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Activation of non-specific cytotoxic cells in *Listeria*-susceptible and -resistant mouse strains

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Summary. An increase in macrophage tumouricidal activity in the spleen was demonstrated following intravenous infection of genetically resistant C57BL/10 mice with *Listeria monocytogenes*, but not after infection of BALB/c mice. However, tumouricidal macrophages appeared in the peritoneal cavity of both strains after infection, while NK cell activity was generally higher in BALB/c mice.

The activation of tumouricidal macrophages and NK cells in the C57BL/B10 mice was T independent and, at least in the peritoneal cavity, radioresistant (600 rads). The relevance of these results to the genetic control of resistance to *Listeria* is discussed.

INTRODUCTION

Activated bactericidal macrophages have long been implicated in resistance of mice to infection with *Listeria monocytogenes* (Mackaness, 1964). However, while the ability of macrophages to lyse tumour cells has been a widely used method of demonstrating activation (Adams, 1982), the relationship between tumouricidal activity and bactericidal activity is unclear (Wing *et al.*, 1977; Hopper & Cahill, 1983).

The tumouricidal activity of macrophages or natural killer (NK) cells has been demonstrated to increase as a result of infection with *Listeria*, or following exposure to *Listeria* extracts. Campbell, Caldwell & Hartman (1979) showed that a *Listeria* cell wall fraction could stimulate peritoneal exudate macrophage *in vitro* to lyse P815 tumour cells. Peritoneal exudates induced by intraperitoneal injection of live *Listeria* were reported to constitute a rich source of NK cells (Holmberg, Springer & Ault, 1981). In a recent paper (Cheers & Wood, 1984) in which we examined the role of NK cells in *Listeria* infection of mice, we noted that *Listeria* induced a population of activated macrophages capable of lysing tumour targets *in vitro*.

In the present paper, we have examined the generation of activated tumouricidal macrophages and NK cells during the early days of *Listeria* infections in mouse strains either genetically resistant or susceptible to intravenously injected *Listeria*. Resistant mice show an ability to restrain early growth of *Listeria* in the liver and an early onset of cell-mediated immunity (Cheers *et al.*, 1978). It was, therefore, of interest to see whether the pattern of macrophage activation and/or NK cell activity correlated with these genetic differences.

Abbreviations: B10, C57BL/10; B/C, BALB/c; c.p.m., counts per minute; E:T, effector/target; HIFCS, heat-inactivated fetal calf serum; i.v., intravenously; NK, natural killer cell.

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MATERIALS AND METHODS

Mice

Inbred C57BL/10 and BALB/c mice were maintained under conventional conditions by strict brother-sister

mating in the Microbiology Animal Breeding Unit, University of Melbourne. Mice were sex-matched within experiments and were 6–10 weeks old when infected.

Bacteria

Listeria monocytogenes was originally obtained from R. V. Blanden (Australian National University) and maintained by weekly subculture on horse blood agar. It was renewed from freeze-dried stock after fewer than 25 passages.

Infection of mice

Twenty four-hour actively growing cultures were washed from trypticase soy agar plates with 1% serum in distilled water (serum water). The inoculum was standardized turbidimetrically using a colorimeter (EEL International Ltd., Bayswater, Australia). The bacteria were injected intravenously in 0·2 ml and the dose checked retrospectively using Miles & Misra (1938) viable counts. The LD_{50} for *Listeria* following i.v. injection was 2×10^3 for BALB/c and 2×10^5 for C57BL/10 mice.

Effector cells

Peritoneal cells were obtained by rinsing the peritoneal cavity with 4·5 ml cold medium containing heparin at a concentration of 10 units/ml. No significant differences were noted to the numbers or percentages of macrophages recovered from the peritoneal cavities of either mouse strain following i.v. infection with *Listeria*. Spleens were cut into fragments and gently pushed through fine stainless steel sieves into cold RPMI medium supplemented with pencillin, streptomycin and 10% heat-inactivated fetal calf serum (HIFCS). After clumps had settled out, the suspended cells were washed twice before diluting in RPMI/10% HIFCS. The viability of cells was checked by eosin exclusion and the cell concentrations adjusted so that equal numbers of viable white cells were added to each well. All assays employed cells pooled from groups of 3–5 mice.

