INFECTION AND IMMUNITY, Oct. 1980, p. 1-4
0019-9567/80/10-0001/04$02.00/0

Vol. 30, No. 1

Enhanced Elimination of *Listeria monocytogenes* at the Site of Delayed Footpad Reaction

MASAO MITSUYAMA[1]*, KIKUO NOMOTO[1], HIDEKI AKEDA[2], AND KENJI TAKEYA[2]

Department of Microbiology[1] and Department of Immunology in Cancer Research Institute[2], School of Medicine, Kyushu University, Fukuoka 812, Japan

The protective activity against a challenge infection with *Listeria monocytogenes* was investigated at the site of a delayed footpad reaction in mice immunized with viable or killed listeria. Delayed footpad reactivity was induced only in mice immunized with viable bacteria. Rapid and marked elimination of challenge bacteria was observed only at the site of reaction in mice immunized with viable bacteria but not in mice immunized with killed bacteria. Macrophage migration inhibitory activity was observed equally in both groups of mice. These results suggest that the delayed footpad reaction contributes directly to the elimination of bacteria irrespective of marcrophage migration inhibitory activity.

It is well known that resistance against *Listeria monocytogenes*, one of the facultative intracellular bacteria, is mediated by cellular immunity but not by humoral immunity (10, 15). Resistance to listeria infection is partly dependent on nonimmune macrophages at an early stage of infection, but complete elimination of bacteria is brought about only by acquired cellular resistance (ACR), which depends on immunologically activated macrophages (3, 17). This kind of immune resistance seems to be induced more readily by infection with viable bacteria than by immunization with killed bacteria (6, 24). Delayed hypersensitivity (DH) also develops in animals infected with viable bacteria (6, 18) and has been reviewed in reference to ACR. DH and ACR are said to have a close correlation with each other from the fact that these two immunological phenomena appear coincidentally in bacterial infection, and protective immunity cannot be adequately expressed in the absence of DH (11, 13). However, it is controversial whether DH has a direct role in ACR or not.

The delayed footpad reaction is one of the assay methods for DH in animals immunized not only with bacteria but also with heterologous erythrocytes (9) or protein antigens (16). It is evident that the delayed footpad reaction is a cell-mediated immunological phenomenon, but its biological significance is not fully elucidated. To clarify the contribution of DH to ACR, it is necessary to know whether the protective activity is actually enhanced at the site of a delayed reaction or not.

In the present study, bacterial challenge was made at the site of a delayed footpad reaction in mice immunized with *L. monocytogenes*, and the correlation was examined between the level of reaction and the degree of bacterial elimination.

MATERIALS AND METHODS

Animals. Female mice of an outbred ddY strain were obtained from the Breeding Unit of Experimental Animals, Kyushu University. Six- to eight-week-old mice were used for the experiments. Each experimental group consisted of five to six mice, and the same protocols were repeated two or three times.

Microorganism. *L. monocytogenes*, strain EGD, was used in all experiments. The bacteria were grown in tryptic soy broth (Difco Laboratories, Detroit Mich.) at 37°C for 20 h, washed repeatedly, suspended in phosphate-buffered saline, and stored at −70°C. Killed bacteria were prepared by adding Formalin to the viable bacterial suspensions followed by repeated washings. The concentration of killed bacteria was adjusted by the use of a photoelectric colorimeter.

Immunization. Mice were immunized intravenously with 2×10^3 viable bacteria or subcutaneously with 4×10^8 killed bacteria in Freund complete adjuvant (FCA).

Assessment of delayed footpad reaction. The thickness of the left hind footpad was measured with a dial-gauge caliper 24 h after injecting an eliciting dose of 10^8 killed bacteria in 0.05 ml of phosphate-buffered saline. The thickness of the right hind footpad was measured 24 h after injecting the same volume of phosphate-buffered saline as a control. The difference in footpad thickness was expressed in 0.1-mm units. Reactions were recorded against the day when eliciting injection was done.

Determination of bacterial growth at the site of delayed reaction. An eliciting antigen was injected into the left hind footpad, and 10^6 viable bacteria suspended in 0.05 ml of phosphate-buffered saline were inoculated into bilateral footpads 24 h after the elicitation. The whole mass of the challenged foot was removed and homogenized in 20 ml of phosphate-buffered saline with a Waring blender (Nihon Seiki,

Co., Tokyo, Japan). The homogenized specimens were diluted serially 10-fold with phosphate-buffered saline, and 0.1 ml of each dilution was spread on nutrient agar containing 0.3% (wt/vol) glucose. Numbers of viable bacteria were estimated by colony-forming units (CFU) and expressed in $\log_{10}$ CFU after incubation for 20 h at 37°C.

