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Two steps in the generation of acquired cellular resistance against *Listeria monocytogenes*: accumulation and activation of macrophages

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Summary. Mice were immunized with 1×10^3 viable *Listeria monocytogenes*, and the mechanism of the acquired resistance against challenge infection with 5×10^4 *L. monocytogenes* was studied by the use of the peritoneal cavity of mice as the site of challenge. An enhanced elimination of bacteria from the peritoneal cavity became detectable on day 5 after immunization, and lasted thereafter. Before day 10 postimmunization, a marked accumulation of macrophages was observed after the challenge but the *in vitro* listericidal activity of macrophages was not so enhanced. After day 15 postimmunization, peritoneal macrophages did not increase in number after the challenge but the *in vitro* listericidal activity of macrophages was the stronger. Accumulation of non-activated macrophages seemed to contribute mainly to the expression of acquired resistance against challenge in the early stage of immunization. So-called activated macrophages appeared to be generated only in the later stage of immunization. Thus it was suggested that there may be at least two steps in the expression of acquired listerial resistance.

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INTRODUCTION

Although various kinds of humoral and cellular factors contribute to the resistance against bacterial infection, phagocytes including macrophages and polymorphonuclear cells seem to be the most important in the resistance against many types of bacteria. At an early stage of primary infection of mice with *Listeria monocytogenes*, the growth of bacteria in organs was markedly enhanced by treatment with blockers of macrophage function as carrageenan (Tatsukawa, Mitsuyama, Takeya, & Nomoto, 1979), dextran sulphate 500 (Hahn, 1974), carbon or silica (Takeya, Shimotori, Taniguchi & Nomoto, 1977). At such a stage, accumulation of normal macrophages to infected sites was suggested to be necessary for the expression of resistance (Mitsuyama, Takeya, Nomoto & Shimotori, 1978).

Cellular immunity has been shown to be required for the complete elimination of *L. monocytogenes* at a later stage of primary infection (Takeya, Shimotori, Taniguchi & Nomoto, 1977). This type of enhanced resistance appears to be governed by cell-mediated immunity, since it cannot be induced in athymic nude mice (Emmerling, Finger & Bockemuhl, 1975; Emmerling, Finger & Hof, 1977). Macrophages were shown to contribute to the expression of such acquired cellular resistance (ACR), and the generation of activated macrophages has been presumed to be

essential for the expression of ACR and to be attributable to macrophage-activating factor (MAF), one of lymphokines, incapable of being distinguished biochemically from macrophage migration inhibitory factor (MIF; Nathan, Remold & David, 1973).

In our previous study (Mitsuyama *et al.*, 1978), the number of *L. monocytogenes* in organs started to decline after day 3 of primary infection and reached an undetectable level by day 10. However, MIF could not be detected before day 10 by the direct assay of MIF (Mitsuyama, Nomoto, Akeda & Takeya, 1980). MIF appears not to be essential for the expression of ACR against *L. monocytogenes* at least under some conditions. The present study was carried out to know the role of an accelerated macrophage accumulation mediated by cellular immunity in the expression of ACR against *L. monocytogenes*. The peritoneal cavity was chosen as the site of challenge infection to follow the cellular accumulation and to obtain macrophages from the infected site.

MATERIALS AND METHODS

Animals

Female mice (7–11 week old) of an outbred ddY strain were purchased from a local breeder (Kyudo Experimental Animal Co., Kumamoto, Japan). Each experimental group consisted of four or five mice.

Micro-organisms

Listeria monocytogenes strain EGD and *Salmonella typhimurium* strain LT-2 were maintained by serial passage in outbred ddY mice. Fresh isolates were obtained from infected spleens, grown in tryptic soy broth (Difco), and stored at −70° in phosphate-buffered saline (PBS) until required. *Mycobacterium bovis*, Bacille Calmette-Guérin (BCG), was grown in Ogawa medium, and stored at −70° in PBS. Killed BCG were prepared by heating at 60° for 30 min.

Immunization

Mice were inoculated intravenously (i.v.) with $1–2 \times 10^3$ viable bacteria.

