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Stimulation of Resistance of Immunocompromised Mice by a Muramyl Dipeptide Analog

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L18-MDP(Ala), a synthetic derivative of muramyl dipeptide, enhanced resistance against *Escherichia coli* and *Candida albicans* infections in aged mice and younger adult mice whose defense mechanism(s) had been depressed by X-ray irradiation or by treatment with cyclophosphamide.

Muramyl dipeptide and its synthetic derivatives (MDPs) have been reported to stimulate the resistance of animals against microbial infections (2, 3, 5, 14–16). Such effects have been confirmed mostly in those animals whose defense mechanisms, including nonspecific and immune systems, were maintained at the normal level. Stimulation of the defense mechanisms by MDPs may result in the enhancement of resistance to microbial infections. However, most patients requiring treatment capable of stimulating the defense mechanisms can be supposed to have some deficiencies in these (11, 12). The present study was conducted to observe the effect of 6-*O*-stearoyl-*N*-acetyl-L-alanyl-D-isoglutamine, L18-MDP(Ala), on resistance against bacterial or fungal infections in mice whose defense mechanisms were disordered to various degrees by X-ray irradiation or treatment with cyclophosphamide (CY).

L18-MDP(Ala) provided by O. Nagase, Chemical Technology Research Center, Research Institute, Daiichi Seiyaku Co., Ltd., Tokyo, Japan, was dissolved in sterile Dulbecco phosphate-buffered saline (PBS; Nissui Seiyaku Co., Ltd., Tokyo, Japan), pH 7.4, at a concentration of 500 μg/ml just before use.

Outbred male mice of strain STD:ddY (Shizuoka Cooperative for Experimental Animals, Hamamatsu, Japan) were used. After a 1-week quarantine for infectious disorders, the mice were moved to a laboratory and were used for experiments at 6 weeks and 17 months of age. The latter group of mice were used as the aged hosts.

The protective mechanisms of younger adult mice against microbial infections were disordered by X-ray irradiation or administration with CY (Endoxan; Shionogi & Co., Ltd., Osaka, Japan). The radiation was delivered from an X-ray generator of type SHT 250M-2 (Shimadzu, K.K., Tokyo, Japan) operating at 200 kV with 0.3-mm Cu and 0.1-mm Al filtration, 100 cm from the target focus, and groups of 10 mice were exposed to X-ray irradiation at 400 or 600 rads. CY was dissolved in PBS and injected at a dose of 100 or 200 mg/kg intraperitoneally (i.p.) into groups of 10 or 20 mice 2 days before infection.

For challenge organisms, *Escherichia coli* ST 0198 and *Candida albicans* D12 were used. *E. coli* and *C. albicans* were cultivated overnight in Trypto-soy broth and Candida GS agar plates (Eiken Chemicals Co., Ltd., Tokyo, Japan) at 37°C, respectively. The latter culture was harvested and suspended in a 5% glucose solution. Adequate dilutions of the bacterial broth culture with PBS or those of the fungal suspension in 5% glucose were used for subcutaneous (s.c.) or intravenous (i.v.) inoculation of normal or immunocompromised young (6 weeks of age) mice and of aged mice to determine the minimal lethal dose (MLD) of each microorganism. The MLDs of *E. coli* for normal, irradiated, and CY-treated mice were 1.0×10^8, 5.0×10^6, and 2.0×10^6 colony-forming units (CFU) per mouse, respectively, and that for aged mice was 1.0×10^6 CFU per mouse. The MLDs of *C. albicans* for normal and CY-treated mice were 5.0×10^6 and 2.5×10^6 CFU per mouse, respectively. In most experiments, 100 μg of L18-MDP(Ala) per mouse was injected s.c. 24 h before infection, and the survival rates of mice were recorded up to 7 days after infection. The effects of the adjuvant were represented as the percentages of mice surviving during this observation period. The significance of results was tested according to the adjusted chi-square method (9).

All of the aged mice treated with 100 μg of L18-MDP(Ala) 24 h before challenge with the

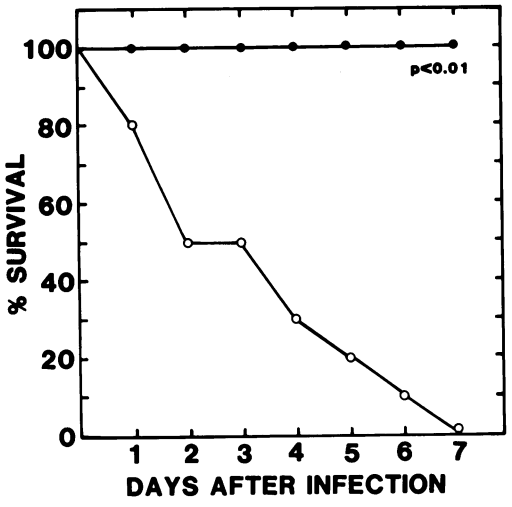

FIG. 1. Effect of L18-MDP(Ala) on resistance of aged mice against *E. coli* infection. Groups of 10 mice at 17 months of age were injected s.c. with 100 μg of L18-MDP(Ala) (●) or PBS (○) per mouse 24 h before s.c. infection with 1.0×10^6 CFU of *E. coli*.

