THE PASSIVE TRANSFER OF ACQUIRED RESISTANCE TO LISTERIA MONOCYTOGENES*

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Acquired resistance to *Listeria monocytogenes* is associated with the development of a delayed type of hypersensitivity to *Listeria* antigens and with the appearance of an abnormal level of antibacterial activity in the mononuclear phagocytes of infected mice (1).

The mechanism responsible for this change in cell function is unknown. In mouse typhoid, in which intracellular parasitism also occurs, it has recently been shown that acquired resistance depends upon the production of specific antibodies which have a marked tendency to become adsorbed to the surface of host cells (2, 3). It seemed likely, therefore, than an antibody with similar properties might be found in animals immunised against *L. monocytogenes*, and that the abnormal functional capacity found in immune macrophages might be due to a specific antibody adsorbed at the cell surface where it would interact with the bacterial cell during the act of ingestion.

Although previous attempts to transfer resistance to this organism passively with serum have been unsuccessful (4, 1), a humoral mediator of immunity must still be considered possible. If the operative antibody were present in low concentration in the serum because of its tendency to be adsorbed at cell surfaces, or if it were rapidly fixed for the same reason when passively transferred to recipient mice, its existence might be difficult to establish. The present experiments have been performed with these considerations in mind.

Materials and Methods

Animals.—Mice of the outbred Swiss-Webster strain were used at 8 to 10 weeks of age.

Organisms.—*Listeria monocytogenes* (NCTC 7973) of serotype I was grown in brain-heart infusion broth (Difco Laboratories, Inc., Detroit). Suspensions were prepared from cultures grown for 16 hours at 37°C. No special precautions were taken to maintain a high level of virulence in the organism during the present series of studies.

Immunisation.—A variety of immune cells and sera was used. In most cases, however, serum was obtained from highly immunised mice which had survived a series of graded doses of living *Listeria*. Blood was obtained from the retroorbital plexus, 6 to 8 days after a final intravenous injection of 10,000 lethal doses of living organisms. Serum was used immediately or stored for

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only a few days at −10°C. Immune cells were obtained from the peritoneal cavity of convalescent mice, 8 to 10 days after the intraperitoneal injection of 0.2 LD_{50} of living *Listeria*. They were recovered by washing out the peritoneal cavity with Hanks' balanced salt solution (BSS) containing 10 I.U. heparin/ml. Before transfer they were washed 3 times in BSS and were finally suspended in BSS, the yield of cells from 3 or 4 mice being contained in a single dose of 0.2 ml. As fewer cells were obtained from unimmunised controls, larger numbers of donors were needed to effect the transfer of equivalent numbers of cells in each experiment.

Preparation of Urea Extracts of Spleen.—A group of 40 mice were given chlortetracycline at a concentration of 1.0 mg/ml of drinking water. After 2 days they were injected intravenously with 5×10^8 living *Listeria*. The immunising inoculum, though unable to multiply in the tissue of chlortetracycline-treated animals, is known to induce a high level of acquired resistance (5). Eight days after immunisation the spleens were removed, pooled, and homogenised in a phosphate-buffered saline containing 4 M urea. After centrifuging for 20 minutes at 20,000 *g* the supernatant was sterilised by membrane filtration (pore size 0.45 μ).

Other Methods.—Procedures used for bacterial enumeration in the spleen, for measuring the rate of clearance of organisms from the peritoneal cavity, and for the preparation and infection of monolayers of mouse macrophages have been described (1).

RESULTS

Survival and Growth of L. monocytogenes in the Spleens of Mice Passively Immunised with Serum.—

A washed suspension containing 3.0×10^6 *Listeria*/ml was diluted 1:3 with normal or immune serum and stood for 3 hours at 4°C. During this period no change occurred in the viable counts. Mice were then injected intravenously with 0.3 ml of one or other of the two suspensions containing 2.0×10^5 viable organisms. Further injections of 0.2 ml of serum were given at intervals of 12 hours. After 4 hours, and then at daily intervals, the bacterial count was determined in the spleens of 5 mice from each group.