In vitro *Cytotoxicity assays*

Two different tumour cells lines were used, P815 (H-2^d) mastocytoma and YAC-1 (H-2^a) lymphoma. All cell lines were maintained as suspension cultures by continuous passage in RPMI medium containing 10% HIFCS. As previously described (Cheers & Wood, 1984), 10^4 target cells labelled with ^{51}chromium were added to microtitration trays along with an appropriate number of viable effector cells. The assay time for the YAC-1 and P816 targets were 4 hr and 18 hr, respectively, with spontaneous lysis varying from 5% to 8% and 20% to 28%, respectively.

All cultures were assayed in triplicate and results expressed as mean percent specific lysis ±SE, calculated as:

$$\frac{\text{test c.p.m.} - \text{spontaneous c.p.m.}}{\text{maximum c.p.m.} - \text{spontaneous c.p.m.}} \times 100.$$

The standard errors obtained were always less than 10% of the mean. The experiments reported here were all repeated several times and the results shown are representative of these obtained.

T.-cell deprived mice

Four-week-old C57BL/10 mice were thymectomized and, 14 days later, lethally irradiated (850 rads) and reconstituted with 6×10^6 syngeneic anti-Thy 1·2-treated bone marrow cells. Mice were then left for 4 weeks to recover before being infected with *Listeria*.

At the same time as cells from these animals were assayed for tumouricidal activity, their spleen cells were examined for the ability to proliferate *in vitro* in the presence of the T-cell mitogen Concanavalin A (Con A). The Con A responsiveness of spleen cells from thymectomized, irradiated, reconstituted mice both before and after *Listeria* infection was markedly reduced (8–10-fold) in comparison to normal controls.

RESULTS

In order to discriminate between activation of NK cells and macrophages, effector cells were assayed against two different tumour targets. NK cell activity was assayed in a conventional 4-hr chromium release assay using the highly NK susceptible YAC-1 cell line as target. For detection of macrophage activity, P815 cells, an NK cell-insensitive target (Roder *et al.*, 1979) was used. The incubation time of this cytotoxic assay was increased to 18 hr to favour the detection of activated macrophages (Keller, 1978; Tagliabue *et al.*, 1979).

Listeria resistant C57BL/10 (B10) mice and susceptible BALB/c mice were infected with approximately 10^4 *Listeria* i.v. 1, 2 and 3 days prior to collection of effector cells. It was not possible to quantitate macrophages or NK cells accurately as a separate population in the spleens, and so the cytotoxic effectors were assayed on the basis of total viable white cell counts.

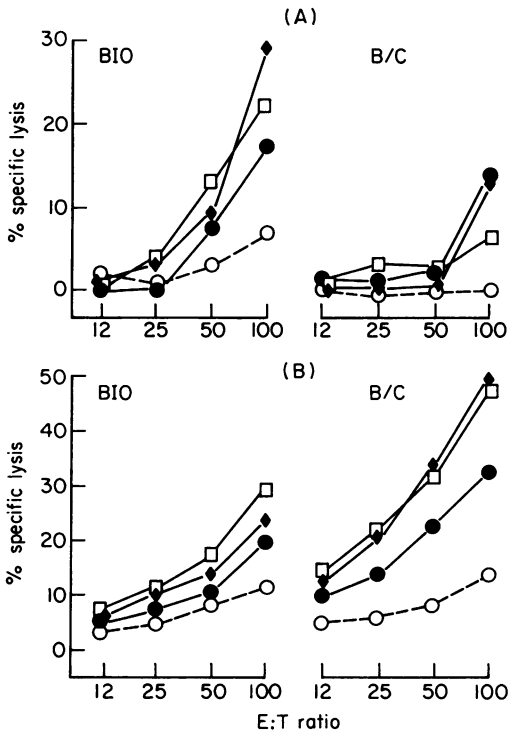

Figure 1. Lysis of P815 (A) and YAC (B) target cells by spleen cells from uninfected mice (O- - -O) and *Listeria*-infected mice day 1 (●——●), day (□——□) and day 3 (▲——▲) post-infection.