Determination of the bacterial growth

Control and immunized mice were challenged intraperitoneally (i.p.) with 5×10^4 viable bacteria suspended in PBS. Twenty-four or forty-eight hours after the inoculation, the peritoneal cavities were washed with 5 ml of Hanks's balanced salt solution (HBSS). The number of viable bacteria in the peritoneal cavity of infected mice was determined by plating ten-fold dilutions of recovered HBSS on nutrient agar containing 0·4% (w/v) glucose.

Preparation of peritoneal cells

Peritoneal cells were recovered by lavage of the peritoneal cavity with HBSS. The number of viable cells was determined by trypan blue dye exclusion method in a haemocytometer. Differential cell counts were performed on Giemsa stained smears of peritoneal cells. The number of macrophages in the total peritoneal cell population, was determined by injecting mice i.p. with 0·5 ml of latex particle suspended in PBS and counting cells with ingested particles.

Phagocytosis and intracellular killing by peritoneal macrophages

Peritoneal cells were washed twice with HBSS and resuspended at 1×10^7 cells/ml in RPMI 1640 medium supplemented with 10% foetal calf serum. Cell suspensions were placed in triplicate plastic petri dishes with washed cover slips. The petri dishes were incubated for 1 hr at 37° in a CO_2 incubator. The petri dishes were washed twice with the medium to remove non-adherent cells, and the monolayer of adherent cells was obtained. The fresh RPMI 1640 medium and 100 units of Penicillin G/ml was added to the petri dishes. The petri dishes were incubated for 16 hr at 37°, and washed twice. Bacterial suspension containing 5×10^6 listeria/ml and 10% fresh autologous serum was added to the petri dishes. The cells were incubated for 20 min at 37° to allow phagocytosis of bacteria, and washed three times to remove free bacteria. The cover slip pieces were stained by Giemsa solution, and examined. For estimating the degree of phagocytosis, the number of phagocytozed bacteria in 100 macrophages was counted. In order to observe the rate of intracellular killing, fresh RPMI was added to the petri dishes, and the cells were incubated for 2 hr. The number of bacteria remaining in 100 macrophages was counted as described above. The rate of intracellular killing was determined according to the following formula: % intracellular killing = [(no. of bacteria after phagocytosis) − (no. of bacteria after 2 hr incubation)/(no. of bacteria after phagocytosis)] × 100.

Statistics

Results were expressed as mean ± standard error of the mean of groups of four or five mice, and results were compared by Student's *t* test.

RESULTS

Time course of bacterial elimination from the peritoneal cavity in immunized mice

In order to assess the ability of mice to eliminate bacteria from the peritoneal cavity mice were challenged i.p. with 5×10^4 viable bacteria on days 5, 10, 15 and 20 after the immunization on day 0, and the number of bacteria was examined 24 hr later. An enhanced elimination of bacteria was observed on day 5 postimmunization. The degree of enhancement became more pronounced after day 10 postimmunization (Fig. 1). With a higher dose of challenge (1×10^6), the same pattern of resistance against challenge infection was observed (data not shown).

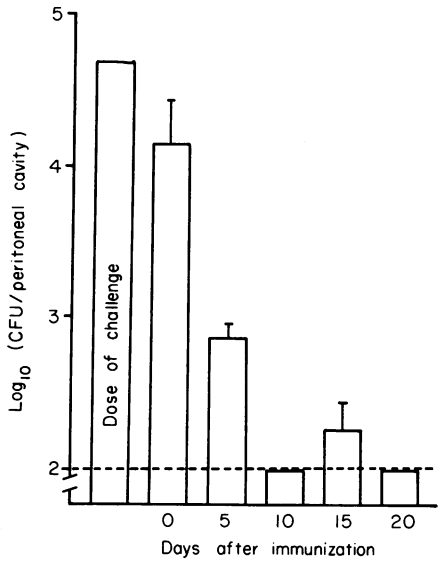

Figure 1. Elimination of *L. monocytogenes* from the peritoneal cavity after challenge infection in mice immunized with 2×10^3 listeria on day 0. Total bactertial count (log) in the peritoneal cavity 24 hr after the challenge is indicated. (Mean ±SEM.)