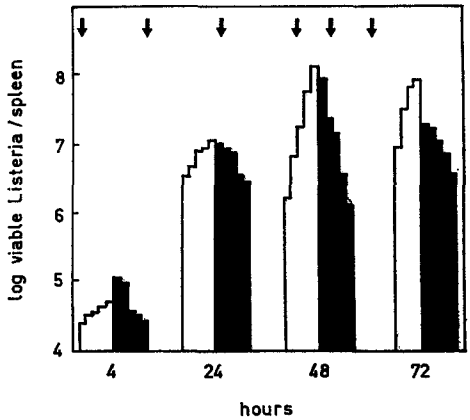

FIG. 1. Histogram showing the effect of repeated injections (arrowed) of normal serum (open) and immune serum (solid) on the viable spleen count in normal recipient mice challenged with presensitised *L. monocytogenes*. The serum injections produced an abnormal growth curve, but no clear indication of any antibacterial effect in the case of immune serum.

The results recorded in Fig. 1 show that the serum of highly immunised mice had no detectable effect on the immediate fate of *Listeria in vivo* and little effect upon its subsequent growth in the spleens of normal recipients.

The Effect of Serum on Bacterial Clearance by the Peritoneal Cavity.—The absence of any obvious effect of immune serum on the immediate fate of intravenously injected *Listeria* could be due to peculiarities in the early distribution of the organism. For instance a majority could be located within granulocytes during the initial stages of infection. Since *L. monocytogenes* ultimately parasitises the mononuclear phagocytes of various organs, it might be more pertinent to examine the effect of serum on organisms introduced into the peri-

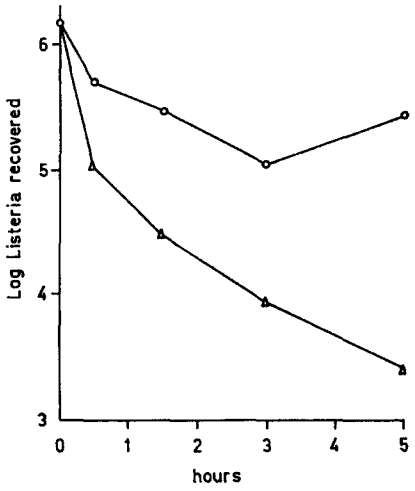

FIG. 2. Viable bacterial counts found in the peritoneal washings recovered at intervals from normal (○——○) and *Listeria*-immunised (△——△) mice injected with approximately 5×10^6 living *L. monocytogenes*. Means of 5 mice per group.

toneal cavity, which normally contains an abundance of free mononuclear cells. The fate of an intraperitoneal inoculum of bacteria can be crudely estimated from the viable organisms present in the peritoneal washings taken at intervals following the intraperitoneal injection of a standard inoculum.

With the object of choosing suitable experimental conditions a preliminary study was made of the sequential changes in the peritoneal population of viable *Listeria* in normal and actively immunised animals injected with equal numbers of organisms. The immune mice were animals which had survived 10 days from a primary peritoneal infection.

The results (Fig. 2) show that in normal mice the bacterial population fell for a period of 3 hours and then began to rise. In immune mice, on the other hand, the fall was faster and progressive.

On the basis of the foregoing result a comparison was made between the viable bacterial populations present at 5 hours in the peritoneal cavity of normal mice injected with a presensitised inoculum containing 2.8×10^5 and 2.1×10^5 viable *L. monocytogenes* in 0.2 ml of normal or immune mouse serum respectively. The two suspensions had been stood at 4°C for 3 h urs prior to injection.

