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Enhanced resistance to *Listeria monocytogenes* in splenectomized mice*

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Summary. Mice infected with live *Listeria monocytogenes* intravenously from 1 week to 3 months following splenectomy exhibit greatly enhanced antibacterial resistance to this micro-organism as compared to normal or sham-splenectomized mice. They survive a dose of *Listeria* 100 times higher than is the LD_{50} of this parasite for normal mice. Initially, the same number of viable micro-organisms lodge in the livers of splenectomized and normal hosts. However, within 24 h after infection, the number of viable *Listeria* which can be recovered from the livers of splenectomized animals is significantly reduced in comparison with control mice. This effect of splenectomy is transient and gradually disappears spontaneously within 3 months following splenectomy. Enhancement of anti-listerial resistance in splenectomized mice can be abrogated by the transfer of normal spleen cells. The presence of a normal splenic cell population that controls macrophage activation is postulated.

INTRODUCTION

The spleen plays an important role in the regulation of cell-mediated immune processes (Battisto & Streilein, 1976). Under certain experimental conditions it can be shown that some types of cell-mediated immunity can be enhanced by neonatal or adult splenectomy. Delayed hypersensitivity to sheep erythrocytes is greatly increased in splenectomized animals (Lagrange, Mackaness & Miller, 1974). Postnatal splenectomy increases the proliferative response of thymocytes to concanavalin A (Papiernik, 1976). With several transplantable tumours, splenectomy of the host has led to an increased number of regressions, reduced tumour growth and lowered the number of metastases (Colmerauer & Pasqualini, 1975). Allograft rejection, in the hands of some authors, can also be enhanced by splenectomy (Streilein, Grebe, Kaplan & Streilein, 1975). There are, at the moment, two possible explanations for such an immunoregulatory action of the spleen. Some authors favour the explanation that splenectomy decreases the production of humoral antibody which, under normal circumstances, exerts feedback regulation of cell-mediated immunity to the same antigen via antibody–antigen complex formation (Lagrange *et al.*, 1974). Others have suggested that the imbalance between humoral and cellular immune responses caused by splenectomy occurs centrally. According to these authors, the spleen normally deflects some T lymphocytes from effector (killer) cells toward helper cells and thus shifts the balance of the total efferent response from cellular towards humoral immunity (Streilein & Read, 1976).

An alternative explanation of the observed enhancement of cell-mediated immunity in splenec-

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tomized animals is the removal of a suppressor T cell population. The existence of such spleen-localized suppressor T cells has been demonstrated both by adoptive transfer experiments in the model of the graft-vs-host reaction (Gershon, Lance & Kondo, 1974) and in the mixed leucocyte reaction *in vitro* (Sampson, Kauffman, Grotelueschen & Metzig, 1976).

It appeared to us that useful information on the feedback mechanism exerted by the spleen on cell-mediated immune reactions would be derived from an *in vivo* experimental model in which the participation of humoral antibody could be excluded. Development of acquired resistance to infection with *Listeria monocytogenes* is dependent on the production of specifically sensitized T cell mediators which are responsible for activation of the effector cells of resistance-macrophages (Mackaness, 1969). The humoral response to *Listeria* is not a significant component of an acquired resistance to this parasite (Mackaness, 1962).

Splenectomized mice have been found to be more resistant to *Listeria* infection than normal mice (Chan, Kongshavn & Skamene, 1977). As the inhibition of humoral feedback by splenectomy is unlikely reason for the increased resistance of these animals, this model provides an opportunity to investigate the possible control mechanisms responsible for the regulation of cellular processes involved in the defence against an infection with a facultative intracellular parasite.

MATERIAL AND METHODS

Mice

Male C_3H/HeJ mice were purchased from Jackson Laboratories, Bar Harbor, Maine.

Splenectomy and sham-splenectomy of mice

Splenectomy was performed in mice anaesthetized with 0·08 mg/g body weight sodium pentobarbital. 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 mobilized outside the abdominal cavity and then returned to the peritoneum. No antibiotics were given.

Bacteria

Listeria 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 standard curve.

Infection of mice and testing of resistance to Listeria monocytogenes

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

(a) *Bacterial growth.* The number of viable organisms in the spleen and livers of infected animals was established by plating out 10-fold dilutions of organ homogenates in saline on tryptose agar, and the colony count performed 18 h later. This was expressed as the number of colony forming units (CFU) of *Listeria.*

(b) *Survival of animals.* Doses of CFU ranging from 10^3 to 10^6 in half log intervals were injected into groups of six experimental animals and animal survival determined. Deaths occurred usually between the 4th and 7th day after infection. All the animals which survived the 7th day usually remained alive and well until the time the experiments were completed.