This was considered to reflect accurately the total picture in the spleens. Macrophage-mediated tumouricidal activity in the spleens of B10 mice rose rapidly after infection, whereas BALB/c mice showed no significant increase over the 3-day period of observation (Fig. 1a). On the other hand, NK cell activity was higher in the infected BALB/c mice than infected B10 mice (Fig. 1b).

Macrophage tumouricidal activity in the peritoneal cavity was significantly higher in uninfected B10 mice compared with BALB/c animals (Fig. 2a). Following infection with *Listeria*, the tumouricidal activity of peritoneal macrophages increased within 1 day in both mouse strains (Fig. 2a). The degree of increased macrophage activity was similar in the two strains until day 3, when BALB/c mice appeared to lose activity (Fig. 2a). This loss of tumouricidal activity was confined to macrophage-mediated lysis, as day 3 BALB/c peritoneal cells showed considerable NK-mediated lysis of YAC-1 target-cells (Fig. 2b). The level of NK cell activity in the peritoneal cavity following injection of *Listeria* was always higher in BALB/c than B10 mice, and increased with time after infection (Fig. 2b).

The higer level of NK cell activity detected with peritoneal cells from *Listeria*-susceptible BALB/c mice did not reflect their greater bacterial load. When animals were infected 48 hr before assaying with widely varying doses of *Listeria*, peritoneal cells from BALB/c mice showed greater NK cell activity than B10 animals, regardless of dose (Fig. 3). Spleen-associated NK activity, which differed little between the strains, showed only a slight increase with increasing bacterial dose between 5×10^2 and 5×10^3, but was suppressed by 5×10^4 *Listeria* (Fig. 4).

The level of macrophage activation in the peritoneal

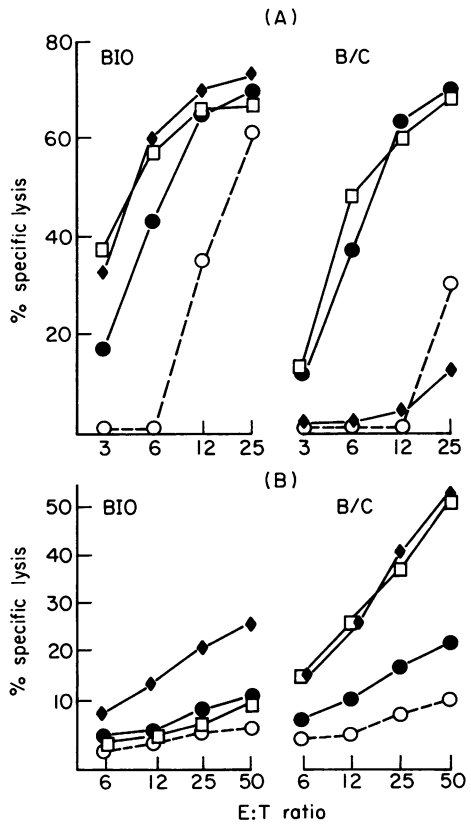

Figure 2. Lysis of P815 (A) and YAC 1 (B) target cells by peritoneal cells from uninfected (O- - -O) and *Listeria*-infected mice day 1 (●——●), day 2 (□——□) and day 3 (▲——▲) post-infection. B10, C57/10; B/C, BALB/c; E:T, effector/target ratio.

