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Cell-Mediated Immune Response to *Salmonella typhimurium* Infection in Mice: Development of Nonspecific Bactericidal Activity Against *Listeria monocytogenes*

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Generation of the cell-mediated immune response of CBA/H mice against *Salmonella typhimurium* (C5S) was monitored by measuring the nonspecifically increased bactericidal activity of macrophages against *Listeria monocytogenes*. The appearance of detectable levels of macrophage activation was inversely related to the initiating infectious dose. With 3×10^3 infecting C5S organisms, significant activity was demonstrable after day 3. Immunity controlled a challenge with a streptomycin-resistant strain of *S. typhimurium* (C5R) successfully only by approximately day 7. In the period of increasing activity against *L. monocytogenes*, growth of C5R was only delayed. Since such an effect could not be demonstrated in *Listeria*-infected mice, these findings suggest that immunity against C5R necessitates specific factors besides macrophage activation.

Specific immunological mechanisms are involved in activating macrophages to nonspecifically increased bacterial activity (6, 17, 18). This has been demonstrated in mice for a variety of infections by facultative intracellular bacteria, i.e., tuberculosis (5), brucellosis (19), listeriosis (18-20), and salmonellosis (6, 9-14), as well as in virus infections with lymphocytic choriomeningitis or ectromelia virus (7) and for graft-versus-host reactions (1). So far T cells have been shown formally to be crucial only in listeriosis (4, 17, 23). However, available circumstantial evidence indicates that they are probably also essential for macrophage activation in the other models mentioned; e.g., T cell-depleted mice are much more susceptible to mycobacterial infection (11, 13, 25).

In salmonellosis, cytophilic antibody rather than activation of macrophages has been evoked to explain cellular resistance (16, 24). Most of the available evidence elaborated by Collins and Mackaness favors the interpretation of cell-mediated immunity being central to protection (6, 9-14, 21). However, antibody may play an important ancillary role. Circumstantial evidence for an essential role of T cells derives from the fact that in salmonellosis infection, immunity is only observed when mice are injected with viable vaccines (9-11), and that delayed-type hypersensitivity develops about day 5 of infection (11, 14). Furthermore, T cell-depleted mice are incapable of mounting anti-salmonella immunity or of developing specific delayed-type hypersensitivity during the first 10 days after infection (11).

Attempts to define the identity of specific effector lymphocytes involved and the kinetics of the generation of effector cells in *S. typhimurium* infections in mice have been unsuccessful as yet, mainly because demonstration of even moderate protection by adoptive transfer experiments is possible but difficult (11). Reasons for this are: (i) the so far unexplained fact that a "carrier state" is rapidly established within a few days; and (ii) the relatively slow growth of the bacteria in mice, necessitating longer intervals between challenge and measurement of protective effects as compared with, for example, *Listeria* (11). A challenge infection may thus cause a carrier state and/or an immune response by itself and make interpretation of results difficult. For similar reasons *L. monocytogenes*, a bacterium that grows much more rapidly in vivo, has been previously used to study the kinetics of cellular resistance to BCG in mice (5).

The same approach was used here to study the development and the dose dependence of the generation of nonspecifically activated macrophages as an indicator of cell-mediated immunity in *S. typhimurium* infections in mice.

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These effects were compared with measurable immunity to *S. typhimurium*. The results indicate that during an early stage of developing immunity the state of activation is either quantitatively insufficient or lacks components that are necessary for the successful protection against an *S. typhimurium* challenge.

MATERIALS AND METHODS

Mice. CBA/H mice from the colony maintained at The John Curtin School of Medical Research were used at 7 to 10 weeks of age.

Bacteria. The strains of *S. typhimurium* were obtained from R. V. Blanden (6, 12, 21). C5S was the streptomycin-sensitive strain, and C5R was a streptomycin-resistant mutant derived from C5S. The virulence for CBA/H mice was comparable for intravenous challenge (mean lethal dose approximately 5×10^3).

The strain of *L. monocytogenes* was orginally obtained from V. P. Ackerman (4). The intravenous mean lethal dose for CBA/H mice is about 3×10^4 (4).

