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FACTORS INFLUENCING THE GROWTH OF LISTERIA MONOCYTOGENES IN THE ORGANS OF MICE

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ABSTRACT

The growth of Listeria monocytogenes in the lungs, liver, spleen, brain, and blood of mice was followed after infection by the respiratory, intravenous, and intraperitoneal routes. The lungs of mice exposed to Listeria aerosols contained about 10^6 cells per cc within 24 hours and the number of organisms remained high for at least 9 days. After exposure by the other two routes, fewer organisms appeared in the lungs. The colony counts of spleen and liver homogenates were similar regardless of the route. Organisms were found sporadically in the brain and blood. When a cell extract of Listeria was injected simultaneously with the organisms, greater numbers of Listeria were found in spleen and liver than when the bacteria were injected alone. On the other hand, the BCG strain of Mycobacterium tuberculosis enhanced the resistance of the mice as shown by the recovery of fewer organisms from the tissues when compared with the tissues of mice receiving L. Monocytogenes alone. Prior administration of BCG also reversed the effect of the Listeria extract.

Five- to six-week-old mice born of Listeria-infected mothers were infected by the intraperitoneal route with the homologous organism. When they were compared with infected progeny of healthy females, fewer organisms were recovered and the gross pathology was less extensive. However, there was no difference in the LT_{50} (time to death for 50 per cent of the animals) between the two groups. In a similar experiment, using seven- to eight-week-old mice born of infected mothers, there was no difference in the bacterial counts, gross pathology, or LT_{50} between the two groups.

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I. INTRODUCTION

Although the pathology of listeriosis has been studied by numerous authors,[1] the dissemination of the organism throughout the host has been observed only indirectly. In a recent report, Kautter *et al*[2] showed that the results of virulence titrations of *Listeria* strains differed when the route of infection was varied. Although exposure by the respiratory route resulted in a reproducible, linear dose response, titrations performed via the intravenous or intraperitoneal routes gave erratic, nonlinear results.

Silverman, Elwell, and Kautter[3] observed that injection of a cell extract of *Listeria*, the so-called mortality-enhancing factor (MEF), reduced the susceptibility of mice and other animals to *Listeria* as well as to other microorganisms. Mycobacterium tuberculosis, BCG, on the other hand, increased the resistance of mice to listerial infection and overcame the effect of MEF.

Because of these observations, it was considered desirable to determine the effect of the route of infection and the previous history of the host on the growth of *Listeria* in the various organs known to be the loci of infection.

II. MATERIALS AND METHODS

Listeria monocytogenes, strain A4413, isolated at the Communicable Disease Center, Atlanta, from a fatal infection in a child, and strain JHH isolated from a case of listerial meningitis at The Johns Hopkins Hospital were used. When injected into Swiss-Webster mice by the intraperitoneal route, 4.8×10^2 cells of strain A4413 and 1.4×10^5 cells of strain JHH were required to kill 50 per cent of the mice within 15 days (LD_{50}). By the respiratory route, the LD_{50} for A4413 was 3.5×10^3 cells and that for JHH was 2.1×10^4 cells. Brain heart infusion broth (Difco) was inoculated with the growth from an eight-hour tryptose agar slant and incubated at 37° C for 16 to 18 hours on a reciprocating shaker (4- to 5-inch stroke, 100 excursions per minute). Suitable dilutions were prepared in one per cent tryptose solution; the bacterial count of the inoculum was determined by quantitation plating on tryptose agar.

Swiss-Webster mice from the Fort Detrick colony were used. Animals were maintained and used in compliance with the principles established by the National Society for Medical Research.[4] Intravenous injections were performed via the caudal tail vein; 0.1 ml contained the challenge dose. Respiratory exposure was made in the modified Henderson apparatus according to the procedures described by Roessler and Kautter.[5] Two-tenths ml was the usual volume injected for intraperitoneal challenge. Animals were sacrificed at intervals after exposure by anesthesia with either chloroform or in an atmosphere of CO_2. Blood was obtained from the jugular vein and cultured in brain heart infusion broth; serial solutions of the blood were prepared in this medium to determine the highest dilution at which growth would occur.