Phagocytosis and intracellular killing of bacteria *in vitro*

Phagocytosis and listericidal activities of peritoneal macrophages were compared among control mice and mice immunized with viable bacteria 5–15 days previously. There was no significant difference in the degree of phagocytosis by macrophages among these three groups of mice (Table 1). The intracellular killing activity of macrophages obtained from mice immunized 15 days previously was significantly greater compared with that of macrophages from control mice and mice immunized 5 days previously. Enhanced elimination of bacteria after challenge at the later stage of infection seemed to be mediated by activated macrophages, but enhanced elimination at the early stage could not be explained in this way. In order to study other factors mediating anti-listerial activity at the early stage, the change in number of peritoneal cells was studied as shown below.

Time course of the change in peritoneal cell number after intraperitoneal challenge in immunized mice

Time course of the change in peritoneal cell number was studied after i.p. inoculation with viable listeria in immunized mice. The total number of peritoneal cells

Table 1. Phagocytosis and intracellular killing of *L. monocytogenes* by peritoneal macrophages *in vitro*

Mice	Number of listeria in 100 macrophages after phagocytosis*	Number of listeria in 100 macrophages after 120 min incubation†	Killing (%) after 120 min incubation
Non-immunized	102·7±11·9‡	68·3±13·5	33·5
Immunized 5 days previously	112·0± 7·1	68·7±15·1	38·7
Immunized 15 days previously	70·3±14·1	17·3± 6·0	75·4§

* Number of phagocytozed listeria in 100 macrophages after incubation with listeria for 20 min.

† Number of listeria in 100 macrophages at further 120 min incubation after phagocytosis.

‡ Mean ±SEM.

§ $P < 0.05$.

was counted after 24 hr and was compared with that in mice injected with PBS (Fig. 2a). In PBS-injected immune mice, no change was observed throughout the observation time. From 5 to 10 days after immunization, a marked increase was observed in the total number of cells following bacterial challenge. After day 15 of immunization, no such increase was observed. The changes in the number of peritoneal macrophages are indicated in Fig. 2b. The changes in the total number seemed to be mainly due to the changes in the macrophage number. The increase of macrophages at the early stage may be caused by an enhanced influx of macrophages into the peritoneal cavity.

Time course of the changes in peritoneal macrophages in number and bacterial elimination from the peritoneal cavity

These results indicated that ACR was expressed after day 5 postimmunization, even in the absence of augmented bactericidal activity of peritoneal macrophages. Only an increase of peritoneal macrophages in number was observed in this early stage. In groups of mice immunized 5 or 15 days previously, the correlation was examined between the changes in peritoneal macrophage number and the ability of bacterial elimination after challenge with 5×10^4 listeria. Twenty-four hours after the challenge (Fig. 3a), the number of macrophages increased in mice immunized 5 days previously, but this increase was not observed in mice immunized 15 days previously. The bacterial elimination was enhanced in both groups of mice as compared with non-immune control. Forty-eight hours after the challenge (Fig. 3b), the same tendency

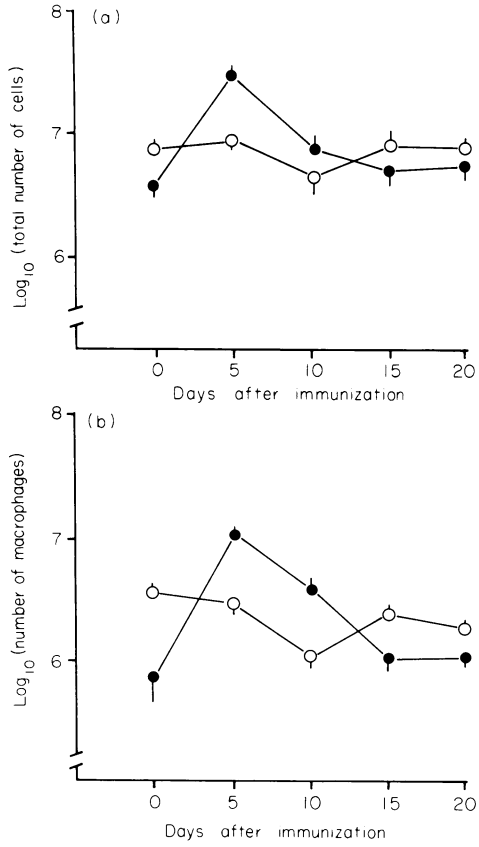

Figure 2. Changes of total peritoneal cells (a) and peritoneal macrophages (b) in number 24 hr after i.p. injection of 5×10^4 *L. monocytogenes* on various days after immunization. Mice were immunized with 2×10^3 viable listeria on day 0. (○) 24 hr after injection of phosphate-buffered saline, (●) 24 hr after injection of listeria. (Mean ±SEM.) The numbers of cells on day 0 show the results from non-immune control mice.

