

THE MODE OF  
IMMUNOPOTENTIATING ACTION  
OF BESTATIN:  
ENHANCED RESISTANCE TO  
*LISTERIA MONOCYTOGENES*  
INFECTION

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UMEZAWA *et al.* have found that bestatin, isolated from culture filtrate of *Streptomyces olivoreticuli*, is a specific inhibitor of aminopeptidase B and leucine aminopeptidase which are located on the surface membrane in various mammalian cells including lymphocytes and macrophages<sup>1,2</sup>. This chemically defined immunopotentiator was shown to enhance delayed type hypersensitivity (DTH) response and antibody production and to exert an antitumor action<sup>3-5</sup>. The present studies were undertaken to study its effect on the resistance to *Listeria monocytogenes* (LM) infection.

Female, specific pathogen free, inbred DDD mice were obtained from the animal supply center in Kyushu University. Eight- to ten-week old mice were used in the experiments.

Bestatin was kindly provided by Prof. S. OKA and Prof. H. UMEZAWA, Institute of Microbial Chemistry, Tokyo, Japan. It was prepared by Nippon Kayaku Co., Ltd. It was dissolved in sterile saline and was administered intravenously, intraperitoneally or orally in a volume of 0.1 ml.

The soluble antigen of LM was prepared from culture filtrates of LM<sup>6,7</sup>. Briefly, protein free medium was prepared by dialysis of 10-fold concentrated solution of Trypticase soy broth against 10 volume of distilled water for 24 hours at 4°C. The dialysate was autoclaved, inoculated with LM and incubated for 18 hours at 37°C with continuous shaking (150 rpm). Bacteria were removed

by centrifugation (3,300 × *g*) and Millipore filter (GS 0.22 μm). The supernatant was concentrated by Diaflo Ultra filtration membrane (PM 30, Amicon) and lyophilized. This powder was used as an antigen for foot pad test (FPT).

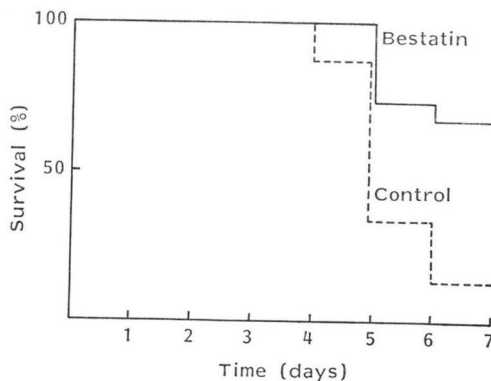
*Listeria monocytogenes* (strain EGD) was provided originally from Dr. G. B. MACKANESS (Trudeau Institute, Saranac Lake, N.Y., USA). Bacterial suspensions were made as described previously<sup>7</sup>. The mean lethal dose (LD<sub>50</sub>) in DDD mice was routinely less than 2 × 10<sup>8</sup> bacteria. Mice were infected *iv* with the dose near the LD<sub>50</sub> in a volume of 0.1 ml. The course of infection was followed quantitatively by bacteria counts in the liver and spleen as described previously<sup>7</sup>. The DTH to LM was measured by FPT using an eliciting dose of 20 μg listerial antigen. The spreading of peritoneal macrophages was measured as described previously.<sup>7</sup>

Test for significance was performed using the Student's *t*-test. The survival rate between experimental groups was analyzed according to the generalized Wilcoxon test. A *P*-value of less than 0.05 was considered statistically significant.

Firstly, mortality studies were performed in bestatin-treated mice. Mice were divided into two groups. One group of mice was administered orally with 100 μg of bestatin daily for 5 days. The other group served as normal control. Each group consisted of 15 mice. All mice were concurrently infected *iv* with 1.1 × 10<sup>8</sup> LM an hour after the last administration of bestatin. The results are presented in Fig. 1. The death of mice

Fig. 1. Per cent survival of bestatin-treated mice infected with LM.

Control and bestatin-treated mice were infected *iv* with 1.1 × 10<sup>8</sup> LM an hour after the last administration of bestatin.



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Fig. 2. Growth of LM in the liver and spleen in bestatin-treated mice. Control (○) and bestatin-treated (●) mice were infected iv with  $1.7 \times 10^3$  LM an hour after the last administration of bestatin.

Each point represents the mean of five.

\*  $P < 0.05$ , \*\*  $P < 0.001$  in comparison with control.

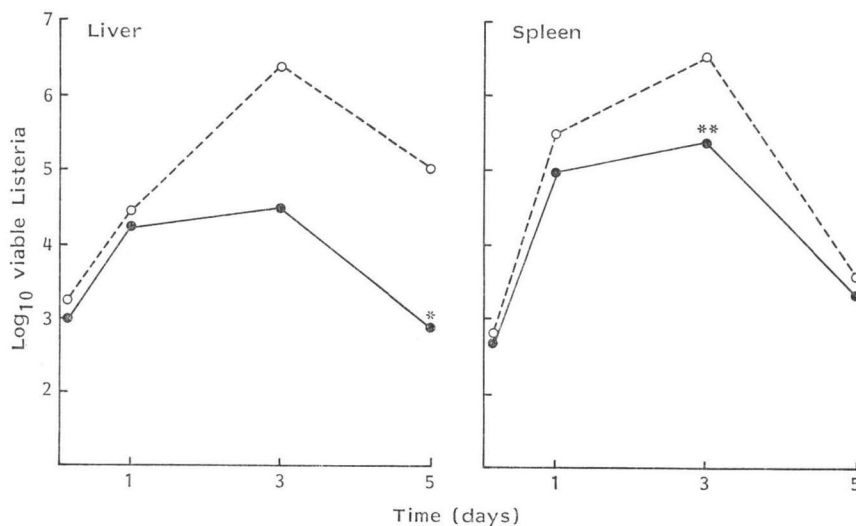


Table 1. Macrophage spreading after LM infection.

