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Enhanced primary resistance to *Listeria monocytogenes* in T cell-deprived mice

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Summary. The present studies reinvestigate the role of the T cell in the development of resistance to *Listeria monocytogenes*. Doubly thymus-deprived adult thymectomized irradiated bone marrow-reconstituted mice (D-AT × BM) were prepared using anti-theta serum-treated bone marrow from AT × BM donors for reconstitution. The growth of the bacteria in spleens and livers of D-AT × BM was inhibited and such animals survived infection with doses which were lethal to normal mice. Since the D-AT × BM animals were T cell-depleted, as evidenced by (a) absence of theta-positive cells in their spleens; (b) inability to mount a primary humoral response to a T-dependent antigen and (c) failure to reject H-2 incompatible skin allografts, their antibacterial resistance was not due to the presence of a residual T-cell population. Further evidence of T-cell depletion in these animals was furnished by the findings that, despite their resistance to *L. monocytogenes*, they failed to exhibit a delayed hypersensitivity reaction to *Listeria* antigens, their splenocytes were unable to transfer resistance to naïve hosts and they did not develop an anamnestic response upon secondary challenge.

We concluded from these findings that primary antibacterial resistance to *L. monocytogenes* need not necessarily depend on the development of specific cell-mediated immunity although under normal circumstances these two processes develop in chronological association. The increased resistance of D-AT × BM animals is interpreted as being due to the enhancement of bactericidal activity of mononuclear phagocytes, possibly caused by the removal of a regulatory T-cell population. This population seems to be radiosensitive and spleen-seeking and requires an intact spleen to mediate its effect.

INTRODUCTION

It is generally believed that resistance to *L. monocytogenes* is a cell-mediated immune response, induced specifically by a T cell-mediated reaction and expressed nonspecifically by activated macrophages with enhanced bactericidal powers (Mackaness, 1969). The evidence to support this hypothesis includes (a) chronological association between the development of antibacterial resistance and specific cell mediated immunity (Mackaness, 1969); (b) decreased resistance to infection of hosts treated with antilymphocyte globulin (Mackaness & Hill, 1969) and (c) T-cell dependence of an adoptive transfer of bacterial resistance using splenocytes from primed donors (Lane & Unanue,

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1972). According to this concept, resistance to *L. monocytogenes* depends upon the development of specific immunity. The T cell is held to be responsible for both phenomena, and antimicrobial resistance would not be detected in the host (or its macrophages) until the tissues have had time to respond immunologically.

It has been shown recently, however, that (a) resistance to infection with *Listeria* does not always develop in the same chronological pattern as indicators of specific immunity (Halliburton & Blazkovec 1975; Osebold, Pearson & Medin, 1974); (b) this resistance can be enhanced by stimuli such as Con A (Lane, Petit, Gordon & Unanue, 1973) or synthetic polynucleotides (Medina, Vas & Robson, 1975), which do not involve the development of specific immunity, and (c) some groups of hosts deficient in T cell populations can resist infection as well as, or even better than, normal animals (Takeya, Mori & Nomoto, 1964; Blanden & Langman, 1972; Campbell, Martens, Cooper & McClatchy, 1974; Chan, Kongshavn & Skamene, 1975; Cheers & Waller, 1975; North, 1973). A major hypothesis which has been put forward to explain the seemingly paradoxical resistance of adult thymectomized, lethally irradiated and bone marrow reconstituted mice (AT × BM) to *Listeria* infection is that such 'T cell-depleted' mice contain a residual T-cell population, sufficient to generate immunity (North, 1973).

The investigations reported in this paper were conducted to clarify the role of T lymphocytes in the development of resistance to *L. monocytogenes*. T cell-deprived mice were prepared very carefully using the 'double deprivation' method and the adequacy of T cell-depletion was determined by three independent criteria.

MATERIALS AND METHODS

Mice

Male CBA and A/J mice were purchased from Jackson Laboratories, Bar Harbor, Maine.

Irradiation

Mice were exposed in a Perspex box to the required dose of total body radiation at an absorbed dose rate of 93·7 rad/min from a ^{60}Cobalt source.