Inocula were prepared from overnight cultures in nutrient broth (BBL, Cockeysville, Md.) for C5S and C5R and in Trypticase soy broth (BBL) for *L. monocytogenes* as described elsewhere (4).

Enumeration of viable bacteria in organs. The method of Mackaness was used (18). Briefly, organs were removed and homogenized individually in nutrient broth (spleens in 2 ml, livers in 10 ml) by mechanical means with Teflon pestles in glass tubes (18-21). Tenfold dilutions were plated on Trypticase soy agar plates (BBL) or nutrient agar plates containing 150 μg of streptomycin per ml. Colonies of *S. typhimurium* and *L. monocytogenes* are easily distinguished morphologically (6).

Evaluation of nonspecific bactericidal activity to *L. monocytogenes* and C5R and immunity to C5R. Mice were infected with C5S. At intervals, groups of 5 to 10 infected mice and 5 to 10 untreated control mice were challenged in different experiments with about 5×10^3 *L. monocytogenes* alone, with the same amount of *L. monocytogenes* with about 1×10^4 C5R, or with about 1×10^4 C5R alone. In one experiment mice were killed after 7 min and after 24 h. In all other experiments, viable counts were determined only after 24 h. Immunity to C5R was measured in one experiment over a prolonged period 1, 3, and 6 days after secondary challenge.

Statistical methods. Results were expressed as mean ± standard error of the mean of groups of five mice, and results were compared by Student's *t* test.

RESULTS

Development of nonspecific bactericidal activity against *L. monocytogenes*. Mice infected with 3×10^3 C5S showed initially some insignificant resistance to *L. monocytogenes* during the first 2 days. Increased resistance became significant in livers between 2 and 3 days; whereas in spleens protection was about 0.5 $\log_{10}$, protection was significant only 1 day later. Maximal levels of protection were reached after 5 days (Table 1). This coincides with titers of infecting C5S reaching maximal levels. Uptake of *Listeria* in spleens of C5S-infected mice and control mice were comparable as indicated by the viable counts measured 7 min after challenge (Ta-

TABLE 1. *Development of bactericidal activity against L. monocytogenes and C5R in mice infected with S. typhimurium (C5S)*[a]

Day of *L. monocytogenes*[b] + C5R challenge after C5S infection	Spleen			Liver		
	C5S	*L. monocytogenes*	C5R	C5S	*L. monocytogenes*	C5R
1	3.90 ± 0.05[c]	4.16 ± 0.15	2.68 ± 0.04	4.36 ± 0.17	5.29 ± 0.27	3.63 ± 0.10
Normal controls	0	4.45 ± 0.06[d]	3.09 ± 0.18[d]	0	5.95 ± 0.39[d]	3.73 ± 0.07[d]
2	4.03 ± 0.2	4.29 ± 0.18	2.15 ± 0.28	4.81 ± 0.23	4.73 ± 0.21	3.07 ± 0.34
Normal controls	0	4.73 ± 0.26[d]	2.90 ± 0.22[d]	0	5.91 ± 0.09[e]	3.60 ± 0.19[d]
3	4.45 ± 0.11	2.53 ± 0.18	2.34 ± 0.07	5.41 ± 0.15	4.08 ± 0.11	3.17 ± 0.23
Normal controls	0	4.62 ± 0.06[f]	3.04 ± 0.06[f]	0	5.92 ± 0.09[f]	3.93 ± 0.08[e]
4	4.95 ± 0.29	1.90 ± 0.17	2.15 ± 0.32	6.17 ± 0.25	3.18 ± 0.31	3.00 ± 0.21
Normal controls	0	4.79 ± 0.08[f]	3.05 ± 0.05[e]	0	5.44 ± 0.14[f]	3.94 ± 0.12[e]
5	5.58 ± 0.09	<1.30	1.34 ± 0.04	5.97 ± 0.42	<2.0	2.47 ± 0.33
Normal controls	0	4.70 ± 0.22[f]	2.51 ± 0.12[f]	0	5.72 ± 0.27	3.60 ± 0.12[e]
6	5.60 ± 0.13	<1.30	1.65 ± 0.35	6.42 ± 0.11	<2.0	2.68 ± 0.19
Normal controls	0	4.62 ± 0.06[f]	2.94 ± 0.08[f]	0	5.52 ± 0.19	3.73 ± 0.03[f]

[a] CBA/H mice were infected with 3×10^3 C5S.