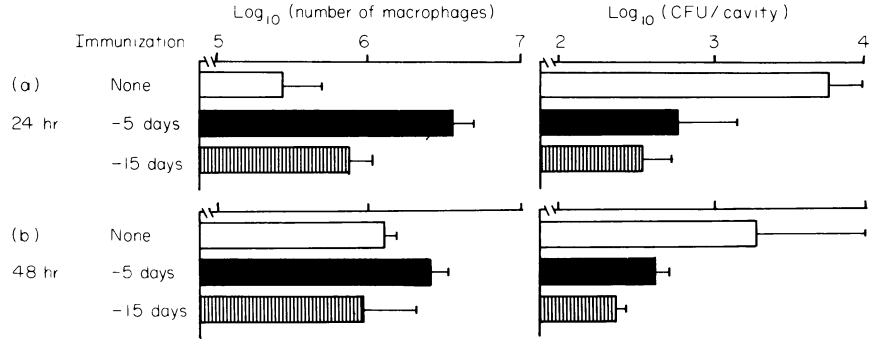

Figure 3. Changes of macrophage number in the peritoneal cavity and the elimination of *L. monocytogenes* from the peritoneal cavity after challenge with 5×10^4 listeria in non-immunized mice (□), mice immunized 5 days previously (■), and mice immunized 15 days previously (▥). Twenty-four hours (a) and 48 hr (b) after challenge infection. (Mean ±SEM.)

Table 2. Changes in numbers of total peritoneal cells and macrophages after injection of various bacterial antigens into mice immunized with *L. monocytogenes*

Intraperitoneal injection with*	No. of total peritoneal cells† ($\times 10^6$/cavity)	No. of peritoneal macrophages ($\times 10^6$/cavity)
PBS	6·38 ± 0·55	1·62 ± 0·13
5×10^4 Viable *L. monocytogenes*	10·70 ± 2·95	3·08 ± 0·28
1×10^5 Viable *S. typhimurium*	2·44 ± 0·70	0·73 ± 0·09
1×10^5 Killed BCG	6·13 ± 1·89	2·13 ± 0·63

* Mice were immunized with 1×10^3 viable *L. monocytogenes* 5 days previously.
† Numbers of cells were examined 24 hr after intraperitoneal injection.
‡ Mean ± SEM.

was observed in both macrophage accumulation and expression of resistance. Even with higher challenge doses (5×10^5, 5×10^6), the same tendency was observed (data not shown).

Effect of other antigens on macrophage accumulation in mice immunized with *L. monocytogenes*

In order to know the effect of other bacterial antigens on macrophage accumulation in the peritoneal cavity, 5×10^4 *L. monocytogenes*, 1×10^5 *S. typhimurium*, and 1×10^5 killed BCG were injected i.p. into mice immunized with listeria 5 days previously. In normal mice, there was no enhancement of macrophage accumulation after the challenge of *L. monocytogenes*, *S. typhimurium*, and BCG (data not shown). In immune mice (Table 2), there was an enhanced accumulation of macrophages after the challenge of *L. monocytogenes*, but no enhancement after the challenge of *S. typhimurium* or killed BCG. This result showed that the challenge with antigenically-specific bacteria is required for the enhancement of macrophages accumulation in listeria-immune mice.