Macrophage migration inhibition test. Peritoneal exudate cells were harvested from 8 mice 72 h after an intraperitoneal injection of 2 ml of sterile liquid paraffin. Pooled peritoneal exudate cells were washed and suspended at a concentration of 2×10^7 cells per ml in RPMI 1640 medium supplemented with 10 % fetal calf serum. The cells were then loaded into capillary tubes, packed by centrifugation, and incubated at 37°C for 20 h in the medium with or without sonicated listeria antigen. The amount of sonicated antigen was equivalent to almost 10^8 CFU/ml, and this dose of antigen exerted only negligible inhibition on the migration of normal peritoneal exudate cells. The areas of migration were measured and expressed as percent migration by the formula described elsewhere (23).

RESULTS

Delayed footpad reactivity of mice after immunization with viable or killed *L. monocytogenes*. Mice were immunized with viable bacteria or killed bacteria in FCA, and their delayed footpad reactions were measured at various days after immunization. In mice immunized with this nonlethal dose of viable bacteria, the numbers of bacteria in the body reached around 10^6 to 10^7 for several days as we have reported (17), so a larger dose was employed in immunization with killed bacteria to equate roughly the dose of immunogen. In mice immunized with viable bacteria, positive delayed reactions were not detected before day 5, but strong reactions of over 12 U were found on day 6 (Fig. 1). Maximal reactions as much as 15 to 20 U were observed on day 9. The degree of delayed footpad reaction decreased to some extent after 9 days, but positive reactions were detectable over a period of 28 days in this group of mice. The reaction showed a peak at 24 h and diminished at 48 h after elicitation at all times of testing. In mice immunized with killed bacteria, on the other hand, significant levels of reaction were not observed, even though FCA was used for immunization.

Elimination of challenged bacteria at the site of delayed reaction. To know whether a positive delayed footpad reaction itself could contribute to protection against bacterial infection, the growth of bacteria at the site of a delayed reaction was measured. Delayed reactions were elicited in the left hind footpad of mice at 8 and 18 days after immunization. The reaction was measured 24 h later, and the challenge infection was given into both nonelicited and antigen-elicited footpads. Counts of viable bacteria in both footpads were determined 24 h after the challenge. An eliciting injection into normal mice did not modify the growth of the challenge bacteria (Table 1). In mice immunized with viable bacteria 8 or 18 days before, an enhanced elimination of bacteria was observed in antigen-elicited footpads which showed marked swelling. The difference of bacterial counts was highly significant between the elicited and the nonelicited footpads in this group of mice. On the other hand, such an enhanced elimination of bacteria was not detected in the elicited footpad of mice immunized with killed bacteria in FCA, which showed an insignificant level of delayed reactivity.

The macrophage migration inhibition test. The macrophage migration inhibition test, one of the in vitro assays for DH, was carried out with peritoneal exudate cells obtained from mice immunized with viable or killed bacteria in FCA. Peritoneal macrophages from nonimmunized mice migrated equally in the presence and absence of antigen. When peritoneal exudate cells obtained on day 8 were examined, the macrophages showed a tendency to overmigrate, and positive inhibition of migration was not observed in the presence of antigen (Table 2). Ten days after immunization, migration inhibitory activity became positive not only in mice immunized with viable bacteria but also in mice immunized with killed bacteria in FCA. The migration inhibition test on days 17 and 24 also gave positive results in both groups of mice. These results indicated a dissociated development of delayed footpad reactivity and macrophage migration inhibitory activity.

DISCUSSION

The delayed footpad reaction was introduced by Gray and Jennings (4) to measure tuberculin

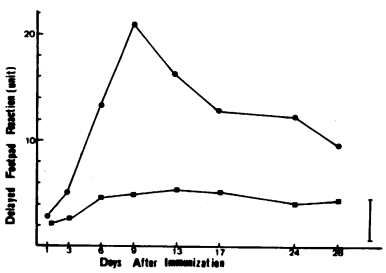

FIG. 1. *Time course of delayed footpad reaction in mice after immunization with L. monocytogenes. Mice were immunized intravenously with 2×10^3 viable bacteria (●) or subcutaneously with 4×10^8 killed bacteria in FCA (■). The bar in the right lower corner indicates the range of reaction in nonimmunized mice. One unit represents 0.1 mm.*

TABLE 1. *Enhanced elimination of L. monocytogenes at the site of delayed footpad reaction*

Immunization	Delayed footpad reaction (× 0.1 mm)	Bacterial count in nonelicited footpad (A)	Bacterial count in antigen-elicited footpad (B)	Log_{10} difference (A) − (B)
None	2.9 ± 0.4	6.50 ± 0.21	6.68 ± 0.12	−0.18
Viable *L. monocytogenes*, intravenous injection				
Day 8	15.8 ± 5.6	5.28 ± 0.53	2.93 ± 0.49	+2.35[a]
Day 18	9.3 ± 2.7	5.82 ± 0.52	3.29 ± 0.48	+2.53[a]
Killed *L. monocytogenes*, subcutaneous injection with FCA				
Day 8	3.8 ± 1.3	6.11 ± 0.20	5.55 ± 0.48	+0.56[b]
Day 18	4.1 ± 1.4	6.30 ± 0.32	5.97 ± 0.40	+0.33[b]

[a] $P < 0.005$.
[b] Not significant.