[b] Challenging doses of *L. monocytogenes* were between 4.5×10^3 and 6×10^3; challenging doses of C5R were between 9.0×10^3 and 1.4×10^4 except for day 5, when it was 2×10^3.

[c] Mean standard error of the mean of viable counts ($\log_{10}$) of bacteria 24 h after challenge, determined from groups of four to five mice.

[d] No significant difference between infected and normal control mice.

[e] Viable counts in C5S-infected mice smaller than in controls; $P < 0.05$.

[f] $P < 0.001$.

ble 2). The observed protection was thus due to increased bactericidal activity of C5S-infected recipients, presumably caused by presence of activated macrophages.

A similar bactericidal effect was observed for simultaneously injected C5R. This protective effect may reflect immunological specificity, since *Listeria*-immune mice infected with a similar (with respect to mean lethal dose) dose of *L. monocytogenes* did not express a protective effect against C5S (Table 3).

The initial infecting dose of C5S largely determined the time at which nonspecific protection against *Listeria* was first observed. Time of appearance of measurable macrophage activation and infecting dose of C5S were inversely related (Table 4).

Comparison of protection against *L. monocytogenes* with immunity to C5R. Protection against C5R as measured 24 h after challenge was significant after 3 days, increasing to maximal levels after 6 days.

To correlate states of activation with immunity, 4- and 7-day-old C5S immune mice representing two different stages of the developing and fully established states of activation were tested for immunity to challenge with C5R. Seven-day-old immune mice controlled growth of C5R effectively but did not eliminate the challenging bacteria completely, whereas in 4-day-old immune mice growth was only delayed (Table 5). This agrees with data obtained in the classical studies by Collins et al. (12).

DISCUSSION

Growth of C5S and development of nonspecific bactericidal activity against *L. monocytogenes* are temporally related. After initially slow growth of C5S and probably nonspecific and insignificant macrophage activation, bacterial titers of C5S increased in parallel with bactericidal capacity (during 24 h) against challenge infections with *Listeria* or C5R. The initial small effect may reflect activation by phospholipids shed by the bacteria (26). The possibility that it is the result of a memory-type reaction due to cross-reaction with gut flora or other environmental organisms remains a possibility.

TABLE 2. *Protection against L. monocytogenes in mice infected with 3 × 10³ S. typhimurium (C5S)*

Time of *Listeria* challenge after C5S infection (days)	Log_{10} mean viable counts of *L. monocytogenes* at:	
	7 min[a]	24 h
2	2.08 ± 0.07[b, c]	4.13 ± 0.33[c]
Control	2.20 ± 0.08	5.09 ± 0.41
4	2.08 ± 0.24[c]	<3.0[d]
Control	2.01 ± 0.07	5.33 ± 0.45

[a] Time after challenge.
[b] Mean ± standard error of the mean of groups of five mice injected with 5 × 10³ *L. monocytogenes*.
[c] Not significantly different from controls.
[d] Significantly less than control; $P < 0.001$.

TABLE 3. *Effect of a primary L. monocytogenes infection on challenging C5S in CBA/H mice*[a]

Time of challenge with C5S after *Listeria* infection (days)	Log_{10} viable bacteria in spleens		
	Listeria immune		Controls
	L. monocytogenes	C5S[b]	(C5S)
2	6.51 ± 0.02	3.68 ± 0.02[c]	3.54 ± 0.06
3	6.79 ± 0.01	4.07 ± 0.11	3.98 ± 0.05
4	6.59 ± 0.11	3.58 ± 0.11	3.43 ± 0.07
5	4.32 ± 0.60	2.46 ± 0.27	2.43 ± 0.02

[a] Mice were infected with 7 × 10³ *L. monocytogenes* on day 0 and challenged with 1 × 10⁴ C5S (days 2–4) or 3 × 10³ C5S (day 5); viable bacteria counts were determined 24 h later.
[b] All values were not significantly different from controls.
[c] Mean ± standard error of the mean of groups of five mice.