As indicated in Fig. 3, treatment with immune serum did not significantly affect the numbers of organisms recovered from the peritoneal cavity (P 0.2). Any small difference that existed was probably due to the lower viable count obtained in immune serum. This in itself was possibly due to bacterial agglutination, but the agglutinin titre of the serum was not determined. From previous

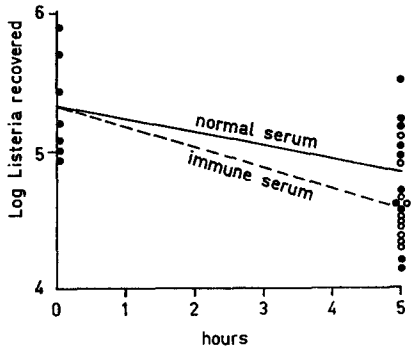

FIG. 3. Viable bacterial counts found in the peritoneal washings of mice at 5 minutes (normal serum only) and at 5 hours after the intraperitoneal injection of *L. monocytogenes* presensitised with normal (●——●) or immune (●-----○) serum. The lines join the means for each group.

experience it is likely to have been high. Since the organisms were observed in smears to have been promptly ingested by peritoneal macrophages, it is concluded that immune serum did not materially influence intracellular survival of *L. monocytogenes*.

The Effect of Serum on Intracellular Survival in Vitro.—In previous experiments a detailed study was made of the behaviour of *L. monocytogenes* in monolayers of mouse macrophages *in vitro* (1). It was shown that *Listeria* was unable to replicate in macrophages obtained during the first 2 to 3 weeks after infection. No observations were made, however, on the effect of serum on the intracellular growth of *Listeria in vitro*.

Monolayers of peritoneal macrophages from normal mice were prepared in open slide chambers. After incubation for 6 hours the cultures were washed, and the medium was replaced with one containing 15 per cent normal or immune mouse serum. After 1 hour the medium was replaced with one of similar composition but containing 10^4 *Listeria*/ml. After 1 hour in contact with the monolayer the excess organisms were removed by flushing each culture over a fountain with 200 ml of sterile saline. Fresh medium (0.4 ml) was then added. Four cultures of each

group were immediately disrupted by introducing the probe of a Mullard-MSE ultrasonicator. The sonicate was diluted and plated to obtain a viable count on the initial intracellular bacterial population. Half of the remaining cultures of each group were incubated with normal medium and half with medium containing 3.0 μg streptomycin/ml. This concentration of antibiotic was chosen for its ability to prevent bacterial multiplication without causing bacterial death (even of extracellular organisms) during the first 12 hours of incubation.

TABLE I

Survival and Growth of L. monocytogenes Following Ingestion by Mouse Macrophages in the Presence of Normal or Immune Mouse Serum

Time	Normal serum		Immune serum	
hrs.				
0	118*		137	
	118		102	
	119		111	
	82		102	
Mean	109		113	
± SD	16		14	
Time	With SIM	Without STM	With STM	Without STM
hrs.				
5	274	373	281	239
	340	264	312	204
	170	265	452	390
	297	392	229	156
Mean	270	324	319	247
± SD	62	59	82	88

* Figures when multiplied by 40 give the absolute counts per culture.

The intracellular multiplication of *L. monocytogenes* after ingestion by normal macrophages in the presence of normal or immune serum is shown in the data of Table I. A threefold increase in bacterial numbers occurred during 5 hours of incubation in the presence and absence of streptomycin whether or not immune serum was present in the culture.

The ability of this method to detect an antibacterial effect of immune serum, had it been present, was established from a comparison of the behaviour of *L. monocytogenes* in cultures of normal and immune cells infected and maintained in the presence of normal serum only. The results of such an experiment are recorded in Table II. The viable count rose in the cultures of normal cells and fell in the cultures of immune cells. The experiment has been repeated

several times. Even in the presence of streptomycin the viable count consistently rose in cultures of normal cells and fell, sometimes to an even greater extent, in cultures of immune cells. This finding is consistent with previous evidence that macrophages of convalescent mice possess marked anti-*Listeria* activity. It is apparent that this property of immune cells cannot be conferred on the cells of normal mice with the serum of hyperimmunised animals. In a

TABLE II

Survival and Growth of L. monocytogenes Following Ingestion by Normal and Immune Mouse Macrophages

Time	Macrophage culture	Normal	Immune
hrs.			
0	1	102*	56
	2	99	43
	3	105	71
	4	69	72
	5	83	22
	6	98	18
Mean		93	47
± SD		13	21
5	1	340	28
	2	280	7
	3	490	24
	4	610	6
	5	590	26
	6	580	—‡
Mean		480	15
± SD		130	10

* Figures multiplied by 40 give the absolute counts per culture.