TABLE 2. *Macrophage migration inhibition test in mice immunized with viable or killed L. monocytogenes*

Time after immunization (days)	% Migration of peritoneal exudate cells obtained from:		
	Mice immunized with v-Lm[a]	Mice immunized with k-Lm/FCA[b]	Nonimmunized control mice
8	122.0	114.7	100.0
10	72.6	70.8	100.0
17	58.1	68.9	100.0
24	75.5	61.8	100.0

[a] v-Lm, Viable *L. monocytogenes*.
[b] k-Lm/FCA, Killed *L. monocytogenes* with FCA.

sensitivity in mice and has been regarded as a reaction similar to the delayed skin reaction of guinea pigs. The development of delayed footpad reactivity is reported in infections with *Mycobacterium tuberculosis* (20), *Mycobacterium lepraemurium* (1), *Salmonella typhimurium* (21) and *L. monocytogenes* (6, 18). All of these bacteria usually survive within macrophages and resist the protection of infected hosts. ACR, the most powerful mechanism of protection, develops in association with DH in the course of active infection with such kinds of intracellularly parasitic bacteria. From the results obtained either in vivo (12, 14) or in vitro (8, 22), it is believed in general that sensitized lymphocytes release some kind of lymphokine after specific stimulation by bacterial antigens, and that the lymphokine alters the activity of nonimmune macrophages to kill the invading microorganisms nonspecifically. In spite of the general acceptance of this interpretation concerning the mechanism of ACR, the contribution of DH to ACR is unclear. It has been suggested that macrophage migration inhibitory factor which is detected in vitro by the migration inhibition test and its allied factors contribute to ACR by immobilization or activation of macrophages (2). The delayed footpad reaction is an in vivo phenomenon, and there has been no evidence to show the direct contribution of this reaction to ACR.

In the present study, a typical form of delayed reaction, as revealed by a delayed onset of marked footpad swelling, was elicited only in the group immunized with viable *L. monocytogenes*. The reaction appeared on day 6 after infection and lasted thereafter. The growth of challenge bacteria was markedly inhibited, and a rapid elimination of bacteria was observed at the site of a delayed footpad reaction on days 8 and 18. The enhancement of elimination on day 8 was almost the same in degree as that on day 18, whereas positive activity was not observed before day 10 in the macrophage migration inhibition test. In mice immunized with killed bacteria in FCA, delayed footpad reactivity was not induced, and the degree of bacterial elimination at the reaction site was not significant. Delayed footpad reactivity and ACR were not induced by immunization of mice with heat-killed listeria (24) or nonvirulent L-forms of listeria (6), regardless of the use of FCA. It is interesting in our result that only macrophage migration inhibitory factor activity was observed in mice immunized with killed listeria in FCA. Dissociated development of delayed footpad reactivity and macrophage migration inhibitory factor activity was revealed. Ohmichi et al (19) showed a dissociation of delayed footpad reactivity and macrophage migration inhibitory factor activity by the use of various methods of immunization with sheep erythrocytes. In experiments with thoracic duct lymphocytes obtained from *Myobacterium bovis* BCG-infected rats, it was shown that the course of macrophage migration inhibitory factor activity differed from that of DH as measured by the radiometric ear assay (7). It seems that lymphocytes which mediate delayed

footpad reaction or delayed ear reaction are somewhat different from those which mediate macrophage migration inhibition.

The enhanced elimination of bacteria at the reaction site clearly demonstrated the direct contribution of delayed footpad reactivity to resistance. Delayed footpad reactivity seems to have a decisive role in protection. However, the idea that the delayed footpad reaction and ACR are mediated exactly by the same T cells was not supported by direct evidence. A report was presented showing that the T cells involved in protection against listeria and delayed footpad reaction to homologous antigen are of the same Ly 123^+ phenotype (5). This report may support the idea mentioned above. The mechanism of bacterial elimination at the reaction site is not clear at a time when macrophage migration inhibitory factor activity is undetectable. This enhanced elimination may be attributable to the function of accumulated macrophages at the reaction site.

