The Effect of *Listeria monocytogenes* on Resistance to Pneumococcal Infection[1] (35094)

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The ability of a host infected with one agent to withstand infection by another lends insight into the basic physiology of host resistance. For this reason, studies have been carried out to investigate the effect of *Listeria monocytogenes* infection on subsequent challenge with *Diplococcus pneumoniae*. Mice inoculated with live or killed *Listeria* were found to be highly resistant to invasion by pneumococci introduced into the peritoneal cavity.

Material and Methods. Animals. Large groups (150–200) of female mice weighing 15–20 g (Charles River Laboratories) were randomized, divided into groups of 8–12/cage, and maintained on Purina Rat Chow Pellets and water, *ad libitum*.

Bacteria. A recent isolate of *Listeria monocytogenes* was passed serially in mice 8 times and maintained on eugon agar; the level of virulence remained unchanged. The LD_{50} was 10^7 organisms by the subcutaneous, and 5×10^4 by the intravenous route. In order to maintain uniformity of inoculum size in sequential studies, a 1:10 dilution of an 18-hr culture was divided into 1-ml aliquots which were stored at $-70°$. One aliquot was thawed at room temperature for use in each phase of the studies.

A single lyophilized culture of Type I *D. pneumoniae* (LD_{50} for mice, 3–7 organisms) was suspended in water, inoculated into brain heart infusion broth containing 3% horse serum (BHI-HS), and incubated for 12 hr at 37°. Aliquots were stored at $-70°$. For each experiment, 0.3 ml was thawed at 37°, inoculated into BHI-HS, and incubated for 6 hr. Serial 10-fold dilutions of the 6-hr culture were made in distilled water (pipettes were changed with each dilution), and mice were inoculated intraperitoneally with 0.1 ml of an appropriate dilution. The animals were injected in random order within a 30-min period. A sample of the inoculum was cultured immediately before and after injection.

Eighteen-hr cultures of *Listeria* and *D. pneumoniae* (10^9 viable organisms/ml of each) were killed by exposure to 0.4% formalin for 1 hr, washed 8 times and resuspended in sterile water to their original concentration. For the purpose of this paper, a killed 18-hr culture is said to contain 10^9 bacteria/ml.

The number of *Listeria* present in spleens was determined in the following manner: the organ was removed aseptically, ground in a sterile porcelain mortar, suspended in 5 ml of sterile water and exposed to sonication for 30 sec (intensity = 50, Biosonik). This degree of sonication was found not to reduce the bacterial count. Serial dilutions were made in distilled water; 0.1 ml of each dilution was spread on eugon agar for surface counting.

Results. Most mice appeared ill 2 days after subcutaneous injection of 0.1 LD_{50} *Listeria*. By the 4th day, as a rule, 85–90% had recovered; the rest were ill or dead. Cultures of spleens at daily intervals revealed that the

[1] These studies were supported by Training Grant No. 276 from the Institcte of Allergy and Infectious Diseases, National Institutes of Health, U.S. Public Health Serivice.

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TABLE I. *Listeria monocytogenes* in the Spleens of Mice Following Subcutaneous Injection of 0.1 LD_{50}.

After injection (days)	No. of organisms/spleen[a]
3	8×10^4
4	3×10^5
5	8×10^3
7	3×10^3
10	0 (<50)
14	0 (<10)
18	0 (<10)

[a] Each number is the average from 5–8 spleens.

number of *Listeria* had increased following injection, reached a maximum at 4 days and declined thereafter. No organisms were detected by the 10th day (Table I).

Four days after subcutaneous injection of 0.1 LD_{50} *Listeria*, healthy appearing animals were inoculated intraperitoneally with doses of pneumococci ranging from 3 to 18 LD_{50}. As shown in Fig. 1, there were fewer deaths in *Listeria*-treated animals than in controls following challenge with either high or low doses of pneumococci. Data from several experiments were combined and survival rates at 96 hr were calculated: 65% of *Listeria*-infected animals survived compared to only 36% of controls. This difference was highly significant ($x^2 = 15.8, p < .0001$). The size of the inoculum of *Listeria* determined

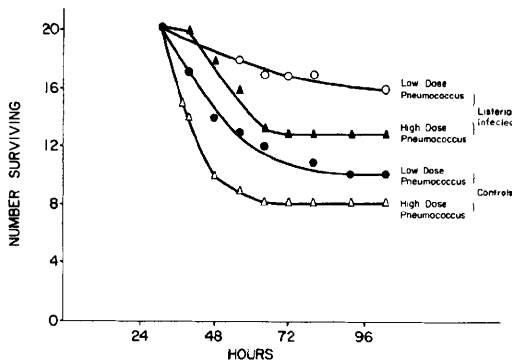

FIG. 1. Mice infected with *Listeria* 4 days previously and controls received 3 LD_{50} (low dose) and 18 LD_{50} (high dose) pneumococci intraperitoneally at time 0. Curves show number surviving in each group by time.