Group	Day 1		Day 3	
	No. of peritoneal cells ( $\times 10^5$ )	% spreading	No. of peritoneal cells ( $\times 10^5$ )	% spreading
Control	$72 \pm 17$	$6.1 \pm 3.3$	$27 \pm 4$	$8.6 \pm 3.6$
Bestatin	$141 \pm 50^*$	$12.4 \pm 4.2^{**}$	$37 \pm 14$	$12.2 \pm 3.8$

All values are expressed as the mean of five  $\pm$  S.D..

\*  $P < 0.01$  in comparison with control.

\*\*  $P < 0.05$  in comparison with control.

was not observed after the 7th day of infection. The survival rates were 67.0% in bestatin-treated mice, 13.7% in normal controls respectively. The difference between both groups was significant to  $P < 0.01$ .

In the next experiment, the course of infection was followed by quantitative enumeration of bacteria in the liver and spleen. Mice were divided into two groups. Hundred  $\mu$ g of bestatin was given iv daily for 5 days. Mice were concurrently infected iv with  $1.7 \times 10^3$  LM an hour after the last injection of bestatin. The results are presented in Fig. 2. The growth of LM in the liver and spleen was clearly inhibited in bestatin-treated mice.

Macrophage spreading in bestatin-treated mice

Table 2. DTH response to LM antigen.

Group	Foot pad test		
	Day 5	Day 7	Day 9
Control	$3.6 \pm 1.8$	$3.5 \pm 1.4$	$5.6 \pm 0.9$
Bestatin	$7.2 \pm 3.6$	$7.5 \pm 2.3^*$	$7.0 \pm 2.9$

The FPT value represents the mean of five  $\pm$  S.D. (0.1 mm).

\*  $P < 0.05$  in comparison with control.

during LM infection was examined. Mice were divided into two groups. One group of mice served as controls. The other group of mice was injected ip with 100  $\mu$ g of bestatin daily for 5 days. All mice were concurrently infected iv

with  $1.6 \times 10^8$  LM an hour after the last injection of bestatin. Spreading of peritoneal macrophages was examined one day and 3 days after infection. The results in Table 1 showed that the numbers of peritoneal macrophages and spread cells were significantly higher in bestatin-treated mice than in normal controls on day 1. However, the difference between them was no more significant on day 3.

Development of DTH to LM in bestatin-treated mice was studied. Mice were divided into two groups. Hundred  $\mu\text{g}$  of bestatin was given iv daily for 3 days. All mice were concurrently infected iv with  $1 \times 10^8$  LM as the immunizing dose an hour after the last injection of bestatin. FPT was carried out on day 5, 7 and 9 after infection. As shown in Table 2, the levels of DTH were higher in bestatin-treated mice than those in controls. The difference between both groups was statistically significant to  $P < 0.05$  on day 7.

The data mentioned above indicate that pretreatment with bestatin enhanced the resistance to LM infection. Such enhanced resistance was associated with increased rate of spreading of macrophages at an early time of infection and with increased level of DTH to LM at a later stage. In general, acquired immunity against LM arises concomitantly with DTH at later stages after the 5th day of infection. It depends on specifically committed T-lymphocytes which activate macrophages as effector cells against LM<sup>8,9</sup>. Our results in Fig. 2 showed that the growth of LM in bestatin-treated mice was clearly inhibited at the early times of infection when the acquired immunity did not arise yet, indicating that bestatin caused strong activation of macrophages without immunological process. BRULEY-ROSSET *et al.* using the *in vitro* cytostatic test described that macrophage activation was obtained after the administration of the high doses of bestatin in aged mice<sup>10</sup>. Moreover our results showed that treatment with bestatin caused the rapid and increased development of DTH to LM. SAITO *et al.* found that bestatin stimulated the blastogenesis of guinea pig lymphocytes triggered with polyclonal mitogen such as phytohemagglutinin, concanavalin A, pokeweed mitogen and lipopolysaccharide<sup>11</sup>. MÜLLER *et al.* described that bestatin increased the incorporation rate of [<sup>3</sup>H]thymidine into DNA only in T cells<sup>12</sup>. ISHIZUKA *et al.* reported that bestatin was mito-

genic on spleen cells and caused the proliferation of T cells probably through the activation of macrophages<sup>13</sup>. It, therefore, seems that bestatin activates primarily macrophages and T-lymphocytes which mediate the DTH and acquired immunity to LM.

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