(c) *Resistance of* Listeria*-primed animals to re-infection.* Animals which survived primary sublethal infection were infected with graded doses of *Listeria* 2 weeks after the primary infection. Subsequently, the bacterial counts were performed on liver homogenates.

(d) *Delayed hypersensitivity to* Listeria *antigens.* Twenty μl of *Listeria* antigens containing 50 μg 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 volume was used.

(e) *Measurement of anti-listerial antibody. Listeria* antigens prepared as described in the previous

paragraph were coupled to the sheep erythrocytes using gluteraldehyde (Ling, 1961) and the mouse anti-listerial antibody was measured using the method of passive haemagglutination. Rabbit anti-listerial antibody obtained from animals immunized with killed micro-organisms was used as a positive control (titre 8192).

Examination of peripheral white blood count

The peripheral white blood count (WBC) was determined on blood taken from the retro-orbital venous plexus. A total white cell count was performed by diluting the blood with Turk's solution. Blood smears were stained with Wright's stain and a differential count made.

Cell transfer studies

Spleen cell suspensions were made by gently tamping fragments of spleen through sterile steel mesh screen. The cells were then filtered through gauze and washed in cold sterile medium 199. Viable cells were counted by using the trypan blue dye exclusion method. One 'spleen equivalent' or about 10^8 viable cells were injected intravenously in 1 ml volume on the day of splenectomy.

RESULTS

Resistance of splenectomized mice to *Listeria* infection

Groups of 6 mice (normal, splenectomized and sham-splenectomized) were injected with different doses of *Listeria* and their survival recorded daily. The LD_{50} dose for normal mice was about 10^4 CFU of *Listeria*. All splenectomized animals survived this dose and also the dose of 10^5 CFU. The LD_{50} dose for splenectomized mice was about 10^6 CFU which was a hundred times more than the LD_{50} for normal mice (Table 1). Sham-splenectomized mice behaved like normal mice.

Uptake of *Listeria* and its proliferation in livers of splenectomized mice

Experiments were performed to ascertain whether there was any difference in the initial uptake of *Listeria* by the livers of splenectomized mice as compared to normal animals. Groups of five mice, normal and splenectomized, were injected with 10^8 CFU of *Listeria*, killed at selected intervals during the first 5 h following infection and the number of CFU in their livers compared (Fig. 1). There was no difference in the number of live bacteria recovered from the livers of normal and splenectomized mice. It should be pointed out that the dose of bacteria (10^8 CFU) far exceeded the lethal dose for both groups of animals. However, since this was only a short-term experiment, terminating in less than one day, this did not create any problem with survival and the number of recovered bacteria was easy to quantify.

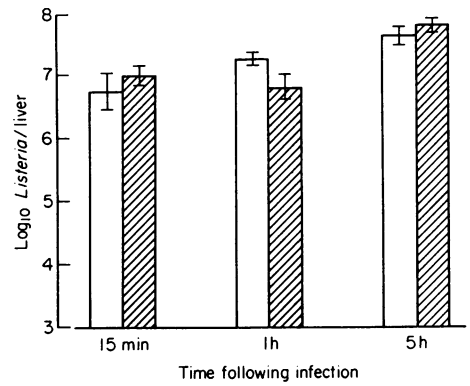

Figure 1. Counts of viable *Listeria* in livers of normal and splenectomized mice. Each bar represents the mean of a group of 5 mice ± standard error of the mean. Open columns, normal; hatched columns, splenectomized.

Table 1. Survival of splenectomized mice infected with *Listeria*

Dose of *Listeria*	10^4			10^5			10^6		
Days following infection	3	6	10	3	6	10	3	6	10
Normal	6/6	4/6	2/6	5/6	1/6	0/6	2/6	0/6	0/6
Sham splenectomized	6/6	3/6	3/6	6/6	0/6	0/6	1/6	1/6	0/6
Splenectomized	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	3/6

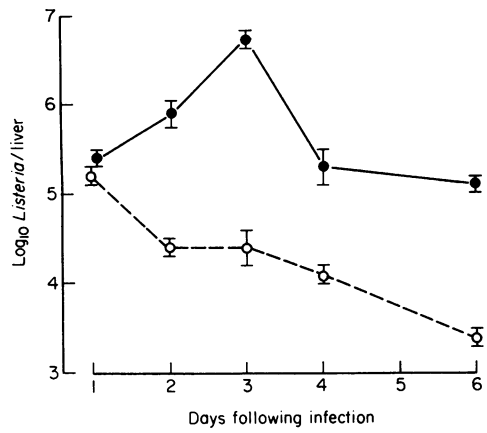

Figure 2. Counts of viable *Listeria* in livers of normal and splenectomized mice. (●) Normal; (○) splenectomized.

