INFECTION AND IMMUNITY, Nov. 1974, p. 1163-1169

Vol. 10, No. 5
Printed in U.S.A.

Induction of Resistance by *Listeria monocytogenes* Cell Wall Fraction

GILBERTO E. RODRIGUEZ,[1] J. KENNETH McCLATCHY, AND PRISCILLA A. CAMPBELL

Division of Allergy and Clinical Immunology, National Jewish Hospital and Research Center, Denver, Colorado 80206

Received for publication 1 July 1974

A crude cell wall fraction of *Listeria monocytogenes* was prepared by sonic disruption and differential centrifugation of viable, washed cultures. When injected into mice, this sterile, crude cell wall fraction protected mice against an intraperitoneal challenge with 18 to 85 50% mean lethal dose of *L. monocytogenes*. Resistance was greatly enhanced when bacterial endotoxin (lipopolysaccharide) was injected along with the cell wall fraction. Resistance was measured both by enumerating the bacteria in the livers and spleens of vaccinated and control mice and by survival studies. Two major lines of evidence suggest that the resistance induced by cell wall fraction is at least in part specific. Unlike nonspecific resistance, the cell wall fraction-induced resistance was relatively long-lived, (i.e., it was demonstrable 6 weeks after the last injection of cell wall fraction and lipopolysaccharide). In addition, cell wall fraction protected against challenge with *L. monocytogenes*, but not against challenge with *S. typhimurium*.

Listeria monocytogenes is a facultative, intracellular bacterium with a variety of susceptible hosts (13). Attempts to prevent listeric infections by active immunization have led to conflicting results. Vaccines composed of sublethal numbers of viable *L. monocytogenes* have been quite successful (14, 25, 28, 29), but these live vaccines sometimes killed an experimental animal (27). Therefore, more recent attempts have been directed toward developing an effective killed vaccine, but unfortunately, these efforts have generally been unsuccessful (2, 7, 11, 12, 14, 18, 25-27). However, in a preliminary trial, Eveleth et al. (10) obtained some evidence that *Listeria* which were killed with formalin or ultraviolet light induced some resistance to naturally acquired infections in sheep. In addition, two other laboratories (4, 17) recently reported that nonviable *L. monocytogenes* preparations can induce resistance to listeric infection in mice. Here, a series of experiments is described in which resistance to *Listeria* was induced in mice by injection of a cell wall fraction (CWF) of *L. monocytogenes*.

MATERIALS AND METHODS

Animals. CF1 and AKR mice from the National Jewish Hospital and Research Center colonies were used throughout these experiments. In individual experiments, 8- to 12-week-old animals of a single strain and sex were used.

[1] Present address: Department of Pediatrics, Medical College of Virginia, Richmond, Va. 23298.

Bacteria. (i) *L. monocytogenes* serotype 1 (kindly supplied by G. B. Mackaness, Saranac Lake, N.Y.) was grown in tryptose phosphate broth (Difco Laboratories, Inc., Detroit, Mich.) with gentle shaking for approximately 36 h at 37 C. The bacteria for challenge were collected by continuous-flow centrifugation, suspended in sterile, pyrogen-free water, and stored frozen at −70 C until used. The 28-day mean lethal dose (LD_{50}) of this bacterium in CF1 mice was found to be 2×10^4 colony-forming units (CFU) after intraperitoneal (i.p.) injection, and in AKR mice it was 9×10^4 CFU. The LD_{50} values were calculated by the method of Reed and Muench (32). (ii) *Salmonella typhimurium* (obtained from the stock culture maintained at this institution) was grown in brain heart infusion broth (Difco), collected by continuous-flow centrifugation, and stored frozen at −70 C until used. The i.p. LD_{50} of *S. typhimurium* in our CF1 mice was 9×10^4 colony-forming units.