‡ Lost during sonication.

series of similar studies serum was taken from mice at 3-day intervals during the course of a primary *Listeria* infection. These sera were also inactive.

The Effect of Immune Cells on Peritoneal Clearance.—The effectiveness of immune cells in restricting bacterial multiplication is not easily demonstrated *in vivo*. When peritoneal cells from immune mice were transferred intravenously to normal mice they produced no effect on the growth of a subsequent inoculum of *Listeria* in the spleens of the recipient animals. It is possible that here, too, a geographical problem exists in that cells and organisms injected separately by

the intravenous route might not make subsequent contact in the tissues. If cells and organisms were transferred to the peritoneal cavity, however, the opportunity for subsequent contact would be greatly increased.

A group of mice was immunised intraperitoneally with 3.6×10^5 *Listeria*. After 9 days some of the survivors were used as cell donors (25 mice); the rest were used to test the efficiency of peritoneal clearance in the donor mice. Two other groups of unimmunised mice were used for the same purposes. The peritoneal cavity of each donor was washed out with 2.0 ml of BSS containing heparin. The cells from normal or immune mice were pooled and washed three times in BSS. They were then brought to an appropriate concentration and transferred to the peritoneal cavity of normal recipients in a dose equivalent to the yield of cells from 2 mice (approximately 1.2×10^7 in an injection volume of 0.2 ml). Five minutes later all mice in the

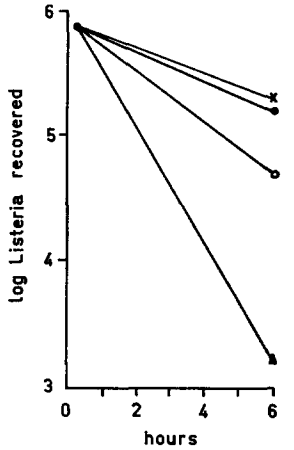

FIG. 4. Viable bacterial counts found in the peritoneal washings 5 minutes after the injection of approximately 10^6 *Listeria* into normal recipients and 6 hours after the same inoculum had been injected into normal control mice (●) or *Listeria*-immune mice (▲), and the recipients of normal (×) or immune cells (○). Means of 10 mice per group.

four groups were challenged intraperitoneally with approximately 1.0×10^6 *Listeria*. To conserve materials the viable counts on the peritoneal washings taken 5 minutes after infection were performed on normal untreated mice, whereas the 6-hour counts were made on 10 mice from each of the four treatment groups.

The results are shown in the curves of Fig. 4. The peritoneal clearance in mice which had received two mouse equivalents of washed cells from normal donors was no more efficient than that of untreated normal mice. Clearance was enhanced in the recipients of immune cells, but the effect was small in comparison with the efficiency of intact immune donors. It should be remembered, however, that the peritoneal cavity of the recipient mice contained a large resident population of normal cells at the time of transfer, and that an unknown part of the bacterial inoculum would have entered these cells rather

than those of the transferred population. This is particularly likely in view of the repeated washing to which the latter cells had been subjected prior to transfer. The small increase in clearance produced by immune cells is, therefore, probably significant.

The Effect of a Urea Extract of Spleen.—Rowley, Turner, and Jenkin (3) have shown that a specific protective antibody can be extracted from the spleens and peritoneal macrophages of mice appropriately immunised against *Salmonella typhimurium.* The antibody was predominantly of 19S type, and could be eluted from cells with 4 M urea. An attempt was made to determine whether a similar protective factor could be obtained from the tissues of mice which had been immunised against *L. monocytogenes.*

TABLE III

Viable L. monocytogenes in the Spleens of Mice Injected with Urea Extracts of Normal or Immune Mouse Spleen

Mouse	Recipients of normal spleen extract		Recipients of immune spleen extract	
	8 hrs.	55 hrs.	8 hrs.	55 hrs.
1	10.4*	58.8‡	13.6*	62.0‡
2	5.2	29.6	7.2	72.0
3	11.2	4.0	10.0	4.0
4	4.4	9.2	6.8	12.0
5	5.2	5.6	4.0	6.8
Mean.....	7.3×10^4	21.0×10^6	8.3×10^4	31.0×10^6

* Numbers multiplied by 10^4 give total spleen count.