A chronological study of bacterial proliferation in the livers of normal and splenectomized mice was then performed (Fig. 2). Groups of five mice were infected with 5×10^3 CFU *Listeria* and selected groups were killed daily and the number of CFU recovered from their livers was calculated. There was no difference in the number of live *Listeria* in the livers of splenectomized and normal mice one day after infection thus confirming the previous experiment (Fig. 1) which showed no difference in bacterial numbers during the first day. However, thereafter there was a dramatic difference in the bacterial kinetics between these two types of hosts. Whereas *Listeria* organisms proliferated logarithmically in the livers of normal animals until the peak on the 3rd day, there was a steady decline in the number of CFU in the livers of splenectomized mice. By the 6th day there was a 100-fold difference in the numbers of bacteria present in the livers of normal and splenectomized mice. No anti-listerial antibody could be detected in either normal or splenectomized animals following primary immunization.

Development of immunity to *Listeria* in splenectomized mice

Groups of normal and splenectomized mice were infected with 5×10^3 CFU *Listeria*. Two weeks later the animals were divided in two subgroups. Half of the animals were tested for delayed hypersensitivity to *Listeria* antigens (footpad swelling) and another half received a secondary dose of 10^3 CFU and were killed 72 h later. The result of this experiment is depicted in Table 2. Splenectomized animals showed only mild degree of delayed hypersensitivity to *Listeria* and their ability to inactivate secondary challenge of *Listeria* was inferior to that of normal primed mice. In fact, the resistance of splenectomized mice to secondary *Listeria* infection was not different from their primary resistance.

Peripheral white blood cell count in splenectomized mice

Peripheral white blood cell counts performed on blood samples of animals at different intervals following splenectomy revealed that no difference from the values obtained in normal mice and remained within the level of 12–18,000 WBC per mm^3. There was no difference in the differential count of peripheral white blood between those 2 groups of animals.

Table 2. Delayed hypersensitivity and secondary anti-listerial resistance of splenectomized mice

	% Footpad swelling†	Log_{10} CFU/liver‡
Normal (primed)*	135 ± 27	1·03 ± 0·09
Splenectomized (primed)*	33 ± 13	3·81 ± 0·34§
Normal	11 ± 3	6·13 ± 0·52
Splenectomized	10 ± 4	4·22 ± 0·21§

* Mice were given 5×10^3 CFU *Listeria* 2 weeks prior to this experiment.
† Compared with contralateral footpad injected with saline only.
‡ Recovery of viable *Listeria* from the liver of mice 3 days after infection with 10^3 CFU *Listeria*.
§ There is no statistical difference between these 2 groups.

Anti-listerial resistance of splenectomized mice at different intervals after splenectomy

Groups of five mice were splenectomized, first at weekly intervals and later at monthly intervals. At the end of a 3 month period all the groups and an additional group of normal mice were injected with 5×10^3 CFU *Listeria*. On the basis of the results presented in Fig. 2, the bacterial count in the liver on the 6th day of infection was chosen as the parameter to measure the degree of host resistance.

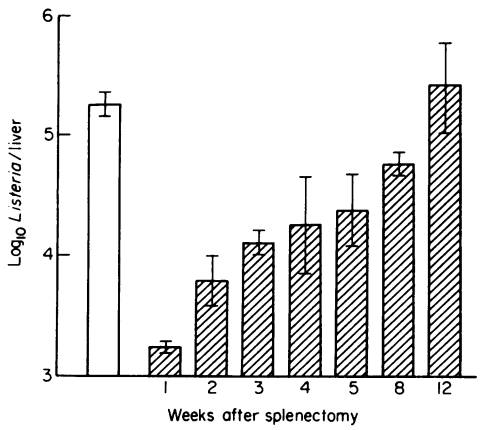

Figure 3. Counts of viable *Listeria* in livers of normal and splenectomized mice at different times following splenectomy. Bacterial counts were performed 6 days after infection with 5×10^3 CFU *Listeria*. Open column, normal; hatched columns, splenectomized.

It was found that the enhanced resistance was most impressive shortly after splenectomy. It could be demonstrated as early as 1 day following the splenectomy and was optimal 1 week post-splenectomy. With time following splenectomy, however, the enhanced resistance gradually returned to normal and by 3 months post-splenectomy, there was no difference in the anti-listerial resistance shown by splenectomized and control mice (Fig. 3).