Irradiated reconstituted mice

Four-week-old CBA mice were irradiated using 1000 rad and reconstituted with 3×10^6 nucleated bone marrow cells. They were used 4 weeks later.

T cell-depleted mice

Three- to five-week-old male CBA mice were thymectomized and 1 week later were irradiated using 1000 rad, and minimally reconstituted with 3×10^6 nucleated bone marrow cells that had been treated at 37° for 1 h with anti-θ serum (14) and guinea-pig serum (GPS) as a source of complement. Six weeks later, the same number of anti-θ-treated bone marrow cells from these AT × BM mice was used to reconstitute a second batch of thymectomized, irradiated mice. The doubly AT × BM mice (D-AT × BM) obtained in this way were used 4 weeks later.

Splenectomized mice

Splenectomy was performed in mice anaesthesized with sodium pentobarbital, giving 0·08 mg/g body weight. The peritoneal cavity was opened by a small incision under the left costal margin, and the spleen was mobilized outside the abdominal cavity. After the splenic vessels were ligated with silk, the spleen was removed, the peritoneum closed with silk and the muscles and skin closed with metal clamps. In the sham-operated animals, the spleen was only mobilized outside the abdominal cavity. No antibiotics were given. The animals were used for experiments beginning 10 days following the operation.

Bacteria

L. monocytogenes, strain EDG, obtained originally from Dr G. B. Mackaness of the Trudeau Institute, Saranac Lake, New York, was used throughout the experiments. The organism was kept virulent by passage through mice. Stock culture, stored frozen at −70° in small aliquots, was thawed and used to seed a fresh culture for each inoculation. The culture was grown up overnight in trypticase soy broth and the number of viable organisms determined before injection by spectrophotometry, using a nephelometric curve.

Infection of mice and testing of resistance to L. monocytogenes

Animals were infected by injection into the tail vein. Their resistance was tested as follows:

1. *Bacterial growth.* The number of viable organisms in the spleens and livers of infected animals was established by plating-out ten-fold dilutions of

organ homogenates in saline on tryptose agar, and the colony count performed 18 h later.

2. *Survival of animals.* Doses of live organisms ranging from 10^3–10^5 in half log intervals were injected into groups of experimental animals and the animal survival determined. Deaths occurred usually between the 4th and 7th day after infection. All the animals which survived the 7th day remained alive and well until the time the experiments were completed (usually 1–2 months).

3. *Resistance of* Listeria*-primed animals to reinfection.* The degree of acquired resistance was quantitated by reinfection of animals surviving a primary sublethal infection, with graded doses of *Listeria*. Subsequently, the bacterial counts were determined.

Testing of specific cell-mediated immunity to L. monocytogenes

1. *Delayed hypersensitivity to* Listeria *antigens.* Twenty μl of protein were injected into the footpads of experimental animals and the footpad swelling was determined 24 h later using the method of Paranjpe & Boone (1974). The antigens were prepared according to the method of Mackaness (1969) from the supernatant of a 24-h culture, by dialysis, ammonium sulphate precipitation and fractionation on a Sephadex G-50 column. The material present in the void column eluate was used.

2. *Adoptive transfer of resistance to* L. monocytogenes. Donor mice were primed with approximately 5×10^3 viable organisms, their spleens harvested 7 days later and the spleen cell suspension prepared according to Mackaness (1969). Recipient mice of the same strain, age and sex as the donor animals were infected i.v. with 5×10^4 bacteria 2 h before cell transfer. The antibacterial effect of an intravenous dose of 10^8 immune cells was then quantitated by determining the number of viable-organisms in the spleens of recipients 48 h after cell transfer.

Assays for T-cell function and presence

1. *Allograft survival.* Normal and D-AT × BM CBA hosts were grafted with H-2 incompatible A strain donor skin using standard techniques. The survival of the skin allografts was followed for 3 months and the end-point of rejection ascertained.

2. *Primary humoral immune response* in vitro *to a T-dependent antigen.* Spleen cells from normal and D-AT × BM mice were cultured *in vitro* with sheep erythrocytes (SE) for 4 days (Marbrook, 1967), and the ability of these cells to form IgM plaque forming cells (PFC) determined using essentially the method of Cunningham & Szenberg (1968) with certain modifications (Kongshavn & Lapp, 1972).