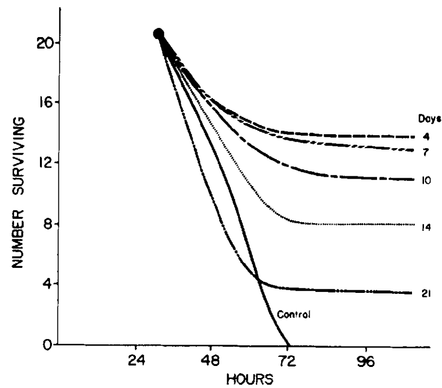

FIG. 2. Mice given 0.1 LD_{50} *Listeria* subcutaneously 4, 7, 10, 14, and 21 days previously, and controls were injected intraperitoneally with 7 LD_{50} pneumococci at time 0. Curves show number surviving by time.

the degree of protection against pneumococci. A dose of 0.01 LD_{50} was less protective than 0.1 LD_{50}, while 0.001 LD_{50} conferred no protection at all.

The effect of varying the interval between injection of *Listeria* and challenge with pneumococci was evaluated as follows: Five groups of 14 mice were given 0.1 LD_{50} *Listeria* subcutaneously 21, 14, 10, 7, and 4 days before inoculation with 7 LD_{50} pneumococci. A control group received no *Listeria*. Figure 2 shows that protection was greatest when the interval between injection of the organisms was 4 and 7 days, less when it was 10 or 14 days and least when it was 21 days.

Protection against pneumococcal infection thus appeared to be greatest 4 days after inoculation with *Listeria*, at a time when the number of *Listeria* in the spleen was highest (Table I). The relation between the numbers of viable *Listeria* in the spleen and resistance to pneumococcal disease was examined in the following manner: Mice were given 0.01 LD_{50} *Listeria* subcutaneously in the thigh. All the animals survived. Four days later, they were inoculated with 5–10 LD_{50} *Listeria* by the same route in the opposite thigh. Ninty percent survied. Bacterial counts in spleens are listed in Table II. After the second injection there was a progressive decline in the number of *Listeria*. By the 4th

TABLE II. *Listeria monocytogenes* in the Spleens of Mice Given 0.1 LD_{50} on Day 0 Followed by 10 LD_{50} on Day 4.[a]

Days after		
0.1 LD_{50}	10 LD_{50}	No. of organisms/spleen[b]
4	0	4×10^4
4.5	0.5	3×10^3
5	1	8×10^2
6	2	1×10^3
7	3	5×10^2
8	4	8×10^1 [c]
9	5	0 (<50)
10	6	0 (<50)

[a] Both injections were given subcutaneously.

[b] Each number is the average from 5–8 spleens.

[c] Organisms were recovered from only 1 of 8 spleens.

day organisms could be recovered from the spleen of only 1, and by the 5th day from none of 8 animals. Pneumococci, 12 LD_{50}, were given to each of 3 groups of mice: Group I received 0.01 LD_{50}, *Listeria* in one thigh followed 4 days later by 5–10 LD_{50} *Listeria* in the opposite thigh. These 2 doses of *Listeria* were given 8 and 4 days, respectively, before pneumococcal challenge. Group II were given a single subcutaneous dose of 0.1 LD_{50} *Listeria* 4 days before challenge. Group III served as controls. As shown in Fig. 3, the fatality rate in Group I was substantially lower than in Groups II or III. It

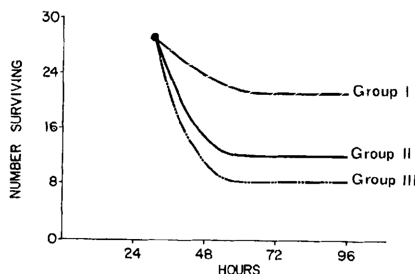

FIG. 3. (Group I) Mice given 0.01 LD_{50} *Listeria* subcutaneously followed 4 days later by 5 LD_{50} *Listeria* and inoculated intraperitoneally with 12 LD_{50} pneumococci 4 days after the second injection of *Listeria*; (Group II) mice given 0.1 LD_{50} *Listeria* 4 days before 12 LD_{50} pneumococci; (Group III) controls, given 12 LD_{50} pneumococci. Curves show number surviving by time.

is striking that the greatest degree of resistance was observed in those animals which had virtually eliminated viable *Listeria* from their spleen by the time of injection with pneumococci.