Cell wall fraction. A crude cell wall preparation was isolated by a modification of previous methods (44). A 24-liter culture of *L. monocytogenes* was grown in tryptose phosphate broth, collected by continuous flow centrifugation, and the pellet was washed 3 times with 250-ml volumes of sterile, pyrogen-free water. Washed *Listeria* (12 to 15 g) was resuspended in 60 ml of water. A 14- to 16-ml amount of the suspension was disrupted by sonic treatment in a stainless-steel chamber that was firmly sealed around a Sonifier probe tip (Bronson cell disruptor, Bronson Instruments, Inc., Stamford, Conn.). The chamber was immersed in an ice bath and sonically treated for 5 min, followed by a 5-min cooling period, until the suspension had been sonically treated for a total of 20 min. After sonic treatment, the viability of these bacteria was determined by plate counts on 5% sheep

blood-agar and was found to be reduced by 95 to 99%. The crude cell wall preparation was obtained by differential centrifugation. An initial centrifugation (at 2,000 × *g* for 15 to 20 min) resulted in a pellet containing whole cells and cell debris. The remaining supernatant fluid contained the cell walls which were collected by sedimentation at 12,000 × *g* for 45 min. The precipitate was then washed 3 times with 200 ml of sterile, pyrogen-free water. An electron micrograph of the precipitate showed the preparation to be rich in rigid cell wall skeletons of disrupted organisms (Fig. 1). This material is referred to as CWF.

The CWF was then lyophilized, weighed, reconstituted with sterile, nonpyrogenic saline to a concentration of 1 mg/ml of saline, and then stored at 4 C in 100-ml bottles. To insure sterility of the CWF, chloroform was added to a concentration of 0.05%. Sterility tests were performed by adding 2.5 ml of CWF to 30 ml of tryptose phosphate broth and incubating with gentle shaking at 37 C for 4 to 5 days. After 48 h of incubation, 0.1-ml samples of CWF in tryptose phosphate broth were plated on each of 12 phenylethyl alcohol-agar plates. Samples occasionally found to have viable *L. monocytogenes* were freeze-thawed several times, and an additional 0.05 ml of chloroform was added. Sterility was then rechecked as described above. As a further confirmation of sterility, eight lethally irradiated mice were each injected i.p. with 0.2 ml of CWF and sacrificed 3 days later. No viable bacteria were recovered when whole livers or spleens were homogenized in a Teflon to glass grinder and 0.1-ml samples were plated. As determined by the above techniques, the CWF employed in the experiments described in this paper did not contain any viable organisms.

Endotoxin. *Salmonella typhosa* lipopolysaccharide (LPS; Difco) was used in some experiments. The dose varied from 25 to 100 μg but was always the same in an individual experiment.

Assay of mice for resistance. Mice were assayed for resistance to infection by determining the number of *L. monocytogenes* cells in their livers, spleens, and blood 72 h after i.p. challenge with varying numbers of *L. monocytogenes*. Mice were anesthetized with chloroform and bled by cardiac puncture. Spleens and livers were removed aseptically and were placed in preweighed, sterile, glass grinding tubes. The tubes were then reweighed, 2 ml of sterile saline was added, and the organs were homogenized for 30 to 60 s with a Teflon to glass grinder. Serial dilutions of the organ homogenates were plated in duplicate on phenylethyl-alcohol agar plates. Heparinized blood was similarly plated to assay for bacteremia. These techniques were described previously (5). After incubation at 37 C for 48 h, *Listeria* CFU were counted. Results are expressed as the $\log_{10}$ *Listeria* CFU per liver or