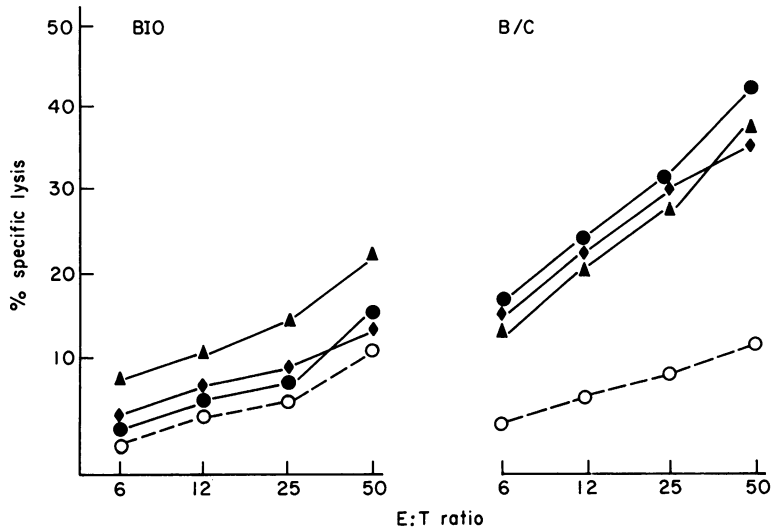

Figure 3. Lysis of YAC 1 target cells by peritoneal cells from B10 (C57BL/10) and B/C (BALB/c) mice either uninfected (O- - -O) or infected with 5×10^2 (●——●), 5×10^3 (▲——▲) or 5×10^4 (♦——♦) *Listeria*. Mice were infected with *Listeria* i.v. 48 hr prior to assay.

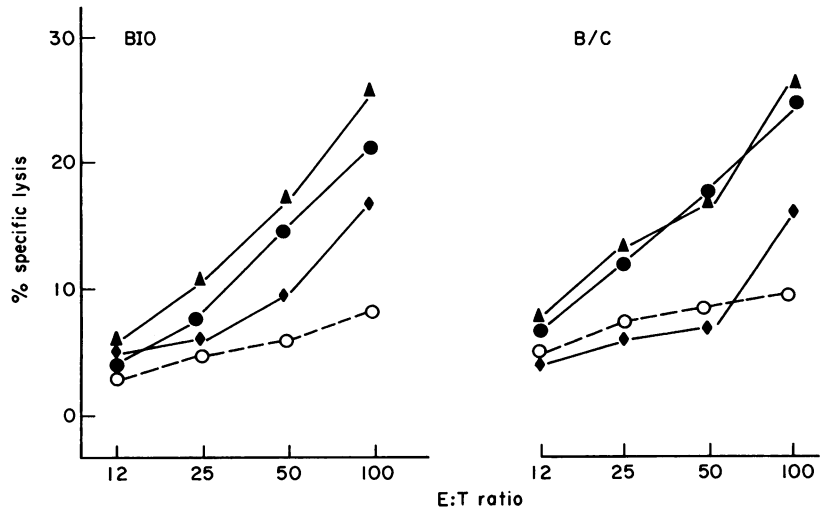

Figure 4. Lysis of YAC-1 target cells by spleen cells from B10 (C57BL/10) and B/C (BALB/c) mice, either uninfected (O- - -O) or infected with 5×10^2 (●——●), 5×10^3 (▲——▲) or 5×10^4 (♦——♦) *Listeria*. Mice were infected with *Listeria* i.v. 48 hr prior to assay.

cavity was independent of the dose of *Listeria* inoculated, except with the highest dose (Fig. 5). The decrease in macrophage activity seen with BALB/c mice infected with 5×10^4 *Listeria* (Fig. 5) was similar to that found with peritoneal cells from day 3 infected mice (Fig. 2a) and may reflect the impending death of these mice. As in Fig. 1, splenic macrophage activation was again present in infected B10 mice only, and increased slightly with increasing doses of *Listeria* (data not shown).