3. *Detection of θ-positive cells in the spleen.* Spleen cell suspensions were prepared from groups of normal, D-AT × BM, *Listeria*-infected normal and *Listeria*-infected D-AT × BM mice, and the percentage of θ-positive cells determined using a cytotoxicity assay with anti-θ antiserum and GPS as a source of complement (Raff, 1969). Cytotoxicity was assessed by the trypan blue dye exclusion method.

RESULTS

Evidence of T-cell depletion in D-AT × BM mice

1. *Acceptance of skin allografts*

Normal CBA mice rejected H-2-incompatible A strain skin allografts in $10{\cdot}5\pm0{\cdot}27$ days. All A strain allografts on D-AT × BM mice survived over the period during which the experiments with *L. monocytogenes* were performed. Nine out of eleven D-AT × BM mice retained their grafts for over 3 months.

2. *Failure to mount a primary humoral response to a T-dependent antigen*

Normal spleen cells cultured *in vitro* with SE developed a primary humoral immune response to SE (1719 ± 182 PFC per culture). Spleen cells of D-AT × BM animals cultured with SE produced only 127 ± 16 PFC per culture, which was within the background levels (normal splenocytes cultured without SE produced 190 ± 6 PFC).

3. *Absence of θ-positive cells in the spleens of D-AT × BM mice*

Twenty-six per cent of nucleated cells in the cell suspension prepared from the spleens of normal mice were sensitive to treatment with anti-θ serum and GPS. On the other hand, no θ-positive cells were detected in the spleen cell suspensions of D-AT × BM mice. Six days after injecting the animals with a sublethal dose of *L. monocytogenes*, 21·8

per cent spleen cells from normal mice exhibited θ-sensitivity, while only 1 per cent of such cells was found in the suspension of splenic cells from D-AT × BM animals. The values of the percentage of stained cells in experimental tubes were corrected by subtracting the percentage of dead cells in control tubes (c) and multiplying by a factor of 100/(100 − c) (Schlesinger & Hurvitz, 1968). The percentage cells dead in the control groups (with no antibody or no GPS) were 6·0–6·7 per cent for the normal spleens and 18·5–18·7 per cent for the D-AT × BM spleens.

Resistance of D-AT × BM to *L. monocytogenes*

1. *Survival of animals injected with live organisms* (Table 1)

Table 1. Effect of dose of *L. monocytogenes* on survival of normal and doubly AT× BM mice

	Dose of *L. monocytogenes*			
Treatment	10^3	5×10^3	10^4	5×10^4
Normal	10/10	3/10	0/10	0/5
Doubly AT× BM	10/10	9/10	5/10	3/5

Doses of live *Listeria* ranging from 10^3–5×10^4 were given i.v. At a dose of 5×10^3 only three out of ten normal mice survived whereas nine out of ten D-AT × BM mice survived. With even higher doses (10^4 and 5×10^4), which killed all normal mice, half of the D-AT × BM mice survived, thus actually showing enhanced resistance to *L. monocytogenes.*

2. *Growth of* L. monocytogenes *in the spleen* (Fig. 1)
Normal and D-AT × BM animals were injected i.v. with a sublethal dose of 10^3 virulent organisms and at timed intervals thereafter the number of bacteria recoverable from the spleens of groups of five mice was determined. Twenty-four hours after injection, significantly fewer organisms were found in the spleens of D-AT × BM animals. For the first 3 days there was a logarithmic bacterial growth in the spleen, similar to that seen in normal mice. The peak was reached on the 3rd day in both groups but the bacterial count never reached as high a value in the D-AT × BM mice. Following the log phase of bacterial growth, the normal mice could eliminate *Listeria* better than D-AT × BM mice, so that by the 7th day, there were fewer residual bacteria in the spleens of normal animals than in the spleens of D-AT × BM animals.