The spleens, livers, lungs, and brains were removed aseptically and the volume of each was determined by displacement. In some experiments, similar organs from the animals sacrificed at the same time were pooled. In most cases, however, each organ was cultured separately. The tissues were ground aseptically with a glass rod and sand in a thick-walled tube. The tissue homogenate was diluted with 1.0 per cent tryptose and plated on tryptose agar plates by the method of Miles and Misra.[6] Two-hundredths ml of the dilution was dropped on the surface of dried tryptose agar from a 0.2-ml pipette graduated in 0.01 ml. After the drop was absorbed into the agar, the plates were inverted and incubated for 18 to 24 hours. Each dilution was replicated six times and the results were based on the mean count of the drops containing 10 to 40 colonies. The results were expressed as the number of organisms per cc of tissue. In some experiments, when the mean weight of the organ was determined, the results were expressed as number of colonies per milligram. When individual organs were cultured, the results were expressed as the geometric mean of counts obtained with three to five specimens.

When the effect of MEF of *Listeria* was studied, two ED_{50}[3] were injected simultaneously with the infecting organism.

Mycobacterium tuberculosis, BCG, was grown for seven to ten days in Dubos' medium. The cells were centrifuged, washed once with saline, and from 10^5 to 5×10^5 viable units were injected into the peritoneal cavity four to seven days prior to infection with Listeria.

The LT_{50} was calculated by the method of Litchfield.[7]

III. RESULTS

Two-, three-, and four-week-old mice were infected by the intravenous route with approximately 400 to 1600 cells of strain A4413. Groups of three animals were sacrificed at 24-hour intervals and similar organs from the three animals were pooled for study. No difference was noted in the response of the three age groups and this factor was not considered in the remainder of the study. The Listeria count in the lungs remained quite low (Table I) but was not reliable quantitatively because of the heavy growth of other organisms in the low dilutions plated. However, few or no colonies of Listeria were present even on plates without contaminants.

Listeria, strain A4413, multiplied more rapidly in the lungs following exposure by the respiratory route than following intraperitoneal injection. In replicate experiments the maximal colony count following intraperitoneal injections ranged from 3.0×10^4 to 2.5×10^6 per cc and appeared between the first and sixth days. After respiratory exposure with this strain, the colony counts one day after exposure reached 1.9×10^6 to 3.7×10^6 per cc and persisted at a high level for the duration of the experiment. The maximal count (1.9×10^8) was reached at about five to six days and was greater than that observed following exposure by either the intravenous or intraperitoneal route.

The colony counts of Listeria obtained with homogenates of spleen or liver of infected mice were similar regardless of the route of infection within the wide range observed among the individual animals (Table I).

The data in Table II indicate that less variation was observed in the colony counts of tissues from mice exposed by the respiratory route than in those obtained from tissue of animals that were challenged by the intraperitoneal route. The standard deviation from the mean ranged from 1.0 to 34.8 colonies with cultures obtained from animals exposed by the respiratory route. With cultures of organs from mice exposed by the intraperitoneal route, the standard deviation was as high as 632.

In the experiments in which the mice were challenged via the intraperitoneal route, the Listeria counts in the organs of those mice receiving 1.5×10^5 cells of strain A4413 reached a maximum within one to three days (Table III). The maximal counts in the organs of mice receiving smaller doses (2.4×10^2 or 1.1×10^3 cells) did not occur until the fifth or sixth day. There was some suggestion that the lower challenge doses

