11 ± 4	<10
	2×10^7	23 ± 10	40 ± 21	<10

[a] Peritoneal macrophages (10^5 cells) from A/J or BDF_1 mice infected with indicated number of bacteria and supernatant from culture were harvested at indicated time. TNF values are expressed as average ± SD for three different experiments.

Table III. Productin of TNF by A/J Mouse Macrophages During Infection with Legionella Avirulent Strain

Infected with[a]	Production of TNF (units/ml) from infected macrophage at[b]		
	Day 0	Day 1	Day 2
None	<10	<10	<10
2×10^5	<10	14 ± 8	<10
2×10^6	11 ± 7	38 ± 17	<10
2×10^7	18 ± 11	850 ± 240	12 ± 8
2×10^8	32 ± 18	990 ± 320	26 ± 14

[a] Peritoneal macrophages (10^5 cells) from A/J mice infected with indicated number of avirulent Legionella and supernatant from cultures were harvested at indicated time.
[b] TNF values are expressed as average ± SD for three different experiments.

It is noteworthy that TNF production in response to Legionella vaccine or LPS in permissive A/J mouse macrophage cultures was two to four times greater than that produced by nonpermissive macrophages from BALB/c or BDF_1 mice. Similar results occurred after *in vitro* infection of the cultures with live Legionella. Whereas A/J mouse macrophages infected with virulent Legionella produced readily detectable TNF, BDF_1 macrophages produced very little TNF under the same conditions. Production of TNF by A/J mouse macrophages infected with the avirulent strain was also similar on Day 1. It should be noted that while the avirulent Legionella did not replicate in the A/J mouse macrophages, the bacteria were taken up as readily as the virulent organisms (data not shown). It is important to note that the Legionella grew vigorously in the macrophages from the A/J mice, but only weakly in the macrophages from the nonpermissive BDF_1 mice. For example, there was a 100- to 1000-fold increase in the number of Legionella in macrophages from the A/J mice over the 48-hr culture period as determined by standard colony-forming unit assay *in vitro* using lysates from infected macrophages. In contrast, macrophages from the BALB/c mice cultured at the same ratio with Legionella *in vitro* for 48 hr only showed a 5- to 10-fold maximum increase in colony-forming unit number over the same 48-hr culture period. Thus, it appears likely that TNF production by macrophages during Legionella infection may be related to an early phase of infection or to initial uptake of the bacteria. This is evident because TNF readily appeared in the macrophage cultures after exposure to the virulent Legionella. Infection of A/J macrophage with 2×10^6 virulent bacteria induced little TNF on Day 1, but high levels

appeared by Day 2, when the number of virulent Legionella had increased markedly within the macrophages. The avirulent strain, which did not grow well within the macrophages, induced only low levels of TNF.

Similar enhanced sensitivity of A/J mouse macrophages to produce TNF when induced by other stimulators was also reported by Lasfargues and Chaby (13). It has also been reported that A/J mice evince a natural resistance to infection with *Mycobacterium bovis*, *Salmonella typhimurium*, or *Leishmania donovani* and that BALB/c mice are susceptible to these pathogens (14–17). However, the susceptibility of macrophages from these mouse strains to infection with Legionella was the opposite. Moreover, it was reported that recombinant TNF could increase host resistance in BDF_1 mice against infection with other intracellular bacterial pathogens such as Mycobacterium, Salmonella, or Listeria monocytogenes (18–20), as well as Legionella (4). For these reasons, it appears likely that TNF production by macrophages from these mouse strains may be related to resistance to these intracellular pathogens. However, the results of the present study with Legionella did not show a relationship in A/J mice between TNF production and resistance to Legionella. Since TNF has been shown to stimulate polymorphonuclear and other lymphoid cells (3), the A/J mice may be deficient in other immune areas. Studies are in progress to correlate induction of TNF *in vivo* in susceptible and resistant mice as compared with the results of this study *in vitro* with macrophages from the same mouse strains. It is anticipated that the results of such *in vivo* studies will provide information as to the role of TNF production in susceptible versus resistant mice in regard to pathogenesis by Legionella.

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