The presence of viable *Listeria* thus appeared to be unnecessary for increased resistance to pneumococcal infection. Studies were, therefore, undertaken to determine the effects of treatment with killed *Listeria*. Injection of 10^8 dead organisms for 4 consecutive days prior to pneumococcal challenge did not enhance resistance. Single doses of killed *Listeria* were only partially protective when given 7, 10, or 14, but not 21 days before pneumococci. When dead *Listeria* were injected twice, 14 and 7 days before pneumococci, a greater degree of protection was noted. However, animals which received 10^8 and 10^6 *Listeria* 30 days apart were not resistant to pneumococci inoculated 5 days after the second dose. In contrast, mice given a single injection of 10^8 killed pneumococci or 0.1 ml of supernatant from an 18-hr culture of this organism, at any time 4 to 30 days previously, were uniformly resistant to 10–15 LD_{50} pneumococci.

The greatest degree of protection against pneumococcal infection by pretreatment with *Listeria* was produced by a single injection of 10^8 killed organisms in Freund's incomplete adjuvant 10 days earlier. Eighty-three percent of 65 mice treated in this fashion survived, compared to 16% of 62 animals which received incomplete Freund's adjuvant alone ($x^2 = 57, p < .0001$).

Protective cross-antigenicity between *D. pneumoniae* (Type I) and *L. monocytogenes* could not be demonstrated. Serum from mice immunized with live or killed *Listeria* did not agglutinate pneumococci or cause capsular swelling. Mice were injected intravenously with serum (0.2 ml) obtained from animals that had received 10^8 killed pneumococci or *Listeria* in adjuvant 10 days previously. Control animals received 0.2 ml of serum from normal mice or 0.2 ml of normal saline. All mice were challenged with 5–10 LD_{50} pneumococci 1–2 hr later. There were no deaths in mice given antipneumococcal serum. In

those given anti-*Listeria* or normal mouse serum the fatality rates were 53% and 49%, respectively, compared to 80% in those receiving normal saline.

Discussion. Infection with *Listeria* has been shown to protect mice against infection by *Brucella* and some strains of *Salmonella* (1). This protection has been related to the ability of each of these organisms to survive and multiply within cells (2). Blanden *et al.* (3) have shown that macrophages from mice infected with *Listeria* are more resistant to infection with other intracellullar organisms. This enhanced marophage resistance was shown to be nonspecific, but it could be recalled by specific immunologic stimulation (4). Dubos and Schaedler (5) demonstrated that infection by staphylococci protected mice to some extent against *M. tuberculosis.* Although they were unable to explain the mechanism for this resistance it may also have been related to macrophage function. Our studies have demonstrated that intracellular infection with *L. monocytogenes* or intense antigenic stimulation with killed *Listeria* rendered mice resistant to infection with the prototypic extracellular organism, *D. pneumoniae.*

The protection against pneumococcal infection resulting from treatment with *Listeria* did not appear to be related to the presence of antibody. Although cross-reaction between these organisms has been demonstrated (6), the common antigen does not appear to be related to the pneumococcal polysaccharide and thus is not likely to confer protection against the pneumococcus. Moreover, the pattern of protection that we observed was not consistent with the action of specific antibody for several reasons: (a) Live or killed *Listeria* injected 4 to 14 days before pneumococcal challenge was partially protective but *Listeria* injected 21 days before pneumococci gave no protection. A single injection of dead pneumococci uniformly protected mice against pneumococci given 4 or more days later. (b) Two injections of killed *Listeria* given 30 days apart in order to elicit high levels of antibody had no effect on survival. (c) Treating mice with large doses of killed *Listeria* given daily for 4 days to simulate the antigenic stimulus of a 4-day infection failed to protect them against pneumococcal challenge. (d) Serum obtained from mice injected with live or killed *Listeria* at a time when they were resistant to pneumocococcal infection did not agglutinate pneumococci or cause capsular swelling and failed to confer immunity when injected into susceptible animals.

Instead, our data suggest that the protection we have observed may be attributable to enhanced macrophage function. Although polymorphonuclear leukocytes probably destroy the majority of pneumococci following injection, especially in the presence of specific antibody, macrophages have also been shown to play a role in this phenomenon (6). In our studies, both the duration and the time of onset of the observed resistance are consistent with those seen in studies of nonspecific cellular immunity (1, 3). The fact that infection with *Listeria* elicited this resistance while simple antigenic stimulation with killed organisms did not, gives further support to this hypothesis. Increased activity of these cells following *Listeria* infection has been found to be important in defense against invasion by other intracellular organisms (1, 2). It is conceivable, therefore, that enhanced macrophage function may also be associated with increased resistance to infection by extracellular organisms.

Summary. Listeria infection has been shown to increase resistance of mice to pneumococcal challenge. This protection does not appear to be related to presence of circulating antibody. Enhancement of macrophage function is proposed as an explanation for the observed phenomenon.

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Received June 15, 1970. P.S.E.B.M., 1970, Vol. 135.