‡ Numbers multiplied by 10^6 give total spleen count.

A group of mice were given chlortetracycline in their drinking water at a concentration of 1.0 mg/ml. Two days later they were infected intravenously with a large dose (10^9) of living *L. monocytogenes.* Although the organisms do not multiply in antibiotic-treated mice they induced a high level of acquired resistance (5). Eight days after infection the spleens were removed, pooled, and homogenised in phosphate buffer containing 4 M urea at pH 7.0. An equal number of spleens from normal mice were treated similarly. The homogenate was clarified by centrifugation and sterilised by membrane filtration (pore size 0.45 μ). Two groups of 10 mice were injected intraperitoneally with a volume equivalent to the extract of 2 spleens from normal or immune animals. Eighteen hours later the mice were challenged by the intravenous injection of approximately 1.0×10^5 *Listeria.* At 8 and 55 hours viable counts were performed on the spleens of 5 mice from each group.

Table III sets out the viable spleen counts 8 and 55 hours after intravenous injection of *L. monocytogenes* into mice that had received urea extracts of the spleens of normal and *Listeria*-immune mice. They were not significantly different. A urea extract of peritoneal macrophages from immune mice was equally ineffective in modifying the growth of *Listeria* under similar conditions of test.

The Effects of Cell and Serum Transfer on the Survival of Lethally Infected Mice.—The tests conducted to this point had all failed to reveal any effect of serum or cell extracts on the growth or survival of *L. monocytogenes* in a variety of experimental situations. It still remained to test for a protective effect of passive immunisation on lethally infected animals in order to exclude any long-term effect that had gone undetected by any of the other methods.

One hundred mice were used as the donors of immune cells and serum. They had been immunised with graded doses of living *Listeria*. Serum and a comparable number of normal cells were obtained from 150 unimmunised mice. The sera were separated immediately and pooled for transfer to normal recipients on the same day. Cells were obtained in the usual way. They were pooled and washed three times with BSS. The organism used for challenge had been

TABLE IV

The Effect of Peritoneal Transfer of Living or Dead Cells, or the Serum of Normal or Immune Mice on the Survival of Mice Challenged Intraperitoneally with L. monocytogenes

Challenge dose	Treatment	Deaths/No. challenged
1.0×10^7 (2 LD_{50})	Control	14/20
	Normal cells (4 mouse equivalents)	6/10
	Immune cells (4 mouse equivalents)	0/10
3.0×10^7 (6 LD_{50})	Control	9/10
	Frozen immune cells (4 mouse equivalents)	10/10
	Frozen normal cells (4 mouse equivalents)	9/10
	Immune serum (0.2 ml)	19/19
	Normal serum (0.2 ml)	19/20

tested for virulence 10 days previously ($LD_{50} = 5 \times 10^6$). The challenging dose of organisms was intended to contain 5 LD_{50}s. It was mixed with cells or serum immediately prior to injection into the peritoneal cavity. Each inoculum contained a volume of serum equivalent to 0.2 ml, or cells equivalent to the yield from 4 mice. The experiment was made feasible by dividing it into two parts as indicated in Table IV. Intact normal or immune cells were transferred on the 1st day, and cells killed by a single cycle of freezing and thawing and the two pooled sera were transferred on the 2nd day. The inocula used were slightly different in the two halves of the experiment (2 and 6 LD_{50}s, respectively).

