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Listeria Pneumonitis: Induction of Immunity After Airborne Infection with *Listeria monocytogenes*

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After implantation of approximately 10^3 *Listeria monocytogenes* organisms into the lungs, mice develop an acute pneumonitis with dissemination of infection to a mediastinal lymph node (MedLN), liver, and spleen. The infections in a MedLN and spleen resolve in approximately 7 days, but the lung infection persists for a few days longer. Pneumonitis is accompanied by a lymphoproliferative response in a MedLN and spleen, and immunity to *Listeria* is conferred adoptively with MedLN and spleen cells but not with mesenteric lymph node cells. Although the spleen appears to be the major repository of sensitized lymphocytes, splenectomized mice combat *Listeria* pneumonitis as effectively as normal mice. It is concluded that the induction of immunity to lung infection with *L. monocytogenes* is efficient and that the cause for the rather protracted pneumonitis is due to a defect in the expression of the cell-mediated immunity effector mechanism.

Listeria monocytogenes is a facultative intracellular parasite that produces, in experimental animals, acute infections controlled by the host through the development of specific cell-mediated immunity. In recent years, few infections have been studied from the immunological standpoint as extensively as experimental listeriosis, and the induction and expression of immunity to this parasite after intravenous or subcutaneous infection have been well characterized (5-8, 11, 12). *L. monocytogenes* also causes an acute pneumonitis after inhalation into lungs, but the host response to such infection is not as well documented (9).

After intravenous (i.v.) or subcutaneous inoculation, *L. monocytogenes* causes a systemic infection, but the organisms do not localize in the lungs, unlike infections with *Mycobacterium tuberculosis*. However, when *L. monocytogenes* is introduced directly into the lungs, it causes an acute pneumonitis, which resolves more slowly than intravenous infection (16). The reason for the more protracted course of *Listeria* infection in lungs remains obscure. It is unclear whether the defect lies in induction or expression of the immune response. A defect in the induction of immunity would result in either a delay in the generation of specifically sensitized lymphocytes or a reduction in their number. On the other hand, a defect in the expression of immunity might arise from impaired migration of sensitized lymphocytes (or monocytes) into the lung tissue or suboptimal microbicidal activity of lung macrophages.

As a first stage of investigating this problem, the induction of immunity after airborne infection with *L. monocytogenes* is reported here.

MATERIALS AND METHODS

Animals. Male or female mice of the B6D2 (C57BL/6 × DBA/2)F_1 inbred strain were used at 6 weeks of age.

Culture. A strain of *L. monocytogenes* (Lm) that is virulent to mice was cultured in Trypticase soy broth, distributed into vials, and stored at −70°C. There was no loss of viability as a result of freezing, and the viability and virulence of the cultures remained stable at −70°C. Immediately before use, a vial was thawed and appropriately diluted in sterile saline.

Aerosol infection. Lm culture was diluted to contain 5×10^8 viable units in a 10-ml volume. This suspension was placed in the nebulizer of a Middlebrook Airborne Infection Apparatus (Tri-R Instruments, Rockville Centre, N.Y.) containing the mice to be infected. The bacterial suspension was nebulized for 30 min, followed by an aerosol decay period of 30 min. The apparatus generates droplet nuclei of 1- to 2-μm diameter, and this procedure implanted 1×10^3 to 5×10^3 viable organisms into the lungs.

Intravenous challenge. Lm culture was diluted to 1×10^5 viable units per ml, and 0.2 ml (2×10^4 viable organisms) was injected into the lateral tail vein.

Radiometry. Tritiated thymidine ([^{3}H]thymidine; radioactive concentration, 5 Ci/mmol [Amersham/Searle]), was diluted in sterile saline to a concentration of 100 μCi/ml. A 1-μCi/g-of-body-weight dose was injected subcutaneously into each mouse. One hour later the mice were killed, and the mediastinal lymph node (MedLN) and spleen were removed. The MedLNs were digested in NCS solubilizer (Amersham/Searle). The spleens were homogenized in 5% cold trichloroacetic acid. The precipitate was washed once in 5% cold trichloroacetic acid and extracted in

5% trichloroacetic acid at 90°C, and the radioactivity of the supernatant was determined. Scintillation fluid was added to the MedLN digests and spleen extracts, and radioactivity was measured in a liquid scintillation counter.