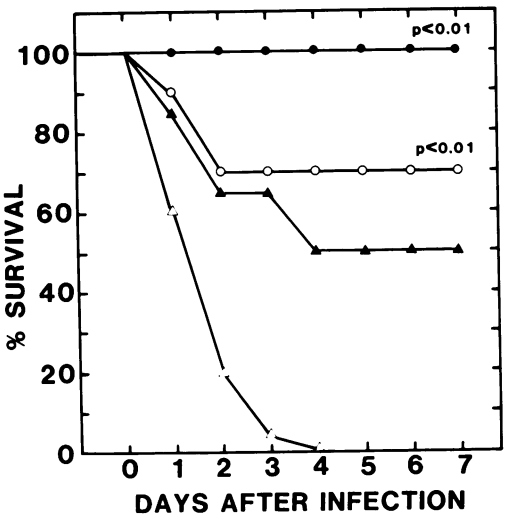

FIG. 3. Effect of L18-MDP(Ala) on resistance against *E. coli* infection in CY-treated mice. Groups of 20 mice were injected i.p. and s.c. on day 1 with 100 mg of CY per kg and 100 μg of L18-MDP(Ala) (▲) or PBS (△) per mouse, respectively, and inoculated s.c. with 2.0×10^6 CFU of *E. coli* on day 0. Simultaneously, CY-untreated (control) mice were also treated s.c. with 100 μg of L18-MDP(Ala) (●) or PBS (○) 24 h before infection.

MLD (1.0×10^6 CFU per mouse) of *E. coli* survived on day 7 (Fig. 1). Mice at 6 weeks of age exposed to X-ray irradiation at 400 or 600 rads on day −2 were treated s.c. with 100 μg of the adjuvant on day −1 and inoculated s.c. with the MLD (5.0×10^6 CFU per mouse) of *E. coli* on day 0. With this inoculum, only 10% of the untreated normal mice died by day 7, irrespective of any treatment with the adjuvant (Fig. 2). On the other hand, all of the irradiated mice died within 3 days in the absence of the adjuvant treatment. When they were treated with the adjuvant 1 day after exposure to 400 and 600 rads, 30 and 10% of them, respectively, survived beyond day 7. Thus, a slight augmentation by L18-MDP(Ala) of the resistance to *E. coli* infection was detected only in mice irradiated with 400 rads.

In another experiment, groups of 20 mice were injected i.p. and s.c. with 100 mg of CY per kg and 100 μg of L18-MDP(Ala) per mouse on day −1, respectively and inoculated s.c. with the MLD (2.0×10^6 CFU) of *E. coli* on day 0. As shown in Fig. 3, 30% of CY-untreated (control) mice in the absence of the adjuvant treatment died by day 2, whereas none of the mice treated with the adjuvant had died by day 7. In contrast, all of the mice treated with CY but without the

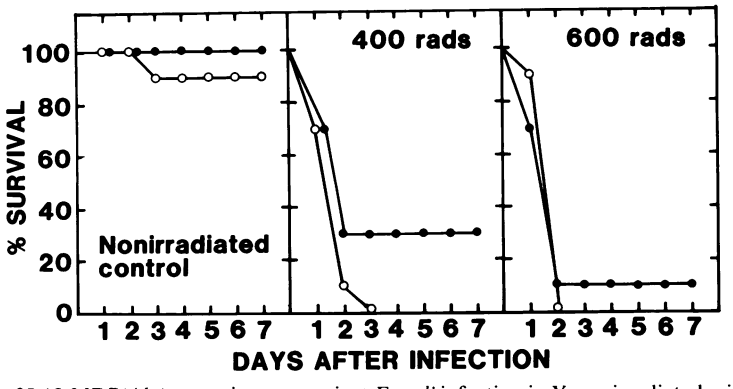

FIG. 2. Effect of L18-MDP(Ala) on resistance against *E. coli* infection in X-ray-irradiated mice. Groups of 10 mice at 6 weeks of age were exposed to 400 or 600 rads on day 2, injected s.c. with 100 μg of L18-MDP(Ala) (●) or PBS (○) per mouse on day 1, and inoculated s.c. with 5.0×10^6 CFU of *E. coli* on day 0. No statistically significant difference in the results between the adjuvant-treated mice and untreated controls was found.