The effectiveness of intact peritoneal cells and the ineffectiveness of serum from immunised animals in conferring protection from a lethal challenge with *L. monocytogenes* is apparent from the survival figures of Table IV. It was interesting to observe that the recipients of immune cells did not display any overt illness at any stage; all other mice, whether they survived or not, were acutely ill by the 4th day and showed the characteristic conjunctivitis that accompanies listeriosis in mice. It seems likely, therefore, that immune cells

were active from the outset in preventing a massive dissemination of organisms from the peritoneal cavity. Their ability to do so, however, was entirely abolished by a single cycle of freezing and thawing of the cells within 5 minutes before their introduction into the peritoneal cavity. This seems to indicate that immune cells owe their effectiveness to the ability, which has been demonstrated *in vitro*, to interfere with the growth of organisms that they ingest, rather than to antibody that is carried into the recipient as a dissociable component of the transferred cells.

In a further experiment of the same type normal and immune cells in a dose of 2 mouse equivalents were injected intraperitoneally 30 minutes *prior* to a small challenging dose (1 LD_{50}) of *L. monocytogenes*. The ratios of survivors to injected mice in untreated controls and the recipients of normal or immune cells were 3:10, 5:10, and 9:10, respectively. The mouse succumbing in the last group was small; it became acutely ill within 24 hours of infection and is unlikely to have died solely as a result of the *Listeria* infection, for none of the other mice in this group showed evidence of illness at any stage of the infection.

DISCUSSION

The present investigation was undertaken in the light of a recent demonstration that acquired resistance of mice to certain enteric pathogens is due to specific antibody that is present not only in serum but also attached to cells (2, 3). The association of protective antibody and macrophage is of singular significance in relation to the problem of acquired cellular resistance. The abnormal antibacterial properties that have been attributed to the cells of animals immunised against *S. enteritidis* for instance (6) can now be rationally explained in terms of adsorbed antibody. In view of the present studies it does not seem likely, however, that a similar mechanism can be held to operate in acquired resistance to *L. monocytogenes*. The application of even more rigorous tests for protective antibody has failed to reveal its presence in serum or attached to cells of animals immunised in a variety of ways. On the other hand, it was readily shown that the macrophages of *Listeria*-immune mice are not only endowed with conspicuous antibacterial activity, but also with the capacity to confer passive protection upon normal recipients.

Two very significant and distinguishing features of the macrophages of *Listeria*-immune mice are the lack of specificity in their antibacterial activity (7), and the fact that the cells must be alive in order to influence the fate of a bacterial population. The first of these may raise doubts as to the immunological basis of this form of acquired resistance. It has been shown, however, that the abnormal cellular activity found in immune mice is due to a specific immunological reaction despite its non-specific nature (7). This must indicate that the alteration in cell function is the end-result of a specific antigen-antibody reaction, occurring perhaps at the cell surface. It is possible, therefore, that antibody will ultimately be shown to play a significant role in acquired cellular

resistance. It appears from the present studies, however, that it is not an antibody with the properties normally associated with protective mechanisms, for it does not appear capable of modifying the bacterial cell in a way which influences its survival and growth in the presence of normal phagocytes.

The second distinctive feature of the immune cells of *Listeria*-resistant mice is their ability to confer protection only when introduced in a living state and under conditions that permit them to interact directly with the infecting bacterial population. This again implies that no dissociable protective factor is associated with the immune cell. In this respect the immunity differs from that developed against *S. typhimurium*.

The possible nature of acquired resistance to facultative intracellular parasites such as *L. monocytogenes* is discussed at greater length in the following paper (7).

SUMMARY

Five methods have been used in an effort to reveal an antibody that could account for the features of acquired resistance to *Listeria monocytogenes*: (*a*) a comparison of the growth rates of *Listeria* in the spleens of mice infused repeatedly with normal or immune mouse serum; (*b*) measurement of peritoneal clearance of *Listeria* in the presence of normal or immune mouse serum; (*c*) the survival rate of *Listeria* in monolayers of mouse macrophages infected in the presence of normal or immune mouse serum; (*d*) the effect of injecting urea extracts of spleens and peritoneal macrophages of normal or immune mice on the survival and growth of *Listeria* in recipient animals; (*e*) a comparison of survival rates in lethally infected mice following the passive transfer of cells and serum from normal or immune donors. The only evidence of passive protection was obtained when intact living cells from immune donors were used for transfer under conditions which permitted them to interact with the parasite population.

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