DISCUSSION

We have studied the relative importance of macrophage accumulation and listericidal activity in the expression of acquired cellular resistance at the site of challenge infection in mice immunized with *L. monocytogenes*. ACR against *L. monocytogenes*, as assessed by the enhanced elimination of bacteria from the peritoneal cavity, was detected from day 5 post-immunization, at the early stage after immunization. The number of peritoneal macrophages showed a remarkable increase after the challenge on day 5 of immunization, while such an increase was not observed on day 15 of immunization. The *in vitro* bactericidal activity of peritoneal macrophages on day 5 of immunization was almost the same as that of macrophages obtained from non-immune mice, but that of peritoneal macrophages on day 15 of immunization was remarkably enhanced. These results indicate that ACR can be expressed even in the absence of augmented bactericidal activity of macrophages. The resistance expressed in the early stage of immunization seemed to be mediated by the enhanced accumulation of non-activated macrophages into the peritoneal cavity, a site of challenge with listeria. Stevenson, Kongshavn & Skamene (1981) reported that mobilization of mononuclear phagocytes to inflammatory sites and chemotactic response of mononuclear phagocytes are responsible for anti-listerial resistance. Their report may support our idea that enhanced elimination of listeria can be brought about by enhanced macrophage accumulation.

Macrophage accumulation can be caused by several mechanisms by which a chemotactic factor is released at the site of infection. It is known that many kinds of bacteria possess or release some bacterial chemotactic factors (Wilkinson, 1980) for polymorphonuclear cells and macrophages. The chemotactic factors derived from complement are well known (Snyderman & Mergenhagen, 1976). Listeria cells may produce a non-specific chemotactic factor or may activate the complement system via the alternative pathway, although there is no report showing the direct

activation of complement by *L. monocytogenes*. However, the contribution of these chemoattractants can be ruled out since a significant degree of macrophage accumulation was observed only in the early stage of immunization and not in normal mice. Enhanced macrophage accumulation on day 5 post-immunization was brought about only by *L. monocytogenes*. This result indicates that the mechanism is antigen-specific. One of the antigen-specific mechanisms to be considered is a chemotactic factor derived from complement activation via the classical pathway. Complement activation via the classical pathway requires specific antigen-antibody interactions. The ineffectiveness of immune serum transfer (data not shown) suggests that this mechanism is not applicable to this case.

It seems that the contribution of another factor, lymphocyte-derived chemotactic factor for macrophage (LDCF), should be taken into account. This LDCF has been studied in an *in vitro* system using Boyden chambers (Snyderman & Mergenhagen, 1976), and was shown to be distinct from a chemoattractant derived from complement (Altman, Snyderman, Oppenheim & Mergenhagen, 1973). Ward, Remold & David (1970) reported a lymphocyte-derived leucotactic factor which was distinct from MIF. In the present study, such a kind of factor released from sensitized lymphocytes might cause the enhanced accumulation of macrophages into the site of challenge only in immunized mice, although there is no direct evidence showing that lymphocytes are contributing to our observation.

Enhanced accumulation of macrophages was observed only in the early stage after immunization. The reason for the lack of increase in macrophage number at the later stage of immunization is not clear. One explanation for this may be that the cells responsible for macrophage chemotaxis are short lived. Another explanation is that MIF or MAF generated in the later stage increase the adhesiveness of macrophages so as to cause a decreased recovery of cells from the peritoneal cavity. In our previous report (Mitsuyama *et al.*, 1980), it was shown that MIF was generated in mice after day 10 postimmunization and not in the early stage, so the latter explanation may be conceivable.

The generation of activated macrophages possessing an enhanced microbicidal activity is attributed to MAF (Nathan, Karnovsky & David, 1971; Nathan *et al.*, 1973). MAF has been reported to be indistinguishable from MIF biochemically (Nathan *et al.*, 1973). In our previous study, MIF activity was observed in the later stage of infection with *L. monocytogenes* in mice (Mitsuyama *et al.*, 1980). In this study, macrophages with augmented bactericidal activity appeared in the later stage of infection. Thus the appearance of activated macrophages in this study may be compatible with the observation by Nathan *et al.* (1971).

It has been generally accepted that the acquired cellular resistance against *L. monocytogenes* is expressed only by the generation of immunologically activated macrophages which are activated by MAF released from sensitized T lymphocytes after interaction with specific antigen (Campbell, 1970; Mackaness, 1962; Nathan *et al.*, 1971; Takeya *et al.*, 1977). In our results however, acquired resistance was expressed when macrophage accumulation was enhanced even in the absence of activated macrophages. The present study leads us to conclude that there are at least two stages in the generation of ACR against *L. monocytogenes*. The ACR in the earlier stage depends upon the enhanced accumulation of macrophages, and that in the later stage depends upon the enhanced microbicidal activity of activated macrophages.

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