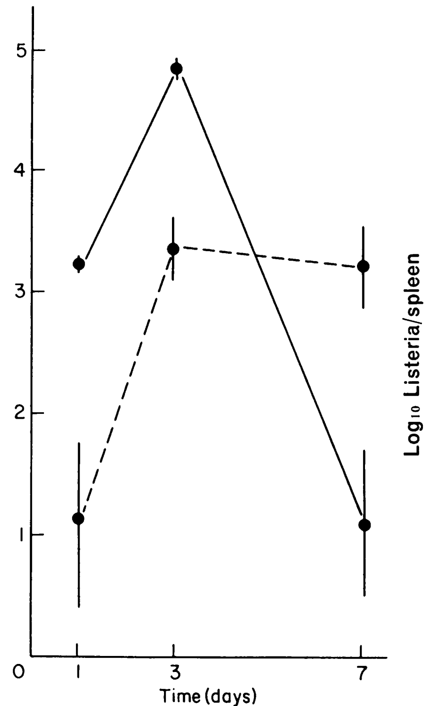

Figure 1. Viable counts of *L. monocytogenes* in spleens of normal (○——○) and doubly AT× BM (●– – –●) mice. Each point represents the mean of a group of five mice. The vertical bars enclose 1 s.e. mean.

Cell-mediated immunity of D-AT × BM animals to *L. monocytogenes*

1. *Delayed-type hypersensitivity (DTH) to* Listeria *antigens as detected by 24-h footpad swelling* (Fig. 2)
Mice immunized with a sublethal dose of live *Listeria* were injected with *Listeria* antigens into the footpad on day 6 when the DTH reaction is known to reach the peak. The footpad reaction was quantitated 24 h later. It can be seen that primed normal mice exhibited a DTH reaction which was 66 per cent greater than that of non-primed mice, whereas primed D-AT × BM mice showed a negligible DTH reaction.

2. *Adoptive transfer of resistance to* L. monocytogenes (Fig. 3)
Cell equivalents of splenic cell suspensions from normal and D-AT × BM animals were injected into naïve hosts which had been primed with *Listeria* 2 h prior to the cell transfer. The 2-h interval between bacterial challenge and the passive cell transfer was

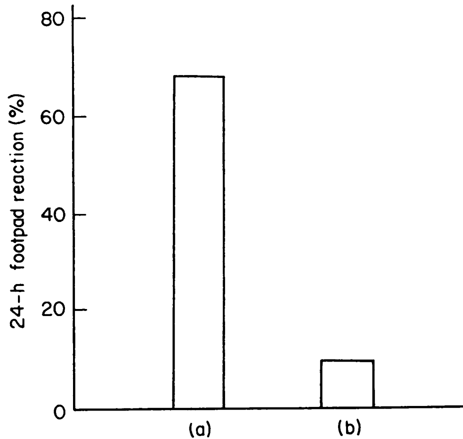

Figure 2. Delayed type hypersensitivity (24-h footpad reaction) to *Listeria* antigens of normal (a) and doubly AT × BM mice primed with 5×10^3 live *L. monocytogenes* (b) 1 week before the footpad assay. Means of a group of five mice.

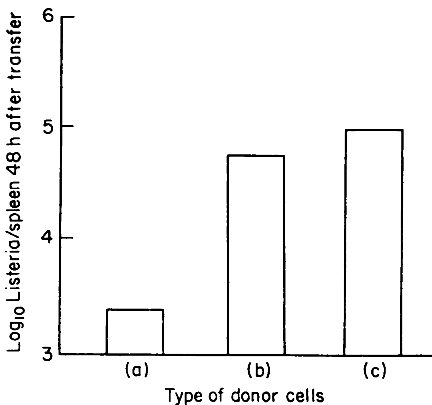

Figure 3. Adoptive transfer of resistance to *L. monocytogenes.* Viable counts of bacteria in spleens of mice injected 48 h earlier with 4×10^4 live Listeria followed in 2 h by injection of 10^8 viable splenocytes from three different groups of donors: (a) normal mice primed with 5×10^3 live Listeria 1 week earlier, (b) doubly AT × BM mice primed with 5×10^3 live *Listeria* 1 week earlier, (c) normal mice, untreated. Means of a group of five mice.

chosen to enable an unopposed lodging of the organisms within target organs. Cells from primed normal mice were able to prevent bacterial multiplication in the spleens of the secondary hosts whereas cells from primed D-AT × BM animals were no more effective in this respect than cells from normal, non-immunized mice.