TABLE I. THE DISTRIBUTION OF LISTERIA MONOCYTOGENES A4413 IN THE ORGANS OF MICE INFECTED BY VARIOUS ROUTES

Route of exposure [a]	Colony count per cc tissue (geometric mean)								
	Day								
	1	2	3	4	5	6	7	8	9
Lung									
Respiratory	$2.8x10^6$	$1.7x10^6$	$1.2x10^7$	$3.1x10^7$	$1.9x10^8$	$9.8x10^7$	$8.7x10^6$	$2.4x10^5$	$8.9x10^6$
Intraperitoneal	$6.2x10^2$	$9.0x10^5$	$1.4x10^5$	$2.2x10^4$	$1.8x10^4$	$9.1x10^2$			
Intravenous	0 [b]	+	+	+	0	0	+	+	
Liver									
Respiratory	$5.4x10^3$	$1.0x10^5$	$7.0x10^4$	$4.7x10^6$	$6.9x10^7$	$1.3x10^7$	$7.9x10^5$	$<1.0x10^3$	$5.8x10^6$
Intraperitoneal	$1.2x10^4$	$4.9x10^6$	$4.7x10^5$	$1.5x10^3$	$5.4x10^4$	$1.3x10^3$		$5.7x10^2$	
Intravenous	$3.3x10^5$	$7.0x10^3$	$1.8x10^4$	$1.7x10^5$	$1.1x10^6$	$2.3x10^6$	$8.1x10^6$	$1.1x10^6$	
Spleen									
Respiratory	$9.7x10^3$	$7.8x10^5$	$7.9x10^6$	$9.4x10^6$	$3.0x10^7$	$6.0x10^5$	$6.3x10^3$	$4.0x10^1$	$1.0x10^2$
Intraperitoneal	$3.4x10^4$	$7.4x10^6$	$5.2x10^6$	$8.1x10^5$	$1.5x10^4$	$6.4x10^3$		$4.3x10^2$	
Intravenous	$6.6x10^5$	$5.9x10^3$	$9.3x10^5$	$6.5x10^6$	$1.1x10^5$	$1.3x10^4$	$1.3x10^3$	$4.0x10^2$	

a. Respiratory dose was $1.2x10^3$ cells; intraperitoneal dose was $1.5x10^3$ cells; and intravenous dose was $1.6x10^3$ cells. Five animals per group were used.

b. Growth of other organisms in cultures from lungs made quantitative results unreliable.

TABLE II. VARIATIONS IN COLONY COUNTS OF TISSUES FROM INDIVIDUAL ANIMALS INFECTED BY RESPIRATORY OR INTRAPERITONEAL ROUTES WITH LISTERIA MONOCYTOGENES A4413

Route [a]	Organ	Colony count per cc tissue (geometric mean)							
		Day							
		1		3		5		7	
		Mean	S.d.[b]	Mean	S.d.	Mean	S.d.	Mean	S.d.
Respiratory	Lung	$3.0x10^6$	2.4	$1.9x10^6$	1.1	$1.9x10^7$	2.3	$1.3x10^6$	11.1
	Liver	$4.5x10^2$	1.02	$6.3x10^4$	2.8	$2.5x10^6$	3.8	$2.1x10^6$	34.8
	Spleen	$1.08x10^3$	1.0	$2.0x10^6$	1.5	$2.1x10^5$	4.6	$1.3x10^4$	2.7
Intraperitoneal	Lung			$6.1x10^4$	4.7	$3.6x10^4$	46.4	$1.6x10^3$	8.6
	Liver			$7.9x10^4$	21.4	$2.3x10^4$	632.4	$1.6x10^3$	496.0
	Spleen			$3.7x10^4$	4.0	$3.6x10^5$	215.0	$8.1x10^3$	11.5

a. Respiratory dose was $4.8x10^2$ cells and intraperitoneal dose was $1.1x10^3$ cells. Five animals per group were used.

b. Standard deviation; (Mean)/(S.d.)=lower limit of distribution; (Mean)x(S.d.)= upper limit.

TABLE III. INFLUENCE OF THE SIZE OF THE CHALLENGE DOSE ON THE NUMBERS OF LISTERIA MONOCYTOGENES IN THE ORGANS OF MICE AFTER INTRAPERITONEAL CHALLENGE

Challenge Cells Per Dose [a]	Maximal colony count per cc tissue (geometric mean)					
	Lung		Liver		Spleen	
	Colonies per cc	Day	Colonies per cc	Day	Colonies per cc	Day
2.4×10^{2}	2.5×10^{6}	5	1.3×10^{7}	6	1.2×10^{8}	5
1.1×10^{3}	1.2×10^{5}	6	3.4×10^{6}	6	4.6×10^{5}	6
1.5×10^{5}	9.0×10^{5}	2	4.9×10^{6}	2	7.4×10^{6}	2
1.5×10^{5}	3.0×10^{4}	1	6.4×10^{3}	2	5.7×10^{5}	3

a. Five mice per group were used.

resulted in a somewhat higher maximal count than was obtained with larger doses.