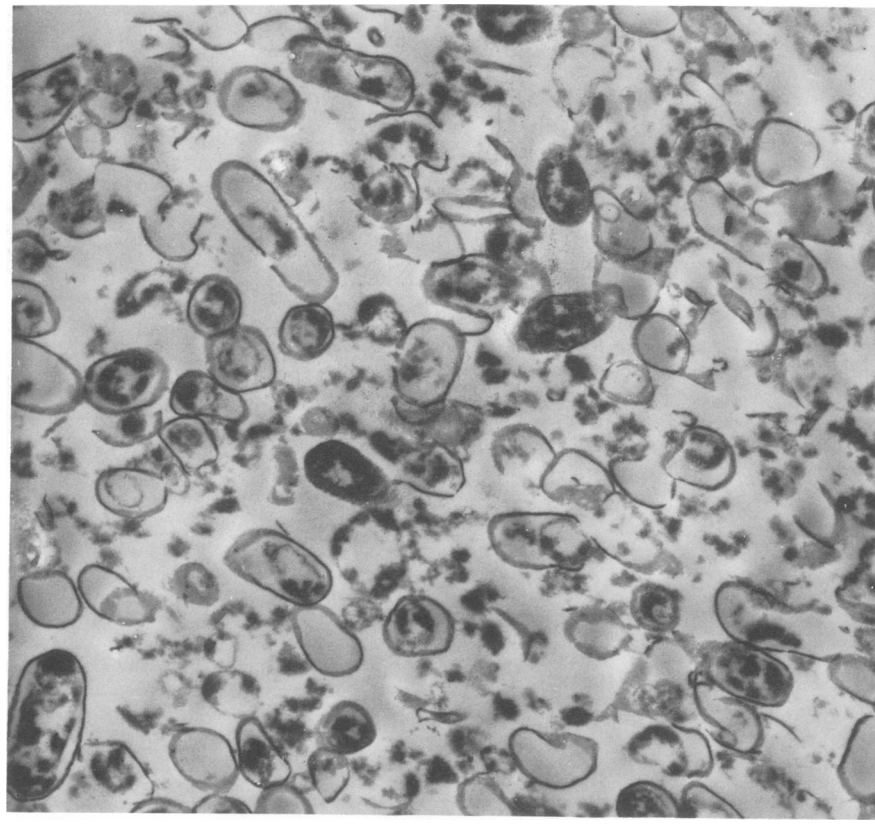

FIG. 1. *Electron micrograph of CWF* (×*12,000*).

spleen or milliliter of blood. In some experiments, resistance was measured by injecting mice with lethal numbers of *Listera* and determining survival.

RESULTS

Effect of CWF on resistance to listeria. In preliminary experiments, one i.v. or i.p. injection of 50 to 250 μg of the CWF (with and without LPS adjuvant) failed to induce resistance in mice which were challenged 7 days later with *L. monocytogenes*. Experiments were then conducted to see if a series of injections of CWF with LPS as an adjuvant could induce resistance. Groups of mice were injected i.v. on day 0 with either 50 μg of CWF plus 100 μg of LPS or with 100 μg of LPS alone. A group of control mice was not injected. The mice which had received CWF plus LPS or LPS alone were reinjected i.p. with the same materials on days 7, 14 and 21. All mice (including controls) were challenged i.p. on day 28 with live *Listeria*. The size of the challenge inoculum in different experiments ranged from 3.5×10^5 to 1.7×10^6 cells of *L. monocytogenes*. All mice were bled under chloroform anesthesia 72 h after challenge, and the number of *L. monocytogenes* colony-forming units in their spleens and livers was determined.

Figure 2 illustrates the results of four such experiments. The bars represent the mean number of *Listeria* cells per liver or spleen expressed as the $\log_{10}$. Mice injected with CWF plus LPS had significantly fewer *Listeria* in their livers and spleens than the uninjected control mice or the mice injected with LPS alone, which indicate that the CWF induces resistance.

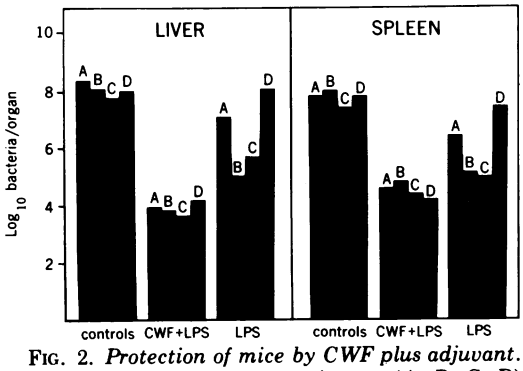

FIG. 2. *Protection of mice by CWF plus adjuvant. Solid bars represent four experiments (A, B, C, D) containing at least five mice per group. Experiment A used AKR mice, and experiments B, C, and D, used CF1 mice. Control mice were uninjected. Inocula were: CWF, 250 μg; LPS, 100 μg. Mice were injected i.v. on day 0 and i.p. on days 7, 14, and 21. They were challenged i.p. on day 28 with 3.5×10^5 to 1.7×10^6 cells of Listeria.*

Results of experiments carried out to determine the optimum dose range of CWF are given in Table 1. The data suggest that a 50-μg dose is more effective than a 1-μg dose or a dose greater than 250 μg; this may indicate high-dose toxicity.