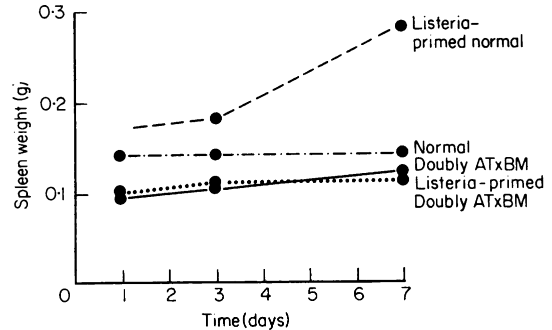

Figure 4. Changes in spleen weight of noninfected normal (O——O) and doubly AT × BM (O——O) mice, and of *Listeria*-infected normal (O- - -O) and doubly AT × BM (O- - -O) mice. Means of a group of five mice.

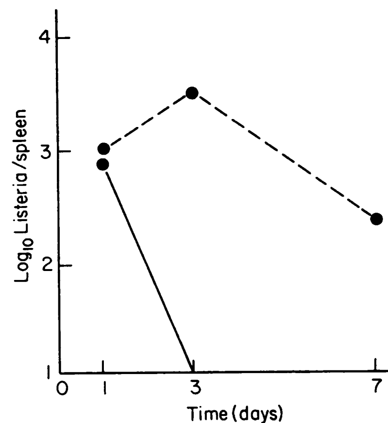

Figure 5. Secondary response to *L. monocytogenes.* Viable counts of bacteria in spleens of primed normal (O——O) and doubly AT × BM (O- - -O) mice following challenge with 10^4 live *Listeria.* Both groups of mice were primed with 10^3 live *Listeria* 2 weeks before secondary challenge. Means of a group of five mice.

3. *Splenomegaly in* Listeria-*primed animals* (Fig. 4)
Primed D-AT × BM mice did not develop any splenomegaly comparable to that seen in primed normal mice.

4. *Resistance to secondary challenge* (Fig. 5)
Groups of normal and D-AT × BM mice were primed with a sublethal dose of 10^3 *Listeria.* Two weeks later they were challenged with a dose of 10^4 organisms and the growth of bacteria in their spleens determined 3 and 7 days following the challenge. Whereas normal mice were able to

Table 2. Effect of dose of *L. monocytogenes* on survival of normal mice, splenectomized mice and splenectomized mice reconstituted with one splenic equivalent of normal splenocytes

	Dose of *L. monocytogenes*			
Treatment	10^3	5×10^3	10^4	5×10^4
Normal	9/10	4/10	0/10	0/5
Splenectomized	10/10	10/10	10/10	1/5
Splenectomized and reconstituted	5/5	10/10	9/10	0/5

eliminate all organisms within 3 days, D-AT × BM animals were much less able to inactivate the micro-organisms.

Resistance of splenectomized mice to *L. monocytogenes* (Table 2).

Splenectomized mice, challenged with a range of different doses of live *Listeira*, proved to be remarkably resistant to infection. All splenectomized animals survived a dose of 10^4 *Listeria*, which was fatal to all normal and sham-splenectomized mice. Splenectomized mice reconstituted with one splenic equivalent of spleen cell suspension were as resistant as the non-reconstituted splenectomized animals. The curves of bacterial multiplication in the livers of splenectomized mice show enhanced elimination of *Listeria* compared with normal mice (Fig. 6). The bacterial count in the livers of sham-splenectomized animals was identical to that of normal animals.