Significant activation was detectable as early as 3 to 4 days after infection with a large dose of 3 × 10³ C5S. This finding is compatible with the measured state of nonspecific macrophage activation being a T cell-mediated phenomenon. Delayed-type hypersensitivity develops at about the same time (11, 14). Also, many T cell functions have been demonstrated to operate at 3 or 4 days after infection, either by cell transfer experiments (e.g., *Listeria* [27]) or in vitro cytotoxicity assays (e.g., ectromelia [15]), and to reach maximal levels by days 5 to 7 (15, 20).

T cells have been shown to be central for protection against *Listeria* infections (4, 17, 23), systemic lymphocytic choriomeningitis (22) or ectromelia infections (3), and the development of graft-versus-host reactions (summarized in reference 8). Circumstantial evidence also exists for their role in salmonellosis (summarized in reference 11). In all of these models macrophages are activated, presumably by specific T cells, to increased bactericidal activity, and it has been suggested that activated macrophages consitute, at least in some of the models, the ultimate effector cells to remove bacteria or viruses (2, 18–20).

Although it has not been shown for salmonellosis that T cells are central to macrophage activation, it can be assumed from the fact that a high (26) degree of macrophage activation is always and only associated with specifically sensitized T cells.

Maximally activated macrophages were ob-

TABLE 4. *Time of appearance of measurable nonspecific bactericidal activity against L. monocytogenes: dependence of infecting dose of C5S*[a]

Time of challenge with *Listeria* after C5S infection (days)	Bacteria	Log_{10} viable bacteria per spleen in CBA/H infected with the following doses of C5S				
		2 to 3	20	200	3,000	Controls
4	C5S	<1.8	<1.8	3.31 ± 0.20	5.01 ± 0.10	
	L. monocytogenes	5.20 ± 0.24[b]	5.11 ± 0.25[b]	5.09 ± 0.25[b]	<2.90[c]	5.25 ± 0.10
8	C5S	<1.8	4.67 ± 0.13	5.26 ± 0.27	5.76 ± 0.25	
	L. monocytogenes	4.32 + 0.06	<2.72[c]	<2.69[c]	<2.0[c]	4.36 ± 0.25
12	C5S	<1.8	5.52 ± 0.48	5.61 ± 0.36	5.70 ± 0.40	
	L. monocytogenes	5.92 ± 0.58	3.30 ± 0.60[c]	3.02 ± 0.19[c]	<2.0[c]	6.01 ± 0.10
16	C5S	2.61 ± 0.82	4.11 ± 1.6	4.83 ± 0.56		
	L. monocytogenes	4.97 ± 0.50[b]	3.01 ± 0.37[c]	<2.80[c]		5.56 ± 0.08

[a] CBA/H were infected intravenously with various doses of C5S; 4, 8, 12 or 16 days later they were challenged with 3×10^3 to 6×10^3 *L. monocytogenes*. Viable bacteria were determined in spleens 24 h later.
[b] Mean ± standard error of the mean of groups of five mice. Not significantly different from control.
[c] Significantly less than control; $P < 0.001$.