Ability of CWF to enhance survival. In addition to the experiments in which resistance was determined by counting the numbers of *L. monocytogenes* CFU in livers and spleens of treated and untreated mice, mortality studies were performed. The immunization schedule and survival rates of one experiment are shown in Table 2. These data, which confirm the bacterial enumeration studies, show that mice injected with CWF plus LPS are resistant to lethal challenge with 25 LD_{50} *L. monocytogenes*. These results have been confirmed in two other experiments in which mice were challenged with 6 and 16 LD_{50}.

Duration of resistance after last injection. Nonspecific resistance is thought to be short-lived, whereas immunologically specific resistance is thought to last several months. Experiments were conducted to determine whether the protection induced by the cell wall fraction was short-lived (i.e., nonspecific) or long-lived (i.e., specific). Mice were injected i.v. on day 0 and i.p. on days 18 and 29 with either 50 μg of CWF, 50 μg of CWF plus 25 μg of LPS, or 25 μg of LPS alone. A fourth group received sterile, nonpyrogenic saline at the same time. Six weeks after the last injection on day 67, all four groups of mice were challenged i.p. with 6×10^5 *L. monocytogenes* and assayed for resistance on day 70. The results (Table 3) indicate that by 6 weeks after the last injection nonspecific resist-

TABLE 1. *Doses of CWF used to induce resistance in mice*[a]

Inoculum	$\log_{10}$ bacteria per liver[b]	$\log_{10}$ bacteria per spleen
None	7.62	7.64
CWF (1 μg/dose [3][c])	7.54	7.13
CWF (50 μg/dose [6])	3.97	5.58
CWF (250 μg/dose [5])	6.19	7.64

[a] Mice were injected i.v. with the doses indicated on days 0 and were boosted i.p. with the same inoculum on days 14, 45, and 93. All mice were challenged on day 103 with 2.4×10^5 cells of *L. monocytogenes*. They were sacrificed 3 days later, and *Listeria* colony-forming units in their livers, spleens, and blood were determined.

[b] $\log_{10}$ of the mean number of *Listeria* per organ in each group.

[c] Numbers in brackets indicate number of mice per group.

TABLE 2. *Ability of CWF to enhance survival*[a]

Inoculum	Survivors (%)	No. survivors per no. challenged
None	4	1/25
CWF (50 μg) + LPS (50 μg)[b]	87	13/15
LPS (50 μg)[c]	23	3/13

[a] All mice were challenged i.p. on day 52 with 5×10^5 cells of *L. monocytogenes* (25 LD_{50}). Percentage of survivors 30 days after challenge.

[b] Mice were injected i.v. on day 0 and i.p. on day 14 with 50 μg of CWF and 50 μg of LPS, and injected i.p. on days 28 and 42 with 50 μg of CWF alone.

[c] Mice were injected i.v. on day 0 and i.p. on day 14 with 50 μg of LPS alone, and with 0.1 ml of sterile, nonpyrogenic saline on days 28 and 42.

TABLE 3. *Duration of resistance after last injection*[a]

Inoculum	Log_{10} *Listeria* per liver	Log_{10} *Listeria* per spleen	Log_{10} *Listeria* per ml of blood	No. survivors/ no. challenged
Saline ([4])[b]	8.45[c]	8.40	5.73	4/13
CWF (50 μg [5])	7.12	7.13	4.30	5/12
LPS (25 μg [5])	8.53	7.81	5.44	7/12
CWF (50 μg) + LPS (25 μg [6])	4.32	5.07	3.23	8/8

[a] Mice were injected i.v. as indicated on day 0 and i.p. on days 18 and 29. All mice were challenged i.p. on day 67 with 6×10^5 (30 LD_{50}) *L. monocytogenes*, and were assayed 3 days later.

[b] Numbers in brackets represent the number of mice assayed.

[c] Log_{10} mean number of *Listeria* colony-forming units per organ.

ance could not be demonstrated. That is, mice injected with LPS had as many organisms in their livers and spleens as did uninjected control mice, whereas only weak resistance was seen in mice receiving CWF alone. However, mice injected with CWF plus LPS were resistant to lethal challenge, as demonstrated both by their increased survival and by the markedly decreased numbers of *Listeria* recovered from their livers, spleens, and blood. Two similar experiments have confirmed these results.