Listeria were isolated from the brain homogenates sporadically regardless of the route of infection. About 25 per cent of the brains cultured contained *Listeria*. A few gave rise to colonies by the first day after injection and *Listeria* were found through seven days. The greatest number of positive cultures were obtained on the fourth day. The number of colonies ranged from 5.0×10^{1} to 3.0×10^{6} per cc of tissue.

Positive blood cultures were obtained from the first through the twelfth day. The greatest number of positive cultures were obtained from samples taken on the second through fourth days. Growth was obtained in dilutions as high as 10^{-4} at about the fifth day, although generally *Listeria* were found only at lower dilutions and during the early stages of infection.

Injection of the mortality-enhancing factor (MEF) simultaneously with *L. monocytogenes* by the intraperitoneal route resulted in increased *in vivo* growth of the organism. The difference between the bacterial counts of animals receiving both MEF and *Listeria* and those receiving the bacteria only was apparent at four hours and persisted for the duration of the experiment (Table IV). Most animals that received MEF were dead by the fourth day.

TABLE IV. EFFECT OF THE INJECTION OF MORTALITY-ENHANCING FACTOR ON THE GROWTH OF LISTERIA MONOCYTOGENES JHH IN THE ORGANS OF MICE

Inoculum	Colony count per cc tissue (geometric mean)						
	Experiment 1			Experiment 2			
	Hours			Hours			
	4	12	24	24	48	72	96
			Liver				
JHH[a]	1.1	1.7	8.1	2.9	95	25	4.7
JHH + MEF	0	200	57	8.2	3.5×10^4	2.7×10^4	1.6×10^8 [b]
			Spleen				
JHH	1.1	2.0	14	3.0	43	100	6.8
JHH + MEF	40	17	59	9.1	7.0×10^4	1.8×10^4	6.5×10^5 [b]

a. Dose in Experiment 1 was 4.5×10^3 cells; dose in Experiment 2 was 4.6×10^3 cells.
b. Only one animal survived at this time.

BCG injected six days prior to challenge with Listeria increased the resistance of the mice so that the bacterial counts obtained from the tissue homogenates were lower than those obtained from the organs of animals receiving Listeria alone (Table V). Listeria were not recovered from the blood of mice that had been injected with BCG. Blood cultures were positive from most mice that were injected with Listeria only. BCG was also capable of overcoming the effect of MEF, so that the number of bacteria recovered from the tissues of animals that received Listeria, MEF, and BCG was somewhat less than that found in the organs of mice receiving only Listeria, strain JHH (Tables V and VI).

Since in utero infection of the fetuses of females infected with Listeria has been reported by numerous investigators,[1] it was considered of interest to determine the fate of the organisms in surviving mice born of infected mothers and to determine the immunological state of these animals. Pregnant mice were infected by the intraperitoneal injection of approximately 3×10^5 cells of strain JHH. Twenty-seven of the 49 progeny born of five females survived and at five to six weeks of age were infected with 1.2×10^3 cells of strain A4413 in the peritoneal cavity.

TABLE V. INFLUENCE OF PRIOR INJECTION OF BCG ON THE GROWTH OF LISTERIA MONOCYTOGENES JHH IN THE ORGANS OF MICE

Inoculum [a]	Colony count per mg tissue (geometric mean)						
	Day						
	1	2	3	4	5	6	7
Lung							
JHH [b]	8.5×10^5	5.9×10^5	2.1×10^5	310	5.9×10^4	920	1,600
JHH + BCG [c]	< 690	1,100	1,800	< 300	< 400	190	< 340
Liver							
JHH	3.3×10^4	5.9×10^5	1.2×10^5	4,200	2.0×10^5	1.9×10^5	3,900
JHH + BCG	< 200	460	2,700	90	< 42	420	< 81
Spleen							
JHH	1,600	1.2×10^7	1.3×10^6	7.7×10^4	3.6×10^5	1.5×10^4	2,000
JHH + BCG	< 1,000	890	1.1×10^5	2,000	110	3,700	< 360
Blood							
JHH	2/3 [d]	3/3	2/3	0/3	1/3	2/3	0/3
JHH + BCG	0/3	0/3	0/3	0/3	0/3	0/3	0/3

a. Three mice per group were used.
b. 7.3×10^4 cells per 0.2-ml dose.
c. 9.1×10^4 living BCG injected into the peritoneal cavity six days before challenge with *L. monocytogenes*.
d. Number of positive blood cultures/total number when one loopful of heart blood was streaked on a tryptose agar plate.