TABLE 5. *Immunity to S. typhimurium*[a]

Time of challenge with C5R after C5S infection (days)	Organ	Log_{10} viable bacteria/organ					
		1 day[b]		3 days		6 days	
		C5S	C5R	C5S	C5R	C5S	C5R
4	Spleen	4.71 ± 0.21	2.62 ± 0.12	5.07 ± 0.39	3.12 ± 0.27[c]	6.26 ± 0.20	4.14 ± 0.13[c]
7		6.60 ± 0.10	2.71 ± 0.21	6.70 ± 0.41	3.29 ± 0.50[c]	6.60 ± 0.14	2.98 ± 0.46[c, d]
Control			2.80 ± 0.04		4.26 ± 0.07		5.77 ± 0.26
4	Liver	5.57 ± 0.31	3.21 ± 0.09[e]	5.91 ± 0.15	3.51 ± 0.17[c]	7.76 ± 0.09	4.82 ± 0.31[c]
7		6.92 ± 0.20	3.08 ± 0.11[e]	6.90 ± 0.37	3.77 ± 0.29[c]	6.43 ± 0.81	3.41 ± 0.31[c, d]
Control			3.67 ± 0.06		5.13 ± 0.11		6.34 ± 0.15

[a] CBA/H mice were infected with 5×10^2 C5S and at intervals challenged with 1×10^4 C5R. Groups of five to six mice were killed 1, 3, and 6 days later, and means ± standard errors of the means of the viable bacteria counts were determined.
[b] Interval after challenge with C5R.
[c] Significantly less than control; $P < 0.001$.
[d] Significantly less than 4-day-old immune mice; $P < 0.01$.
[e] Significantly less then control; $P < 0.01$.

served at (depending on the dose of the initiating infection) the time when growth of the infecting C5S or of a challenging C5R infection was effectively controlled. It is interesting that the titer of bacteria reached in spleens was about the same when mice were infected intravenously with 20 C5S or 100 times more. This may indicate that the control of further growth is dependent on the extent of the stimulation of immunological mechanisms, which is only reached at the observed bacterial titers.

The development of these immunological control mechanisms is somewhere between *L. monocytogenes* (18) and BCG (5) infections in mice, where maximal levels of growth are reached at around 3 and 12 days, respectively.

Presence of activated macrophages capable of effectively controlling *L. monocytogenes* infections is apparently not sufficient to control a challenging infection with *S. typhimurium*, as indicated by the failure of 1- to 5-day-old *Listeria*-immune or 3- to 5-day-old C5S-immune mice to control growth of *S. typhimurium* successfully. In a similar experiment using 7-day-old *Listeria*-immune mice, Blanden et al. (6) observed a small protective effect against C5R. This may have been due to the later stage of immunity and/or the slightly higher dose of *Listeria* used.

Thus, the fact that there is a rapid establishment of a carrier state in *S. typhimurium* infections and that immunity is observed only when a uniformly high level of infection is reached may indicate the need for additional specific factors. Possible explanations are: (i) free antibody may be needed to opsonize bacteria for effective uptake and/or destruction by activated macrophages (16); (ii) macrophage activation

may be rendered specific either by cytophilic antibody (24) or non-immunological, specific induction of enzymes dealing more effectively with *S. typhimurium;* or (iii) the state of activation of individual macrophages or the overall state may differ quantitatively, depending upon time and/or antigen.

In conclusion, both the fact that nonspecifically activated macrophages can be demonstrated in *S. typhimurium* infections in mice and the kinetics of the development of this cell-mediated immune response, measurable as early as 3 to 4 days after infection, add circumstantial evidence for cell-mediated immunity being strongly involved in immunity to infection. However, additional specific factors appear to be necessary for protective immunity against *S. typhimurium*.