Inability of Listeria CWF to protect against infection by S. typhimurium. If the CWF were acting only through nonspecific mechanisms, it might be expected to induce resistance to other intracellular bacteria. To test this, it was first determined whether our live *L. monocytogenes* strain could induce active resistance to *S. typhimurium*. Groups of mice were injected i.p. with a sublethal dose of either live *Listeria* or live *Salmonella* on day 0 and day 28. A control group was not injected. Seven days after the second injection, the mice were challenged i.p. with lethal numbers of either *Listeria* or *Salmonella*. Table 4 shows one of two experiments in which mice immunized with live *Listeria* developed resistance to *L. monocytogenes* but not to *S. typhimurium*, whereas mice immunized with *Salmonella* were resistant to infection with either *L. monocytogenes* or *S. typhimurium*. The capacity of *Listeria* CWF to induce resistance to infection by *S. typhimurium* was then studied. One group of mice was injected i.v. on day 0 with 50 μg and another group with 100 μg of *Listeria* CWF. The same inocula were repeated i.p. on days 21, 35, and 49. A third group of uninjected mice served as controls. On day 56, one-half of each group was challenged with *L. monocytogenes* and the other half with *S. typhimurium*. Mice inoculated with either dose of *Listeria* CWF were resistant to challenge with *L. monocytogenes* but not to challenge with comparable numbers of virulent *S. typhimurium*. The results of these experiments are shown (Table 5).

Inability of CWF to induce short-term, nonspecific resistance. Rotta et al. (34) have shown that a single i.p. injection of 250 μg of group A streptococcal cell walls, followed 24 h later by i.p. challenge, will cause a nonspecific resistance in mice against lethal challenge with homologous and heterologous strains of streptococcus. To determine whether a single injection of *Listeria* cell walls could act similarly, 12 mice were injected i.p. with 250 μg of *Listeria* CWF, whereas 12 control mice received i.p. 0.1 ml of sterile, nonpyrogenic saline. Twenty-four hours later, both groups were challenged i.p. with 3.2×10^5 *L. monocytogenes* (16 LD_{50}). The 30-day survival rate for the control group was 25%

TABLE 4. *Specificity of immunization with live organisms*[a]

Inoculum	Challenge	Survivors[b] (%)	No. of survivors/no. challenged
Saline	*Listeria*	0	0/10
Listeria	*Salmonella*	10	1/10
Listeria	*Listeria*	100	10/10
Saline	*Salmonella*	0	0/10
Salmonella	*Listeria*	87	8/9
Salmonella	*Salmonella*	87	8/9

[a] Mice were injected i.p. on days 0 and 28 with 2×10^3 cells of *Listeria* or 4×10^3 cells of *Salmonella* and were challenged i.p. on day 35 with 3.2×10^5 (16 LD_{50}) *Listeria* or 4.7×10^5 (5 LD_{50}) *Salmonella*.

[b] The percentages of survivors were calculated from the number surviving 30 days after challenge divided by the number challenged.

versus 0% for those mice that received CWF. Therefore, we could not demonstrate the short-term (presumably nonspecific) resistance seen with gram-positive streptococcal cell walls.

DISCUSSION

The data presented in this paper provide evidence that a CWF of *L. monocytogenes* can protect mice against lethal challenge with this organism. This CWF induced more effective resistance when it was injected with the adjuvant, endotoxin (LPS). Although at present the mechanisms by which this fraction induces resistance are not clear, at least three possible modes of action should be considered. (i) The CWF may be nonspecifically activating macrophages to enhance phagocytic and bactericidal activity, as has been reported in experiments using endotoxin, double-stranded RNA, synthetic polyanions, and various particulate materials (1, 3, 33, 42). (ii) The CWF could be manifesting its effect by acting as an antigen to induce opsonic or cytophilic antibodies which in turn could cause increased phagocytosis and killing of the challenge bacteria by macrophages. These mechanisms have been shown to be active in *Salmonella* infections (36, 37, 43), but evidence against their importance in *L. monocytogenes* infections has been presented (20, 24). (iii) The CWF might induce cell-mediated immunity in thymus-derived lymphocytes which could then mount a specific cell-mediated immunologic response against the challenge organism. Mackaness (21, 22) has suggested that specific resistance to *Listeria* is mediated by immunologically specific, thymus-derived lymphocytes. These cells are thought to respond to antigen by secreting lymphokines which activate macrophages nonspecifically to phagocytose and kill the invading bacteria.