TABLE VI. EFFECT OF PRIOR INJECTION OF BCG ON THE <u>IN VIVO</u> GROWTH IN MICE OF <u>LISTERIA MONOCYTOGENES</u> JHH IN THE PRESENCE OF MORTALITY-ENHANCING FACTOR

Inoculum [a]	Colony count per cc tissue (geometric mean)						
	Day						
	1	2	3	4	5	6	7
				Liver			
JHH + MEF [b]	$3.5x10^3$	$7.3x10^5$	$1.2x10^7$	$2.0x10^5$	$5.2x10^3$	$6.3x10^1$	$4.5x10^4$
JHH + MEF + BCG [c]	$4.8x10^4$	$2.7x10^4$	$4.0x10^4$	$8.2x10^3$	$8.7x10^4$	$1.1x10^3$	$3.0x10^2$
				Spleen			
JHH + MEF	$1.2x10^4$	$5.1x10^6$	$6.1x10^7$	$5.3x10^6$	$2.0x10^5$	$2.0x10^4$	$3.8x10^4$
JHH + MEF + BCG	$7.0x10^4$	$1.5x10^4$	$2.8x10^5$	$4.4x10^5$	$1.2x10^5$	$4.8x10^5$	$1.4x10^3$
				Blood			
JHH + MEF	0/3 [d]	3/3	3/3	1/3	1/3	0/3	1/2
JHH + MEF + BCG	0/3	1/3	1/3	1/3	2/3	0/3	0/3

a. Three animals per group were used.
b. $6.7x10^4$ cells of strain JHH injected by intraperitoneal route simultaneously with 2 ED_{50} amounts of mortality-enhancing fraction.
c. $9.1x10^4$ living cells of BCG injected into peritoneal cavity six days prior to challenge.
d. Number of positive blood cultures/total number plated.

Although no significant difference in the LT_{50}, the time in which 50 per cent of the exposed mice died, was noted between the test group and mice of the same age from uninfected females, a marked difference was noted in both the extent of the gross pathological changes and in the number of organisms recovered from the spleens and livers. The LT_{50} for the mice born of infected females was 4.5 days (95 per cent confidence limits: 3.4 to 6.0 days); that for the mice from the uninfected parent was 3.8 days (95 per cent confidence limits: 3.0 to 4.8 days). The control animals, however, showed much more extensive pathology and the cell counts per cc of spleen or liver were higher (Table VII). However, in a similar experiment using mice seven to eight weeks old, no difference was observed between the test group and the control group. The number of organisms per cc of spleen or tissue was essentially the same in both groups and the changes in cell population paralleled each other quite closely.

IV. DISCUSSION

Infection of mice by the respiratory, intravenous, or intraperitoneal routes produced the same amount of growth of L. monocytogenes in the spleen, liver, and brain. Following aerosol exposure, however, the colony counts obtained from lung suspensions were high within the first 24 hours after infection and remained at a high level for up to nine days. Infection by the other routes not only resulted in a somewhat lower colony count but also in a more gradual increase in the number of Listeria in the lungs. The standard deviation from the geometric means of the colony count was more uniform and smaller with the cultures obtained from animals infected by aerosol than with the cultures of mice infected by the other routes (Table II). This observation may explain the more uniform results of virulence titrations observed by Kautter et al following respiratory exposure and the erratic response observed with intraperitoneal or intravenous infection.

The difference between the in vivo growth of Listeria after respiratory exposure and that following infection by the other routes also may be explained in part by unpublished observations by Kautter. He observed that following the introduction of Listeria into the lungs there was an immediate increase in the number of organisms. Two hours after exposure, the mean increase in Listeria was approximately 1.6 times those present at zero hours, and by four hours the bacterial count increased about fivefold. In the peritoneal cavity, however, the organisms disappeared rapidly for the first six hours and then began to increase (Silverman, unpublished data). In agreement with this, we have observed that when Listeria were infected into the peritoneal cavity 24 hours after the injection of sodium caseinate, the accumulated polymorphonuclear cells rapidly ingested the organism so that within 10 to 15 minutes the leucocytes were completely filled with bacteria.