Cell transfers. MedLN, mesenteric lymph nodes (MesLN), and spleens were teased apart in phosphate-buffered saline and passed through stainless-steel gauze sieve. The spleen cells were washed once in ammonium chloride-tris(hydroxymethyl)aminomethane buffer to lyse erythrocytes (1) and were again suspended in phosphate-buffered saline. Total lymphoid cell counts were made, and cell viability was estimated by trypan blue exclusion. The cells were injected i.v. in an appropriate volume of phosphate-buffered saline. Cells were transferred on the basis either of donor equivalents or absolute numbers of cells. A donor equivalent is that number of cells obtained from the spleen, MedLN, or MesLN of a single donor.

Assay of adoptive immunity. After cell transfer, groups of five mice were challenged with 2×10^4 Lm i.v. Forty-eight hours later, the mice were killed and viable counts of Lm were made from the spleens.

Viable counts. Each MedLN, spleen, and pair of lungs was homogenized in sterile saline by using a Teflon pestle-glass tube, motor-driven homogenizer. Appropriate dilutions of the homogenates were plated on Trypticase soy agar and incubated at 37°C for 24 h, after which bacterial colonies were counted.

Splenectomy. Mice were anesthetized with ether, and the skin and peritoneum were opened with a left subcostal incision. Each splenic pedicle was ligatured; the spleen was excised; and the skin was closed with Michel clips. In the case of sham-splenectomized controls, the skin and peritoneum were incised and closed, but the spleen was not disturbed.

Statistics. The geometric mean viable counts of Lm from each group of five mice was estimated. Group means were compared by analysis of variance and the Newman-Keuls modification of the Q test (14). The data is expressed either as absolute geometric mean viable counts or as "resistance to *Listeria*." The latter was estimated by subtracting the geometric mean viable count of a test group from that of the control group.

RESULTS

Course of lung infection with Lm. In preliminary experiments, differing numbers of viable *Listeria* were implanted in the lungs, and the course of infection was followed by counting the viable Lm in the lungs, spleen, and intrathoracic lymph nodes. It was found that an implantation of 5×10^3 Lm or more into the lungs killed a variable proportion of mice. Consequently, in all the reported experiments the implanted inoculum was 1×10^3 to 3×10^3 organisms. It was also observed that after lung infection one intrathoracic lymph node consistently underwent enlargement and became infected with Lm. This node lies laterally to the junction of the trachea with the right bronchus and is designated in this text as the MedLN. Other intrathoracic lymph nodes were not usually enlarged or infected as a result of *Listeria* pneumonitis, and their possible contribution to the immune response has been disregarded.

Approximately 3×10^3 Lm were implanted in the lungs, and the number of viable organisms in the lungs, MedLN, and spleen were monitored for 14 days (Fig. 1). Lm grew rapidly in the lungs for 24 to 48 h, after which the number of viable organisms was sustained at approximately 10^6 until days 6 to 8. From that time, the organisms were rapidly eliminated, and the lungs were virtually sterile by day 14. Small numbers of viable *Listeria* were found in the MedLN and spleen 24 h after infection, and numbers increased rapidly during the next 24 h. It is notable that the phase of rapid elimination of *Listeria* from the MedLN and spleen was initiated on days 4 to 5, predating analogous events in the lungs by 3 to 4 days.

Lymphoproliferation in the MedLN and spleen. At each time point in the experiment illustrated in Fig. 1, the proliferative responses in the MedLN and spleen were measured. The kinetics of the response in the two organs were closely similar (Fig. 2), commencing on day 4, peaking at days 5 to 6, and then subsiding rapidly. Note that the scales on the two ordinates of Fig. 2 differ, so the magnitude of DNA synthesis in the spleen greatly exceeded that in the MedLN. Thus, the peak incorporation of [^{3}H]thymidine was 3.25×10^6 cpm per spleen as compared to 6.6×10^3 cpm per MedLN, a 500-fold difference.