Since the time of Metchnikoff and Pasteur, resistance to bacterial infections has been divided into specific and nonspecific states of resistance. Resistance to *L. monocytogenes* (as well as to *M. tuberculosis*) is believed to consist of both specific and nonspecific components (7, 8, 18, 19, 21, 22). However, specific resistance to *Listeria* is often difficult to analyze, since *Listeria* organisms are extremely readily phagocytized and killed by nonspecifically activated macrophages (6, 23, 38, 39). Three sets of experiments reported here suggest that the resistance induced by *Listeria* CWF was, at least in part, specific. (i) In earlier experiments, the experimental mice were challenged 7 days after the last injection, since that was the time at which Coppel and Youmans (7) had shown protection to be maximal after immunization with attenuated *Listeria*. However, the nonspecific protection conferred by LPS was in many experiments still demonstrable 7 days after injection. Therefore, mice in later experiments were challenged 6 weeks after the last injection. At this time, nonspecific resistance induced by endotoxin was minimal or not demonstrable. These experiments showing long-term protection after injection of CWF plus LPS suggested that resistance was at least partly specific. (ii) If specific resistance were involved, *Listeria* immunization should have been most effective against *Listeria* challenge because of an anamnestic recall of acquired resistance brought about by the specific challenge inoculum. Such an anamnestic response is shown in Table 3. (iii) Specific resistance after challenge with the immunizing organism (*Listeria*) should be of much greater magnitude than resistance after challenge with a different organism (*Salmonella*). Further indirect evidence suggesting specificity was given by the experiment in which infection by *L. monocytogenes* did not protect against challenge with *S. typhimurium* (Table 4). Similarly, CWF protected against challenge with *Listeria*, but not with *Salmonella* (Table 5). If the CWF were acting mainly through nonspecific mechanisms, it would be expected to protect against challenge with other similar bacteria.

The nonspecific immunity conferred on mice by endotoxin against lethal challenge with both gram-positive and gram-negative organisms has been well confirmed (9, 16, 35, 40). The mechanisms by which LPS modify host reactions to infection involve many biological systems, both humoral and cellular, and have been well re-

TABLE 5. *Effect of Listeria CWF on resistance to Salmonella typhimurium*[a]

Inoculum	Challenge			
	Listeria		*Salmonella*	
	Log_{10}[b] bacteria per liver	Log_{10} bacteria per spleen	Log_{10} bacteria per liver	Log_{10} bacteria per spleen
None	4.88	5.20	4.26	3.60
CWF (50 μg)	2.67	3.24	3.48	3.09
CWF (100 μg)	2.84	2.84	4.21	3.56

[a] Mice were injected i.v. on day 0 and i.p. on days 21, 35, and 49. They were challenged i.p. on day 56 with either 10^4 cells of *L. monocytogenes* or 1.2×10^4 cells of *S. typhimurium*. Both groups were assayed 3 days after challenge.

[b] Log_{10} of the mean number of bacteria per liver or spleen.

viewed by Shilo (40) and Thorbecke and Benacerraf (42). It has been shown that LPS obtained in an identical manner from different bacteria produced a biphasic change in resistance irrespective of its origin, but that the degree of activity varies greatly with the source of LPS and is dose-dependent (16, 35). We could not find similar biphasic activity with our CWF (unpublished data) suggesting that CWF may not have activities similar to LPS.

Although induction of resistance to *Listeria* by nonliving *Listeria* antigens has been extremely difficult to demonstrate, two recent reports concerning this problem have appeared in the literature. Bartlema and Braunius (4), working with penicillin-induced *L. monocytogenes* L-forms, found an impressive resistance against *Listeria* challenge in mice immunized i.p. with the L-form culture fluid and *Bordetella pertussis* adjuvant. Giving the L-form culture fluid alone without adjuvant as a booster provoked a very rapid reactivation of the resistance, which suggested a specific mechanism. Klasky and Pickett (17) disrupted live virulent *Listeria* cultures by passing them through a French press and then collected the cell walls by differential sucrose centrifugation. A single i.p. dose of cell wall preparation (containing 1 mg of protein) was injected into mice, followed 10 days later by i.p. challenge with 250 LD_{50} *Listeria*. These investigators reported that 95% of these mice survived challenge. They considered the immunogen to be protein in nature and to be located in the *Listeria* cell wall.