LITERATURE CITED

1. **Alexander, J., and J. Curtis.** 1979. Development of delayed hypersensitivity responses in *Mycobacterium lepraemurium* infections in resistant and susceptible strain of mice. Immunology **36**:563–568.
2. **Campbell, P. A.** 1976. Immunocompetent cells in resistance to bacterial infections. Bacteriol. Rev. **40**:284–313.
3. **Emmerling, P., H. Finger, and J. Bockemühl.** 1975. *Listeria monocytogenes* infection in nude mice. Infect. Immun. **12**:437–439.
4. **Gray, D. F., and P. A. Jennings.** 1955. Allergy in experimental mouse tuberculosis. Am. Rev. Tuberc. **72**:171–195.
5. **Kaufmann, S. H. E., M. M. Simon, and H. Hahn.** 1979. Specific Lyt 123 T cells are involved in protection against *Listeria monocytogenes* and delayed-type hypersensitivity to listerial antigens. J. Exp. Med. **150**: 1033–1038.
6. **Kerckhaert, J. A. M., F. M. A. Hofhuis, and J. M. N. Willers.** 1977. Influence of cyclophosphamide on delayed hypersensitivity and acquired cellular resistance to *Listeria monocytogenes* in the mouse. Immunology **32**:1027–1032.
7. **Kostiala, A. A., M. J. Lefford, and D. D. McGregor.** 1978. Immunological memory in tuberculosis. 2. Mediators of protective immunity, delayed hypersensitivity and macrophage migration inhibition in central lymph. Cell. Immunol. **41**:9–19.
8. **Krahenbuhl, J. L., L. T. Rosenberg, and J. S. Remington.** 1973. The role of thymus-derived lymphocytes in the in vitro activation of macrophages to kill Listeria monocytogenes. J. Immunol. **111**:992–995.
9. **Lagrange, P. H., and G. B. Mackaness.** 1975. A stable form of delayed-type hypersensitivity. J. Exp. Med. **141**:82–96.
10. **Lane, F. C., and E. R. Unanue.** 1972. Requirement of thymus T lymphocytes for resistance in Listeriosis. J. Exp. Med. **135**:1104–1112.
11. **Lefford, M. J.** 1975. Delayed hypersensitivity and immunity in tuberculosis. Am. Rev. Respir. Dis. **111**:243–246.
12. **Mackaness, G. B.** 1964. The immunological basis of acquired cellular resistance. J. Exp. Med. **120**:105–120.
13. **Mackaness, G. B.** 1967. The relationship of delayed hypersensitivity to acquired cellular resistance. Br. Med. Bull. **23**:52–54.
14. **Mackaness, G. B.** 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J. Exp. Med. **129**:973–992.
15. **Miki, K., and G. B. Mackaness.** 1964. The passive transfer of acquired cellular resistance to *Listeria monocytogenes*. J. Exp. Med. **120**:93–103.
16. **Mitsuoka, A., S. Morikawa, M. Baba, and T. Harada.** 1979. Cyclophosphamide eliminates suppressor T cells in age-associated central regulation of delayed hypersensitivity in mice. J. Exp. Med. **149**:1018–1028.
17. **Mitsuyama, M., K. Takeya, K. Nomoto, and S. Shimotori.** 1978. Three phases of phagocyte contribution to resistance against *Listeria monocytogenes*. J. Gen. Microbiol. **106**:165–171.
18. **North, R. J.** 1969. Cellular kinetics associated with the development of acquired cellular resistance. J. Exp. Med. **130**:299–314.
19. **Ohmichi, Y., K. Nomoto, H. Yamada, and K Takeya.** 1976. Relationships among differentiated T-cell subpopulations. I. Dissociated development of tuberculin type hypersensitivity, Jones-Mote type hypersensitivity and activation of helper function. Immunology **31**:101–110.
20. **Patel, P. J., and M. J. Lefford.** 1978. Antigen specific lymphocyte transformation, delayed hypersensitivity and protective immunity. I. Kinetics of the response. Cell. Immunol. **37**:315–326.
21. **Plant, J., A. A. Glynn, and B. M. Wilson.** 1978. Protective effects of a supernatant factor from *Salmonella typhimurium* on *Salmonella typhimurium* infection of inbred mice. Infect. Immun. **22**:125–131.
22. **Simon, H. B., and J. N. Shaegren.** 1971. Cellular immunity in vitro. I. Immunologically mediated enhancement of macrophage bactericidal capacity. J. Exp. Med. **133**:1377–1389.
23. **Terasaka, R., K. Nomoto, K. Himeno, and K. Takeya.** 1979. Relationships among differentiated T-cell subpopulations. IV.Preferential induction of delayed footpad reaction to xenografts and its radioresistant nature. Immunology **36**:699–705.
24. **Van der Meer, C., F. M. A. Hofhuis, and J. M. N. Willers.** 1979. Delayed-type hypersensitivity and acquired cellular resistance in mice immunized with killed *Listeria monocytogenes* and adjuvants. Immunology **37**:77–82.