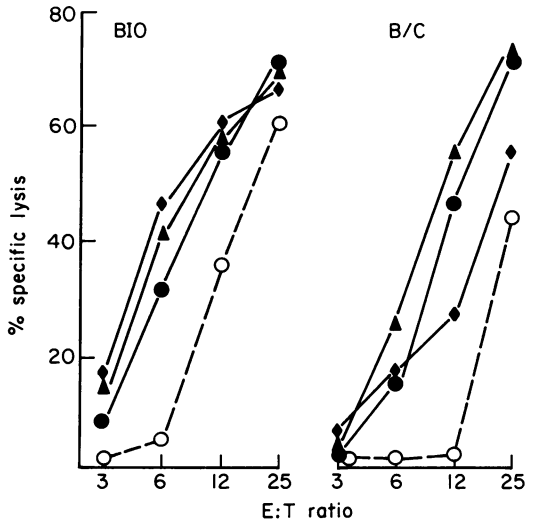

Figure 5. Lysis of P815 target cells by peritoneal cells from B10 (C57BL/10) and B/C (BALB/c) mice, either uninfected (O- - -O) or infected with 5×10^2 (●——●), 5×10^3 (▲——▲) or 5×10^4 (♦——♦) *Listeria*. Mice were infected i.v. with *Listeria* 48 hr prior to assay.

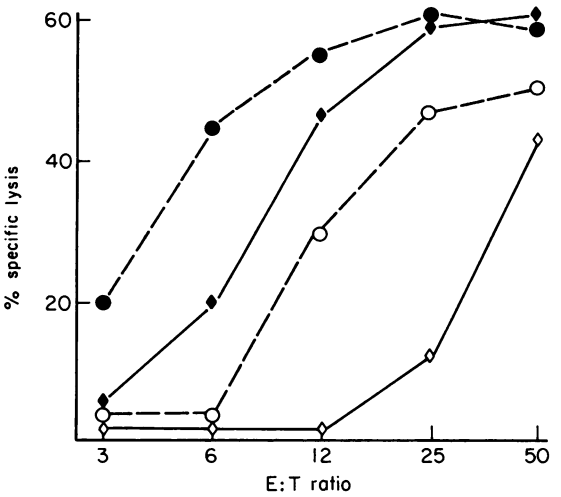

Figure 7. Lysis of P815 target cells by peritoneal cells from uninfected control (◊——◊) or irradiated mice (O- - -O), and *Listeria*-infected control (♦——♦) or irradiated mice (●- - -●). C57BL/10 mice were infected with 10^4 *Listeria* 48 hr prior to assay.

In order to examine the role of T cells in the early activation of macrophages by *Listeria*, B10 mice were thymectomized, irradiated and reconstituted with anti- Thy 1-treated bone marrow. This treatment did not effect the ability of peritoneal macrophages to lyse P815 cells 48 hr after *Listeria* infection (Fig. 6). *Listeria*-induced activation of tumouricidal macrophages in the spleen was also unaltered in T-cell depleted mice (data not shown).

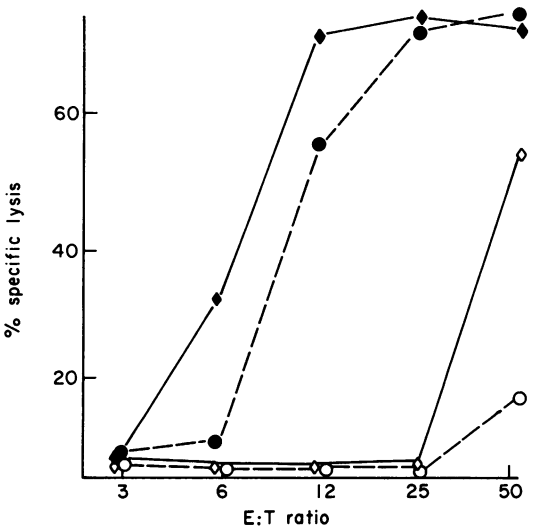

Figure 6. Lysis of P815 target cells by peritoneal cells from uninfected control (◊——◊) or AT × BM-treated mice (O- - -O), and *Listeria*-infected control (♦——♦) or AT × BM-treated mice (●——●). C57BL/10 mice were infected i.v. with $1{\cdot}4 \times 10^4$ *Listeria* 48 hr prior to assay.