Transfer of adoptive immunity. Figure 2

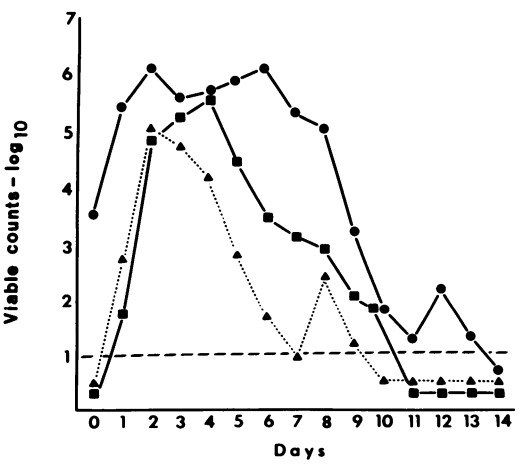

FIG. 1. *Growth of Lm in normal mice after inhalation into the lungs. Symbols: ●, lungs; ▲, MedLN; □, spleen. The horizontal broken line denotes the lower limit of the viable count procedure.*

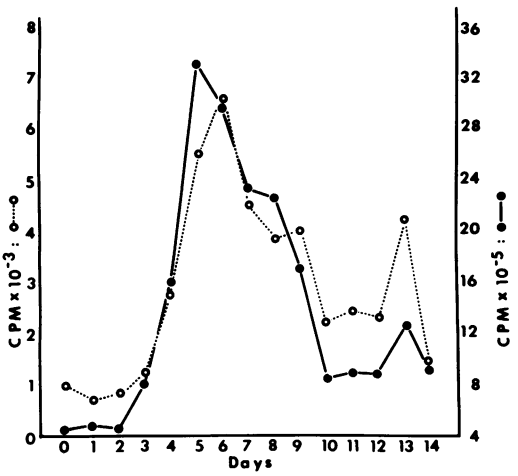

FIG. 2. *Lymphoproliferation in the MedLN* (○) *and spleen* (●) *during the course of pulmonary infection with Lm.*

suggested that lymphocytes responsive to Lm were generated in the MedLN and spleen. Accordingly, an attempt was made to confer immunity to Lm upon syngeneic recipients by transfer of MedLN and spleen cells.

Donor mice were immunized by implantation of approximately 10^3 viable *Listeria* into the lungs. At 2-day intervals for 10 days, MedLN and spleen cells were obtained from immunized mice and injected i.v. into groups of five recipient mice, which were then challenged with 2×10^4 Lm i.v. Viable counts of Lm in the spleens of recipient mice were obtained 48 h after challenge (Table 1). Recipient mice received either no lymphoid cells (controls), five donor MedLN equivalents (2×10^7 to 4×10^7 cells), or one donor spleen equivalent (6×10^7 to 21×10^7 cells). The viability of the cells did not vary materially from day to day, being approximately 80% for both MedLN and spleen cells.

A modest level of protection was conferred by MedLN cells from day 4 onward. The differences, although small, were significant ($P < 0.01$) due to the low intragroup variation, as shown by the standard error of the means (Table 1, column 6). A much higher level of protection was conveyed by spleen cells, from days 6 to 10, but it should be kept in mind that much larger numbers of spleen cells than MedLN cells were transferred. It is notable that the earliest measurable protection was conferred by lymphoid cells obtained on the day on which DNA synthesis in the corresponding lymphoid organ was maximal: day 4 for MedLN and day 6 for spleen cells (Fig. 2). Day 6 was also the time at which the spleen became greatly enlarged, as indicated by the lymphoid cell yield (Table 1). The MedLN of aerosol-infected mice were also swollen, but this is not evident from Table 1, because enlargement occurred as early as day 2. Table 1 also shows that some lymphoid cell populations significantly enhanced *Listeria* infection. Such results are commonly observed when nonprotective or normal cells are transferred.