Abrogation of enhanced anti-listerial resistance by transfer of normal splenocytes

Groups of mice were splenectomized and within 6 h were repopulated by syngeneic spleen cells in the amount of one 'spleen equivalent' ($0{\cdot}8$–$1{\cdot}3 \times 10^8$ of viable mononuclear spleen cells) per mouse. They were infected with *Listeria* at predetermined intervals following repopulation and the bacterial count in the liver was enumerated 3 and 6 days later. When the mice were infected shortly after splenectomy and repopulation (1–2 weeks), no effect was observed. However, when 4 weeks were allowed to elapse before the infection, the splenectomized mice which had been repopulated with normal spleen cells showed decreased anti-listerial resistance when compared with splenectomized mice that had not been infused with spleen cells (Table 3). Such abrogation of enhanced resistance by passive transfer of normal spleen cells was not always successful in repeated experiments when a sublethal dose of *Listeria* (5×10^3 CFU) was used. Successful passive transfer as demonstrated in Table 3 was usually achieved when a lower dose of *Listeria* (5×10^2 CFU), resulting in only 10-fold difference between the numbers of bacteria in the livers of normal and splenectomized animals, was used.

DISCUSSION

Our observation of an increased resistance of splenectomized mice to *Listeria* is an unusual one in the repertoire of known infectious consequences of splenectomy. Asplenic states are usually characterized by defects in defence, rather than by enhancement. It is well known that patients, mainly

Table 3. Effect of splenocyte infusion of anti-listerial resistance of splenectomized mice

Day	Normal mice		Splenectomized mice*	
		+ spleen cells		+ spleen cells
3	$4{\cdot}65 \pm 0{\cdot}12$	$4{\cdot}66 \pm 0{\cdot}23$	$3{\cdot}41 \pm 0{\cdot}15$	$4{\cdot}51 \pm 0{\cdot}33$
6	$3{\cdot}45 \pm 0{\cdot}15$	$3{\cdot}74 \pm 0{\cdot}11$	$2{\cdot}26 \pm 0{\cdot}09$	$3{\cdot}52 \pm 0{\cdot}34$

Log_{10} *Listeria* in the liver of mice infected with 5×10^2 CFU.
* Splenectomy performed 4 weeks before infection with *Listeria*.

children, who have undergone splenectomy suffer from severe, often fatal infections with micro-organisms possessing polysaccharide capsule such as *Pneumococcus*, *Meningococcus* and *Hemophilus Influenzae* (Editorial, 1976). This effect is the result of defective formation of opsonins in the absence of the spleen, with resulting inability of polymorphonuclear leucocytes to phagocytose such pyogenic bacteria (Bogart, Biggar & Good, 1972). It is interesting that asplenic patients do not suffer from infections with micro-organisms *Mycobacterium tuberculosis* or *Listeria monocytogenes* which are destroyed by the concerted action of lymphocytes and macrophages.

The natural course of experimental *Listeria* infection in mice is such that the liver captures over 90% of an intravenously injected inoculum of bacteria (the remaining 10% can be found in the spleen) and can destroy about 50% of this bacterial growth within 12 h after infection as a result of the bactericidal activity of natively resistant macrophages (North, 1974). The remaining bacteria begin to multiply in a logarithmic fashion (and some of these will again be destroyed by the continuous bactericidal activity of normal macrophages) until such time when liver macrophages become strongly activated, most likely through the action of *Listeria*-immune T cell mediators (Simon & Sheagren, 1971). This occurs about 3 days after the infection (Blanden & Langman, 1972). Increased anti-listerial resistance of splenectomized mice as described in this paper can therefore be caused either by a decrease in the number of micro-organisms that reach the liver in the first phase of the infection, or by an increased macrophage activity in the liver, either during the non-immune or the immune phase of the response.