The difficulty in developing killed vaccines which induce resistance to listeric infection may be due to lability of the vaccine (unpublished data) and to two toxic mortality-enhancing factors as reported by Silverman et al. (41) and Patocka et al. (30, 31).

ACKNOWLEDGMENTS

We appreciate the skilled technical assistance of Barbara Martens and H. Rebecca Cooper. We also appreciate the help of R. S. Farr, P. Minden, and C. Schuffler in the preparation of the manuscript. Special thanks are due to A. G. Farr for making the electron micrographs.

This investigation was supported by Public Health Service research grants AI 11240, AI 08190, and Allergy Disease Center grant AI 10398, from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Alexander, P., and R. Evans. 1971. Endotoxin and double stranded RNA render macrophages cytotoxic. Nature N. Biol. **232**:76-78.
2. Armstrong, A. S., and C. P. Sword. 1964. Cellular resistance in listeriosis. J. Infect. Dis. **114**:258-264.
3. Auzins, I., and D. Rowley. 1962. On the question of specificity of cellular immunity. Aust. J. Exp. Biol. Med. Sci. **40**:283-291.
4. Bartlema, H. C., and R. Braunius. 1971. Studies on vaccination against listeria infections. Antonie van Leeuwenhoek; J. Microbiol. Serol. **37**:261-262.
5. Campbell, P. A., B. L. Martens, H. R. Cooper, and J. K. McClatchy. 1974. Requirement for bone marrow-derived cells in resistance to listeria. J. Immunol. **112**:1407-1414.
6. Collins, F. M., D. L. Boros, and K. S. Warren. 1972. The effect of *Schistosoma mansoni* infection on the response of mice to *Salmonella enteritidis* and *Listeria monocytogenes*. J. Infect. Dis. **125**:249-256.
7. Coppel, S., and G. P. Youmans. 1969. Specificity of acquired resistance produced by immunization with *Listeria monocytogenes* and listeria fractions. J. Bacteriol. **97**:121-126.
8. Coppel, S., and G. P. Youmans. 1969. Specificity of the anamnestic response produced by *Listeria monocytogenes* or *Mycobacterium tuberculosis* to challenge with *Listeria monocytogenes*. J. Bacteriol. **97**:127-133.
9. Dubos, R. J., and R. W. Schaedler. 1956. Reversible changes in the susceptibility of mice to bacterial infections. I. Changes brought about by injection of pertussis vaccine or of bacterial endotoxins. J. Exp. Med. **104**:53-65.
10. Eveleth, D. F., A. I. Goldsby, F. M. Bowlin, G. C. Holm, and J. Turn. 1953. Field trials and laboratory tests with listeria bacterins. Proc. Book Amer. Vet. Med. Ass. p. 154-155.
11. Graham, R., and N. D. Levine. 1943. Studies on listerellosis. V. A note on the use of large doses of formalin killed broth cultures in experimental prophylaxis of ovine encephalitis. J. Amer. Vet. Med. Ass. **102**:365-367.
12. Graham, R., C. C. Morrill, and N. D. Levine. 1940. Studies on listerella. IV. Unsuccessful attempts at immunization with living and dead Listerella cultures. Cornell Vet. **30**:291-298.
13. Gray, M. L., and A. H. Killinger. 1966. *Listeria monocytogenes* and listeric infections. Bacteriol. Rev. **30**:309-382.
14. Hasenclever, H. F., and W. W. Karakawa. 1957. Immunization of mice against *Listeria monocytogenes*. J. Bacteriol. **74**:584-586.
15. Howard, J. G., G. Biozzi, B. N., Halpern, C. Stiffel, and D. Mouton. 1959. The effect of *Mycobacterium tuberculosis* (BCG) infection on the resistance of mice to bacterial endotoxin and *Salmonella enteritidis* infection. Brit. J. Exp. Pathol. **40**:281-290.
16. Iida, T., and M. Tajima. 1971. Stimulation of nonspecific resistance by heterologous endotoxins and experimental immunity to *Bordetella pertussis* in mice. Immunology **21**:313-322.
17. Klasky, S., and M. J. Pickett. 1968. Immunogenicity and specificity of *Listeria monocytogenes* cell walls. J. Infect. Dis. **118**:65-75.
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. 1971. Resistance to intracellular infection. J. Infect. Dis. **123**:439-445.
22. Mackaness, G. B., and R. V. Blanden. 1967. Cellular immunity. Progr. Allergy **11**:89-140.
23. Mackaness, G. B., R. V. Blanden, and F. M. Collins. 1966. Host-parasite relations in mouse typhoid. J. Exp. Med. **124**:573-583.
24. Miki, K., and G. B. Mackaness. 1964. The passive transfer of acquired resistance to *Listeria monocytogenes*. J. Exp. Med. **120**:93-103.
25. Olafson, P. 1940. Listerella encephalitis (circling disease)