Comparison of efficacy of MedLN and spleen cells. In the previous experiment, spleen cells appeared to convey much more protection than MedLN cells, on the basis of donor organ equivalents. However, since one donor equivalent of spleen contained approximately 25 times as many cells as one donor equivalent of MedLN, it was uncertain whether spleen cells were superior to MedLN cells on a cell-for-cell basis. An experiment was designed to elucidate this point.

Donor mice were infected with 10^3 Lm into the lungs. MedLN, spleen, and MesLN cells were obtained 7 days later and were injected i.v. into groups of recipients which were then challenged with 2×10^4 Lm i.v. Recipients received either no cells (controls), five donor equivalents of MedLN, 2 donor equivalents of MesLN, or 1/2, 1/4, 1/8, or 1/16 donor spleen equivalents. The results are shown in Fig. 3, in which resistance to Lm ($\log_{10}$) is plotted against the number of cells transferred ($\log_{10}$). A straight line was fitted to the spleen cell data by the method of least squares and extrapolated to zero cells. The results obtained with MedLN and MesLN cells are also plotted in Fig. 3, from which it is clear that spleen and MedLN cells are closely similar in protective ability when compared on a numerical basis. It is noteworthy that MesLN cells were completely inert in this assay.

Immunity in splenectomized mice. The above experiments indicate that, although immune spleen cells are no more protective than

TABLE 1. *Transfer of immunity to Lm with MedLN and spleen cells at intervals after pulmonary infection*

Day after immunizing infection	No. of cells transferred		Resistance to *L. monocytogenes* ($\log_{10}$) in recipients of:		
	MedLN	Spleen	MedLN	Spleen	SE[a]
2	3.4×10^7	8.8×10^7	−0.59[b]	−0.04	0.1486
4	3.0×10^7	6.0×10^7	0.42[b]	−0.50[b]	0.1198
6	2.2×10^7	1.5×10^8	0.81[c]	3.38[c]	0.1498
8	2.7×10^7	2.1×10^8	0.85[c]	2.00[c]	0.1238
10	3.9×10^7	1.9×10^8	0.67[b]	3.10[c]	0.2021

[a] Standard error of any group mean.
[b] $P < 0.05$ compared with mean from control mice that received no cells.
[c] $P < 0.01$ compared with mean from control mice that received no cells.

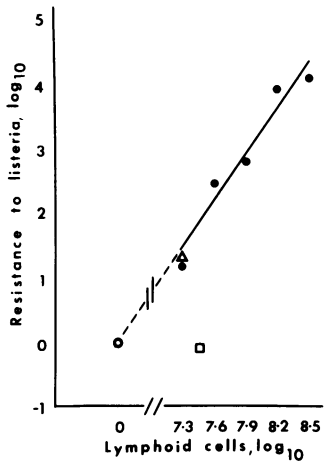

FIG. 3. *Adoptive immunity to Lm transferred by graded doses of spleen cells (●) and single doses of MedLN (△) and MesLN (□) cells. Normal controls (○).*

MedLN cells on a cell-for-cell basis, the spleen is the major repository of sensitized lymphocytes, by virtue of its size. Moreover, the course of pulmonary listeriosis (Fig. 1) suggests that elimination of the organisms from the lungs does not occur until a lymphoproliferative response is generated in the spleen, and the infection is first controlled in the latter organ. Consequently, it was thought that splenectomized mice might be less able to combat a pulmonary infection.

Accordingly, mice were either splenectomized or sham-splenectomized. One week later these animals were infected with Lm into the lungs, and the course of the infection was followed in the lungs and MedLN of both groups of mice, in the combined liver and spleen of sham-splenectomized mice, and in the liver of splenectomized mice (Fig. 4). The course of infection was closely similar in sham-splenectomized and splenectomized mice, and such differences as are apparent are not statistically significant.

Transfer of adoptive immunity with cells from splenectomized mice. The preceding experiment suggested that either the events in the spleen were irrelevant to the control of *Listeria* pneumonitis, or that the absence of the spleen was counterbalanced by increased numbers of sensitized lymphocytes in the MedLN and/or other lymphoid organs. These alternatives were examined in the following experiment.