It is possible that the absence of the spleen leads to an alteration in the normal pattern of *Listeria* traffic after intravenous injection. The spleen normally provides a hospitable environment for 5–10% of the total load of intravenously injected *Listeria* which lodges there and it contributes significantly to the total numbers of bacteria which can be recovered from mice at the peak of infection. However, the fact that splenectomized animals lose their enhanced antilisterial resistance after a certain period of time (3 months) following splenectomy argues against the possibility that the anatomic abnormality itself is responsible for the described effect, and suggests that host–parasite interactions within the spleen are not crucial in the final outcome of this bacterial infection. A similar argument can be advanced to rule out the possibility that significant numbers of *Listeria* are destroyed in the splenectomized host before they can reach the liver. The spleen is the major haemopoietic organ in the mouse and its removal may be expected to cause an increased output and turnover of haemopoietic precursors from other foci of haemopoiesis, e.g. from the bone marrow. This may possibly lead to monocytosis or to an increased monocyte-associated phagocytosis in the peripheral blood. This possibility seems unlikely in our model. First of all, the peripheral white blood count and the differential count has not changed significantly following splenectomy. Moreover, the number of *Listeria* organisms which lodge in, and can be recovered from, the liver of splenectomized animals is very similar to that found in normal animals when measured 1, 5 or 24 h after infection. This observation also argues against the other possible explanation of increased antilisterial resistance in splenectomized mice, i.e. that there is an increase in the macrophage activity in splenectomized animals even *before* the infection with *Listeria*. The presence of such activated macrophages has been documented in other models of immunologically deprived animals such as thymectomized (Takeya, Mori & Imaizumi, 1968), thymectomized and irradiated (Cheers & Waller, 1975) and nude mice (Zinkernagel & Blanden, 1975). This phenomenon had been usually explained by the presence of inapparent infection, either exogenous or endogenous, in the immunodeficient animals resulting in nonspecifically increased 'native resistance' (Meltzer, 1976). Our data (both the absence of any appreciable decrease in the numbers of *Listeria* in the livers of experimental animals compared with controls between 1–24 h post-infection, and the loss of the enhanced resistance of splenectomized mice with time) suggest that this was not the mechanism of protection in our model.

On the basis of foregoing discussion it is therefore possible to conclude that there is no difference in the handling of *Listeria* between the normal and the splenectomized host for at least 24 h following the infection, i.e. during the phase of the liver uptake of *Listeria* and during the first wave of bacterial killing by natively resistant liver macrophages. The question then arises whether, from that time on, the more efficient inactivation of *Listeria* in splenectomized mice is due to the enhancement of specific

antilisterial immunity or to an enhanced activity of natively resistant mononuclear phagocytes within the liver brought about by the bacterium itself, without T cell participation. Our results favour the latter mechanism although the evidence presented here is admittedly indirect: (1) As mentioned above, the bacterial proliferation in the liver until the 3rd day post-infection, as detected by the recovery of viable *Listeria* from the organ homogenate, is the result of two opposite processes: bacterial multiplication and bactericidal action of natively resistant macrophages. If this macrophage population is destroyed, as happens for example in animals treated with Dextran 500, the rate of bacterial proliferation is greatly increased (Hahn, 1975). Specific antilisterial immunity resulting in the production of activated macrophages with decisively more potent bacterial powers than the natively resistant macrophages needs at least 3 days for its development (Blanden & Langman, 1973). Since splenectomized mice already show greater antilisterial efficiency in their liver between 24 and 72 h following infection, the participation of the specific immune reaction in this process is unlikely. (2) The parameters of cell-mediated *immune* response to *Listeria*, e.g. delayed hypersensitivity to *Listeria* antigens and increased resistance to secondary infection (Mackaness, 1969), could not be demonstrated in splenectomized mice. It is likely that splenectomy removes a cell population responsible for the development of cell-mediated immunity to *Listeria*, similar to that postulated as being responsible for the delayed hypersensitivity of mice to sheep erythrocytes and ovalbumin (Kettman & Lubet, 1976). As expected, no antilisterial antibody was found in either the splenectomized or normal mice following intravenous infection with live *Listeria*. The absence of significant antilisterial immunity in splenectomized animals favours, therefore, the idea that their increased resistance to *Listeria* results from enhanced antimicrobial activity of natively resistant macrophages expressed within 24 h following the exposure of the host to *Listeria*.

The results of experiments to test this hypothesis reported elsewhere (Skamene & Chayasirisobhon, 1977) suggest that this, indeed, is the case. Splenectomized animals exhibit increased readiness for blood clearance and liver uptake of foreign particles (e.g. Cr^{51}-labelled sheep erythrocytes) following infection with *Listeria* when compared with normal *Listeria*-infected mice. Furthermore, there is an increased proportion of actively phagocytosing cells among the peritoneal macrophages of splenectomized mice exposed to *Listeria* for 24 h in comparison with peritoneal exudate cells of normal, *Listeria*-stimulated animals.

We interpret our findings as showing that there is a cell population and/or factor within the mouse spleen which under normal circumstances regulates activity of natively resistant macrophages. This population and/or factor is replaced spontaneously in adult mice within three months of splenectomy and can be passively transferred with a normal suspension of spleen cells. Whether this is a suppressor T cell (or its product) as is known from the models of B cell and T cell mediated responses in other systems remains to be elucidated.

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