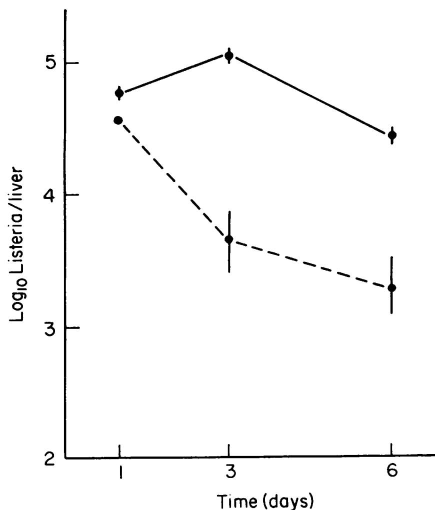

Figure 6. Viable counts of *L. monocytogenes* in livers of normal (O——O) and splenectomized (O– – –O) mice. Means of a group of five mice. Vertical bars enclose 1 s.e. mean.

DISCUSSION

In this study we have demonstrated that T cell-depleted mice are able to resist infections with *L. monocytogenes*. In fact, their resistance is superior to that of normal mice. Our results thus confirm the findings of other investigators in similar models of antilisterial resistance (Takeya, Mori & Nomoto, 1964; Blanden & Langman, 1972; Campbell, Martens, Cooper & McClatchy, 1974; Chan, Kongshavn & Skamene, 1975; Cheers & Waller, 1975; North, 1973) and also of resistance to *Brucella abortus* (Cheers & Waller, 1975) and *Mycobacterium leprae* (Prabhakaran, Harris & Krichheimer, 1975). The present studies provide strong evidence against the possibility, raised by others (North, 1973), of the presence of a residual T-cell population in 'T cell-depleted' mice, sufficient to activate macrophages which then express antibacterial activity. The D-AT × BM mice used in our experiments were shown to be T cell-depleted, by at least three independent criteria: their spleens lacked θ-positive lymphocytes; they were unable to mount a humoral immune response to a T-dependent antigen, SE; and they failed to reject H-2 incompatible skin allografts. The fact that they lacked the anamnestic response to secondary challenge with *Listeria*, that they exhibited no DTH to its antigens, and that their spleen cells lacked the ability to transfer adoptively antibacterial resistance to naïve hosts furnishes further indirect evidence to support the contention that the animals were functionally T cell-depleted. We conclude, therefore, that antibacterial resistance to primary infection with *L. monocytogenes* need not depend upon T cell-mediated reactions.

We interpret the above findings as being the result of enhanced native (primary) resistance to *Listeria* in D-AT × BM mice. The primary resistance is responsible for inactivation of the bacteria in the time period before a specific immune response develops. It is most likely that enhancement of this

primary resistance results in lowering the number of viable organisms which remain available for multiplication in the liver and spleen during the phase of logarithmic growth (the first 1–2 two days following i.v. infection by *Listeria*). The number of proliferating bacteria, therefore, never reaches a level high enough to cause death of the host. Subsequently, the normal cell-mediated immunity (which cannot develop in D-AT × BM animals due to the absence of T cells) is not necessary in this case for adequate protection. Thus, although under normal circumstances, acquired resistance and a specific immune response to *Listeria* develop in chronological association, the primary resistance can be manifested in the absence of detectable cell mediated immunity.

If, as we believe, residual T cell-mediated macrophage activation is not the cause of antilisterial resistance in D-AT × BM hosts, two other possible explanations remain. Either (1) the resistance is due to factors other than macrophage activation, or (2) the macrophage activation can be induced by mechanisms other than specifically activated T lymphocytes and/or their products.

With respect to the first possibility, it occurred to us that the resistance of D-AT × BM animals might be due to a 'functional absence' of the spleen, since the spleens of these animals are smaller than those of normal mice and the splenomegaly, characteristic of the response of normal mice to *Listeria* infection, is much diminished in *Listeria*-primed D-AT × BM mice (Fig. 4). When the organisms are administered i.v. a significant portion of the inoculum is normally taken up by the spleens where the bacteria replicate inside the splenic macrophages during the growth phase (Mackaness, 1962). As described earlier, the number of organisms found in the spleens of D-AT × BM mice in primary infection is much lower than that found in normal mice. Even by 24 h after injection of an identical dose, the number of bacteria recovered from the spleens of D-AT × BM animals is 100 times lower than that recovered from normal animals. Perhaps the spleen normally provides a hospitable environment, enabling the *Listeria* organisms to multiply and kill the host in the case of a lethal dose. The spleen in the D-AT × BM mice might thus constitute a relatively hostile environment giving the host a greater than normal resistance. This hypothesis is substantiated by the finding that splenectomized mice, like D-AT × BM mice, are also highly resistant to *Listeria* infection. At the moment we have no data to disprove this hypothesis although, as will be discussed later, it does not seem to be the only mechanism responsible for the increased resistance.