The infrequent occurrence of Listeria in brain homogenates is in accordance with the observations of various authors. Seeliger[1] mentions that

TABLE VII. COMPARISON OF GROWTH OF <u>LISTERIA MONOCYTOGENES</u> A4413 IN ORGANS OF MICE BORN OF <u>LISTERIA</u>-INFECTED MOTHERS AND MICE BORN OF HEALTHY MOTHERS

Mous · Litter	Organ	Colony count per mg tissue (geometric mean)				
		Days				
		1	2	3	4	5
5 - 6 weeks old, from: a/						
Infected mother	Liver	410	550	6,500	3,600	1,700
Normal mother	Liver	$1.4x10^4$	$5.7x10^4$	$5.4x10^6$	$3.8x10^6$	$4.3x10^5$ c/
Infected mother	Spleen	910	380	440	6,100	$1.2x10^4$
Normal mother	Spleen	$3.5x10^4$	$3.7x10^4$	$7.0x10^6$	$6.1x10^6$	$9.4x10^6$ c/
7 - 8 weeks old, from b/						
Infected mother	Liver	440	$2.3x10^4$	$1.2x10^4$	$8.2x10^6$	$1.1x10^5$
Normal mother	Liver	$9.2x10^2$	$1.5x10^4$	1.2x10[illegible]	$2.4x10^7$	$1.2x10^6$ c/
Infected mother	Spleen	730	$2.0x10^4$	$5.1x10^4$	$2.9x10^6$	$3.2x10^4$
Normal mother	Spleen	400	$1.5x10^4$	$1.1x10^6$	$1.2x10^5$	$2.5x10^6$ c/

a. $1.3x10^3$ cells per dose; 3 mice per group.
b. $5.4x10^2$ cells per dose; 4 mice per group for infected progeny; 2 mice per group of controls.
c. Single survivor.

only after intracranial or intrathecal injection was neurolisteriosis consistently produced in rodents. He suggests that the short time interval between infection and death in septic listeriosis precludes infection of the brain.

The greater number of organisms recovered after the injection of MEF agreed with previous observations of the greater mortality that followed injection of the cell extract with the organism. The reverse effect induced by the prior injection of BCG and the neutralization of the effect of MEF by injection of the acid-fast organism suggests that MEF exerts its effect in part, at least, on the reticuloendothelial system (RES). Lurie[8] reported enhanced activity by the cells of this system during infection with *Mycobacterium*. The exact role of MEF in *Listeria* infections is yet to be elucidated. It may be, as with the toxin of plague,[9] that as the host responds to the infection by destroying the organism, the cellular component (MEF) is liberated. Thus, the host indirectly enhances the infection. On the other hand, in view of the latent nature of *Listeria* infections,[1] it may be that when resistance is reduced because of stress and if *Listeria* is present in an inapparent infection, the host becomes more susceptible to the MEF produced by the organisms already present in the tissues and an acute infection is initiated. These hypothesis require further investigation of the pathogenesis of the disease and of the role of cellular components of the parasite.

The increased resistance following the injection of BCG is in accord with previous observations by Pullinger,[10] Henderson *et al*,[11] Dubos and Schaedler,[12] and Sulitzeanu *et al* with *Brucella abortus*, *Bacillus anthracis*, *Staphylococcus aureus*, and *Mycobacterium fortuitum*. Elberg *et al*,[14] studying monocytes obtained from animals exposed to *Mycobacterium*, also observed increased resistance toward infection with *Brucella*.

The comparative studies with progeny of *Listeria*-infected females and those of healthy animals indicate that prenatal exposure of *Listeria* produced a temporary degree of immunity rather than a state of immune tolerance. Rees and Garbutt[15] reported immunity in mice infected *in utero* with mycobacteria. They observed a shortened survival time in their control group. Although *in utero* infection failed to increase the LT_{50}, the gross pathology and bacterial growth was less extensive in this group than in the control group. The temporary nature of the immunity suggests that it may have been passive.

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