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LITERATURE CITED

1. **Blanden, R. V.** 1969. Increased antibacterial resistance and immunodepression during graft-versus-host reactions in mice. Transplantation 7:484-497.
2. **Blanden, R. V.** 1971. Mechanisms of recovery from a generalized viral infection: mousepox. III. Regression of infectious foci. J. Exp. Med. 133:1090-1104.
3. **Blanden, R. V.** 1974. T cell response to viral and bacterial infection. Transplant. Rev. 19:56-88.
4. **Blanden, R. V., and R. E. Langman.** 1972. Cell-mediated immunity to bacterial infection in the mouse. Thymus-derived cells as effectors of acquired resistance to *Listeria monocytogenes*. Scand. J. Immunol. 1:379-391.
5. **Blanden, R. V., M. J. Lefford, and G. V. Mackaness.** 1969. The host response to Calmette-Guérin bacillus infection in mice. J. Exp. Med. 129:1079-1107.
6. **Blanden, R. V., G. B. Mackaness, and F. M. Collins.** 1966. Mechanisms of acquired resistance in mouse typhoid. J. Exp. Med. 124:585-600.
7. **Blanden, R. V., and A. C. Mims.** 1973. Macrophage activation in mice infected with ectromelia or lymphocytic choriomeningitis viruses. Aust. J. Exp. Biol. Med. Sci. 51:393-398.
8. **Cerottini, J. C., and K. T. Brunner.** 1974. Cell-mediated cytotoxicity, allograft rejection and tumor immunity. Adv. Immunol. 19:67-132.
9. **Collins, F. M.** 1971. Mechanisms in anti-microbial immunity. J. Reticuloendothel. Soc. 10:58-99.
10. **Collins, F. M.** 1973. Immunogenicity of living and heat-killed *Salmonella pullorum* vaccines. Infect. Immun. 7:735-742.
11. **Collins, F. M.** 1974. Vaccines and cell-mediated immunity. Bacteriol. Rev. 38:371-402.
12. **Collins, F. M., R. V. Blanden, and G. B. Mackaness.** 1966. Infection immunity in experimental salmonellosis. J. Exp. Med. 124:601-619.
13. **Collins, F. M., C. C. Congdon, and N. E. Morrison.** 1975. Growth of *Mycobacterium bovis* (BCG) in T lymphocyte-depleted mice. Infect. Immun. 11:57-64.
14. **Collins, F. M., and G. B. Mackaness.** 1968. Delayed hypersensitivity and Arthus reactivity in relation to host resistance in *Salmonella*-infected mice. J. Immunol. 101:830-845.
15. **Gardner, I., N. A. Bowern, and R. V. Blanden.** 1974. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. I. Specificity and kinetics. Eur. J. Immunol. 4:63-67.
16. **Jenkin, C. R., and D. Rowley.** 1963. Basis for immunity to typhoid in mice and the question of "cellular immunity." Bacteriol. Rev. 27:391-423.
17. **Lane, F. C., and E. R. Unanue.** 1972. Requirement of thymus (T) lymphocytes for resistance to listeriosis. J. Exp. Med. 135:1104-1112.
18. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. 116:381-406.
19. **Mackaness, G. B.** 1964. The immunological basis of acquired cellular resistance. J. Exp. Med. 120:105-120.
20. **Mackaness, G. B.** 1969. The influence of immunologically committed lymphoid cells on macrophage activity *in vivo*. J. Exp. Med. 129:973-992.
21. **Mackaness, G. B., R. V. Blanden, and F. M. Collins.** 1966. Host-parasite relations in mouse typhoid. J. Exp. Med. 124:573-583.
22. **Mims, C. A., and R. V. Blanden.** 1972. Anti-viral action of immune lymphocytes in mice infected with lymphocytic choriomeningitis virus. Infect. Immun. 6:695-698.
23. **North, R. J.** 1973. Importance of thymus-derived lymphocytes in cell-mediated immunity to infection. Cell. Immunol. 7:166-176.
24. **Rowley, D., K. J. Turner, and C. R. Jenkin.** 1964. The basis for immunity to mouse typhoid. III. Cell-bound antibody. Aust. J. Exp. Biol. Med. Sci. 42:237-248.
25. **Sher, N. A., S. D. Chaparas, L. E. Greenberg, E. B. Merchant, and J. E Vickers.** 1975. Response of congenitally athymic mice to infection with Mycobacterium bovis (strain BCG). J. Natl. Cancer Inst. 54:1419-1426.
26. **Zinkernagel, R. M., and R. V. Blanden.** 1975. Macrophage activation in mice lacking thymus derived (T) cells. Experientia 31:591-593.
27. **Zinkernagel, R. M., R. V. Blanden, and R. E. Langman.** 1974. Early appearance of sensitized lymphocytes in mice infected with *Listeria monocytogenes*. J. Immunol. 112:496-501.