of sheep, cattle, and goats. Cornell Vet. **30**:141-150.
26. Olson, C., Jr., R. H. Cook, and V. Bagdonos. 1951. An attempt to immunize sheep during an outbreak of listeriosis. Amer. J. Vet. Res. **12**:306-313.
27. Osebold, J., A. Njoku-Obi, and J. M. Abare. 1959. Acquired resistance of sheep to *Listeria monocytogenes* and pilot studies on vaccination. Amer. J. Vet. Res. **20**:966-972.
28. Osebold, J. W., and M. T. Sawyer. 1955. Listeriosis-factors in immunity and pathogenesis. Proc. Book Amer. Vet. Med. Ass. p. 189-195.
29. Osebold, J. W., and M. T. Sawyer. 1957. Immunization studies on listeriosis in mice. J. Immunol. **78**:262-268.
30. Patocka, F., M. Mara, and J. Schindler. 1959. Studies on the pathogenicity of *Listeria monocytogenes*. II. Influence of substances isolated from cells of *Listeria monocytogenes* on experimental listerosis in white mice. Zentralbl. Bakteriol. **174**:586-593.
31. Patocka, F., J. Schindler, and M. Mara. 1959. Studies on the pathogenicity of *Listeria monocytogenes*. I. Protein substances isolated from cells of *Listeria monocytogenes* enhancing listeria infection. Zentralbl. Bakteriol. **174**:573-585.
32. Reed, L. J., and H. Muench. 1938. A simple method of estimating 50 percent endpoints. Amer. J. Hyg. **27**:493-497.
33. Remington, J. S., and T. C. Merigan. 1970. Synthetic polyanions protect mice against intracellular bacterial infection. Nature (London) **226**:361-363.
34. Rotta, J., T. J. Prendergast, W. W. Karakawa, C. K. Harmon, and R. M. Krause. 1965. Enhanced resistance of streptococcal infection induced in mice by cell wall mucopeptide. J. Exp. Med. **122**:877-890.
35. Rowley, D. 1956. Rapidly induced changes in the level of non-specific immunity in laboratory animals. Brit. J. Exp. Pathol. **37**:223-234.
36. Rowley, D., K. J. Turner, and C. R. Jenkin. 1964. The basis for immunity to mouse typhoid. I. The carrier state. Aust. J. Exp. Biol. Med. Sci. **42**:215-228.
37. 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.
38. Ruskin, J., J. McIntosh, and J. S. Remington. 1969. Studies on the mechanisms of resistance to phylogenetically diverse intracellular organisms. J. Immunol. **103**:252-259.
39. Ruskin, J., and J. S. Remington. 1971. Resistance to intracellular infection in mice immunized with toxoplasma vaccine and adjuvant. J. Reticuloendothel. Soc. **9**:465-479.
40. Shilo, M. 1959. Non-specific resistance to infections. Annu. Rev. Microbiol. **13**:255-278.
41. Silverman, S. J., L. Elwell, and D. A. Kautter. 1961. A mortality enhancing extract isolated from *Listeria monocytogenes*. J. Immunol. **86**:669-674.
42. Thorbecke, G. J., and B. Benacerraf. 1962. Reticuloendothelial system and immunological phenomenon. Progr. Allergy. **6**:559-598.
43. Turner, K. J., C. R. Jenkin, and D. Rowley. 1964. The basis for immunity to mouse typhoid. II. Antibody formation during the carrier state. Aust. J. Exp. Biol. Med. Sci. **42**:229-236.
44. Works, E. 1971. Cell walls, p. 361-418. *In* J. R. Novis and D. W. Ribbons (ed.), Methods in microbiology, vol. 5A. Academic Press Inc., New York.