The cells responsible for early resistance to *Listeria* in B10 mice are known to be radiosusceptible (Sadarangani, Skamene & Kongshavn, 1980; Cheers & Macgeorge, 1982). Therefore, attempts were made to determine the radiosusceptibility of *Listeria*-induced cytotoxic cells. A dose of irradiation (600 rads), given 2 hr prior to infection with *Listeria*, actually somewhat increased the activation of cytotoxic macrophages in the peritoneal cavity 2 days later (Fig. 7). However, it was not possible to determine accurately the effect of irradiation on *Listeria*-induced spleen cell activity due to the poor recovery of viable cells (less than 5%) and the finding that irradiation alone greatly increased NK cell activity in the spleen (data not shown).

DISCUSSION

During the course of *Listeria* infections in mice, two distinct non-specific cytotoxic cells were generated: NK cells and activated macrophages. Using tumour

targets with different sensitivity for lysis by these two effector cells, we examined their activation in *Listeria*-susceptible (BALB/c) and -resistant (C57Bl/10) mice. The degree of increased NK cell activity detected in the peritoneal cavity and spleen following an i.v. injection of *Listeria* was generally higher in BALB/c mice compared with B10 animals. The level of NK activity induced was largely independent of the dose of *Listeria* injected but increased with time after infection.

This finding of higher levels of NK cell activity in *Listeria*-susceptible BALB/c mice compared to resistant B10 animals supports our earlier conclusion (Cheers & Wood, 1984) that NK cells do not play a role in early defence against *L. monocytogenes*.

The degree of activation of macrophages following infection with *Listeria* differed in the two anatomical sites examined. In the spleen, macrophage activation was only detected with cells from resistant B10 mice following *Listeria* infection. However, peritoneal cells from infected BALB/c and B10 mice both showed significantly increased macrophage-mediated lysis of P815 targets. These data suggest that, while the activation of tumouricidal macrophages in the spleen may correlate with the expression of resistance to *Listeria in vivo* (Cheers *et al.*, 1978), the tumouricidal activity of macrophages in the peritoneal cavity does not. It was a curious and unexplained observation that the peritoneal cavity of infected BALB/c mice contained tumouricidal macrophages, but the spleen, the site of infection, did not.

The T-cell independence of *Listeria*-induced macrophage tumouricidal activity is consistent with data on the mechanism of early genetic control of *Listeria* (Cheers *et al.*, 1980; Newborg & North, 1980). Campbell *et al.* (1979) found that *Listeria* cell wall components activated thioglycollate-induced peritoneal exudate cells to lyse P815 targets *in vitro*, and that this was also a T-cell independent phenomenon.

The cells responsible for early resistance to *Listeria* in B10 mice are radiosensitive (Sadarangani *et al.*, 1980; Cheers & Macgeorge, 1982). In contrast, the development of tumouricidal cells in the peritoneal cavity was radioresistant. We were unable to determine, for technical reasons, the radiosensitivity of the activation of tumouricidal macrophages in the spleen. This, therefore, leaves open the question of the relevance of this phenomenon to genetic control of resistance to *Listeria*.

The possible role that activated tumouricidal macrophages may have in controlling a *Listeria* infection has been questioned by the finding that macrophages with bactericidal activity against *Listeria* were distinguishable from those with tumouricidal activity (Hopper & Cahill, 1983; Campbell, Czuprynski & Cook, 1984). Whether the tumouricidal and bactericidal activities of macrophages activated during the early stages of *Listeria* infections are dissociable is still to be determined, as Wing *et al.* (1977) found that differences in the functional capacity of macrophages depended on the method used to activate these cells. This study casts further doubt on the wisdom of equating tumouricidal activity, often assayed with cells from the peritoneal cavity, with macrophage bactericidal activity at the site of infection (the spleen).

ACKNOWLEDGMENTS

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