Mice were either splenectomized or sham-splenectomized and were infected 1 week later with airborne *Listeria*. On the day 7 after infection, MedLN and MesLN cells were obtained from both groups of infected mice, and spleen cells were obtained from sham-splenectomized mice. Recipient mice were given either no cells, five donor equivalents of MedLN cells, two donor equivalents of MesLN cells, or one donor equivalent of spleen cells i.v. and were then challenged with Lm by the same route. Viable *Listeria* were enumerated in the spleens of recipient mice 48 h after challenge (Table 2).

The yield of cells from the MedLN and MesLN of splenectomized mice was no greater

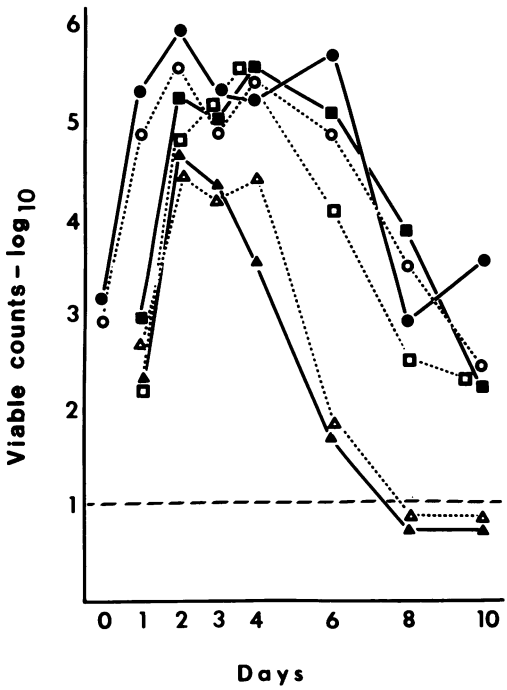

FIG. 4. *Course of airborne Listeria infection in sham-splenectomized (open symbols) and splenectomized (closed symbols) mice. Symbols: ○, lungs; △, MedLN; ■, liver; □, liver and spleen combined.*

TABLE 2. *Effect of splenectomy on transfer of immunity to Lm with MedLN and MesLN cells*

Donor	Cell type	No. of cells	Viable Lm (log_{10}) in spleen of recipient
None	None	0	6.54
Splenectomized	MedLN	3.9×10^7	4.21[a]
Sham-splenectomized	MedLN	4.2×10^7	4.11[a]
Splenectomized	MesLN	2.7×10^7	6.44
Sham-splenectomized	MesLN	2.9×10^7	6.30
Sham-splenectomized	Spleen	1.6×10^8	2.66[a]

[a] Differs significantly ($P < 0.01$) from counts obtained from control mice.

than that obtained from sham-splenectomized animals, showing that there was no compensatory hypertrophy of these lymphoid organs in the former group. Moreover, the protective capacity of the MedLN cells was similar regardless of the type of donor. The MesLN cells of both groups of mice failed to transfer protection, indicating that the absence of the spleen was not compensated by the diversion of sensitized lymphocytes to a lymphoid organ, the MesLN, in which they were not normally present. As expected, the spleen cells from sham-splenectomized mice were highly protective.

DISCUSSION

The predilection of *M. tuberculosis* to localize in lungs and produce progressive disease is well known and has long been a source of interest (3, 4, 9, 13). There appear to be two alternative explanations of this phenomenon: that it is peculiar to *M. tuberculosis,* which finds the lung an unusually suitable environment for replication; or that there is a failure of the host to induce or express its immune response in the lungs. The observation that Lm also produces a relatively protracted infection when implanted into the lungs was important in that it suggested that host factors may be as much to blame for the chronicity of some lung infections as the supposed peculiarities of the pathogen (16).