With respect to the second possibility, it seems very likely that nonspecifically induced increased bactericidal activity of mononuclear phagocytes in T cell-depleted and splenectomized animals is the major reason for their enhanced resistance. It was noted sometime ago by Takeya, Mori & Imaizumi (1968), that multiplication of *L. monocytogenes* is suppressed within macrophages from neonatally thymectomized mice. This observation was later extended by Zinkernagel & Blanden (1975) who showed that peritoneal macrophages of nude mice were more efficient at phagocytosis of *Listeria* than normal control macrophages and by Cheers & Waller (1975) who demonstrated enhanced macrophage activity and increased resistance to *Listeria* infection by nude mice. In addition, recent findings in this laboratory suggest that adult splenectomized mice possess peritoneal exudate macrophages with enhanced ability both to phagocytose and to kill [^{3}H]thymidine-labelled *Listeria* organisms (unpublished results). These observations are in agreement with our finding that there is a greater reduction in numbers of viable bacteria in the livers of splenectomized mice than in the livers of normal mice (Fig. 6) and with the observation that the clearance of *L. monocytogenes* from the bloodstream of AT × BM animals is much more efficient than in normal mice (Zinkernagel & Blanden, 1975). In the latter case, however, the contribution of polymorphonuclear leucocytes to enhanced bacterial clearance cannot be ruled out, especially since it has been noted that AT × BM mice contain twice as many polymorphonuclear leucocytes in their peripheral blood (Campbell, Martens, Cooper & McClatchy, 1974).

If immunodeprived mice (nude, neonatally thymectomized, sublethally irradiated, AT × BM, D-AT × BM, splenectomized) possess macrophages with enhanced bactericidal properties before the introduction of *Listeria* organisms, the question then arises as to what is the stimulus for such macrophage activation. One proposal has been that leakage of the gut flora through the gut wall, damaged either by irradiation (Campbell, Martens, Cooper & McClatchy, 1974) or by bacterial penetration in the absence of T-dependent IgA antibodies in nude mice (Cheers & Waller, 1975) allows the

phospholipid cell walls of enteric bacteria to non-specifically stimulate the macrophages (Fauve & Hevin, 1974). However, such a mechanism would be highly unlikely in the case of splenectomized animals since no gut damage or enteric bacterial overgrowth can there be postulated. Another possibility is that cells other than T cells can produce factors leading to macrophage activation, and that T cells themselves can under normal circumstances suppress the activity of such cells and/or of macrophages themselves. Participation of B cells in such a macrophage activating role has already been proposed (Campbell, Martens, Cooper & McClatchy, 1974). T cell-directed suppression of macrophage activation has not yet been demonstrated, although there is now ample experimental evidence for a suppressive role of T cells in other immune processes (Katz, 1972). Based on our experimental results, such a T-cell population responsible for suppressing macrophage activation would be localized in the spleen [similar to suppressive T cells controlling graft-*vs*-host reaction (Gershon, Lance & Kondo, 1974) and homograft rejection (Streilein, Grebe, Kaplan & Streilein, 1975)] and require an intact spleen for its function.

It seems probable that the initial steps of macrophage activation following an immunodeprivation procedure would involve an increased mitotic activity of macrophage precursors. In this respect, it has been noted by Van Furth & Cohn (1968) in the course of their studies on the origin and kinetics of mononuclear phagocytes that such procedures as splenectomy, or lethal irradiation combined with shielding part of the bone marrow, result in a greatly increased percentage of newly divided macrophages appearing in the peritoneal cavity. It is likely that those macrophages which are activated before infection with *Listeria* are the effector cells of primary resistance in the initial encounter with bacteria in the T cell-deprived mice.

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