As a first step in determining what component of the immune response was at fault, the induction of immunity to Lm has been studied in mice infected by inhalation. It has been found that the course of lung infection was closely similar to that described previously (16). In addition, it has been established that infection spreads to the draining MedLN and subsequently to the spleen, presumably via blood. Although the infection reaches the MedLN and spleen later than the lungs, the bacilli are eliminated from these lymphoid organs more rapidly than they are from the lungs.

After i.v. and subcutaneous infection, the onset of killing of *Listeria* is temporally associated with maximum lymphoproliferation in the appropriate lymphoid organ (12). In this study, the association between DNA synthesis in the MedLN and spleen and bacterial killing in the spleen was good. However, the reduction of viable organisms in the MedLN predated measurable lymphoproliferation, and the elimination of *Listeria* from the lungs lagged considerably behind DNA synthesis in the lymphoid organs.

When MedLN and spleen cells were used to transfer immunity adoptively, little protection was conferred by cells obtained before days 4 to 6, the days when DNA synthesis in the lymphoid organs was high. However, cells obtained on day 10, when lymphoproliferation had declined to almost normal levels, were also protective. This result suggests that not only immunoblasts convey immunity but also their nonreplicating progeny. The spleen contained a larger number of protective, sensitized lymphocytes than the MedLN but, on a cell-for-cell basis, lymphocytes from these organs were closely similar in activity. In contrast, MesLN cells were totally unprotective. These observations are consistent with earlier studies which have shown that the lymphocytes that mediate anti-*Listeria* immunity are non-recirculating cells that would not be expected to occur in lymphoid tissue which is neither the site of *Listeria* infection nor drains such a site (5). The ability of MedLN and spleen cells from mice exposed to Lm aerosol to convey immunity within 6 to 7 days of infection indicates that the induction of immunity via the lungs is as efficient as that induced by i.v. or subcutaneous infection (5, 12).

There was strong indirect evidence that the spleen was involved in the control of *Listeria* pneumonitis. The spleen was the major source of newly formed, sensitized cells, and elimination of *Listeria* from the lungs did not occur until the organism was substantially eliminated from the spleen. However, the course of *Listeria* pneumonitis in splenectomized mice did not materially differ from that in sham-splenectomized controls. More interesting is the observation that, in the absence of the spleen, the MedLNs were neither compensatorily enlarged nor enriched with sensitized cells. Neither did the MesLN assume the functions of the absent spleen. These findings indicate that the sensitized cells that were generated in the MedLN and possibly other, less conspicuous, intrathoracic, draining lymph nodes were sufficient to combat infection and that the cellular proliferation in the spleen was superfluous.

The efficiency with which cell-mediated immunity was induced after airborne infection is consistent with other investigations in which animals have been immunized via the lower respiratory tract. These studies were concerned in part with humoral immunity, but cell-mediated immunity, as measured by cells that produce migration inhibitory factor, can also be generated after pulmonary immunization (2, 10, 15, 17).

The course of *Listeria* pneumonitis suggests that both nonspecific and specific factors play roles in the control of the infection. After implantation of Lm into the lungs, the organisms multiply rapidly for 1 to 2 days, and then their numbers remain fairly stable for 6 to 7 days.

During the latter period, the multiplication of Lm is contained, but an effective bactericidal mechanism appears to be lacking. Analogous events occur in the MedLN and spleen, but the period of bacterial containment is shorter, approximately 2 days. This initial containment of infection occurs before the generation of sensitized lymphocytes and is probably nonspecific in nature. This type of resistance appears to be local in distribution, since there was rapid growth of Lm in the MedLN and spleen on days 1 to 2, when the growth of the organism in the lungs was inhibited. This state of bacterial containment was succeeded by rapid killing of Lm in the MedLN and spleen that coincided with cellular proliferation in those organs. It is concluded that the generation of specifically sensitized lymphocytes mediated this more effective antibacterial mechanism. Compared to the MedLN and spleen, there was a delay of 3 to 4 days before Lm was eliminated from the lungs. One possible reason for this is that the migration of sensitized lymphocytes from the lymphoid organs to the lungs may take several days, a hypothesis that is now being investigated.

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