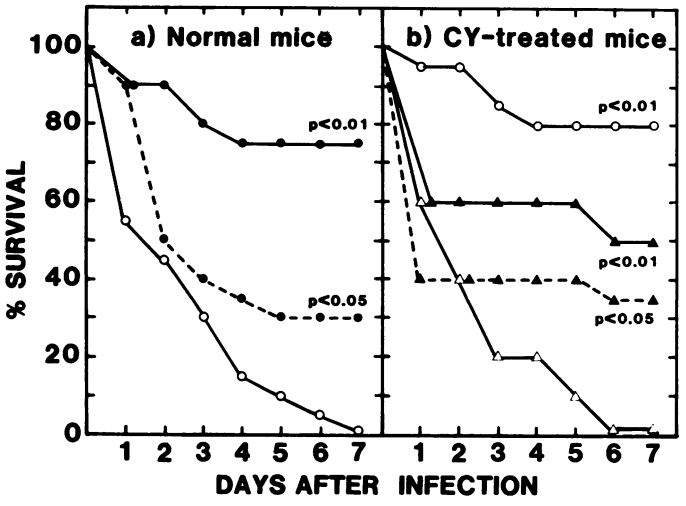

FIG. 4. Effect of L18-MDP(Ala) on resistance against *C. albicans* infection in Cy-treated mice. (a) Groups of 20 normal mice were injected s.c. with 100 μg of L18-MDP(Ala) (●) per mouse 24 h before (solid line) or 6 h after (dotted line) i.v. inoculation with 5.0×10^6 CFU of *C. albicans*. A group of mice was given PBS (○) 24 before infection as a control. (b) Mice were injected i.p. with 200 mg of CY per kg 24 h before infection and injected s.c. with 10 (▲ [dotted line]) or 100 (▲ [solid line]) μg of L18-MDP(Ala) per minute 2 and 24 h before i.v. inoculation with 2.5×10^6 CFU of *C. albicans*. As controls, CY-treated (△) and CY-untreated (○) mice were given PBS at corresponding times.

adjuvant had died by day 4 after challenge with the same amount of *E. coli*. Treatment with the adjuvant, on the other hand, enabled 50% of CY-treated mice to survive beyond day 7.

Groups of 20 normal young mice were injected s.c. with L18-MDP(Ala) 24 h before or 6 h after i.v. challenge with the MLD (5.0×10^6 CFU) of *C. albicans*. All of the untreated mice died by day 7 (Fig. 4a), whereas 75 and 30% of mice receiving the adjuvant 24 h before and 6 h after infection, respectively, survived beyond day 7. Thus, in normal mice, it was demonstrated that the adjuvant enhances the resistance to *C. albicans* infection. The treatment was more effective before infection than after.

When mice were injected i.p. with 200 mg of CY per kg 24 h before infection, the MLD of *C. albicans* was reduced to 2.5×10^6 CFU per mouse. With this inoculum, only 20% of CY-untreated mice died by day 7. To the CY-treated mice, 10 or 100 μg of the adjuvant per mouse was injected s.c. 2 and 24 h before infection. As a result, the mortalities on day 7 were reduced to 65 and 50% by s.c. treatment with 10 and 100 μg of the adjuvant, respectively (Fig. 4b).

In previous reports (14, 17), we found that L18-MDP(Ala) augmented the resistance of normal young mice to *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus*, or *C. albicans* infection. In aged mice, the resistance of *E. coli* infections appears to be depressed since all of the mice at 17 months of age died by day 7 after s.c. challenge with 10^6 CFU of *E. coli*, which is a sublethal dose for younger adult mice. With the same inoculum, on the other hand, all of the mice treated with L18-MDP(Ala) survived beyond day 7. As reported by our study group (15), the function of macrophages as scavenger cells was more accentuated in mice at 16 to 24 months of age than in younger adult mice. In these aged mice, the resistance to an early phase of infection with *Listeria monocytogenes*, which depends upon scavenger macrophages, was greater than that in young adult mice. Furthermore, we have found that L18-MDP(Ala) stimulated the resistance of immunosuppressed guinea pigs against pseudomonal pneumonia (Y. Osada, T. Otani, T. Une, H. Ogawa, and K. Nomoto, J. Gen. Microbiol., in press). The phagocytic activities of polymorphonuclear cells (PMNs) derived from the animals receiving the adjuvant were significantly higher than those of the cells from the untreated animals. Migration of PMNs to the infection site was also significantly greater in the adjuvant-treated animals than in untreated controls. Thus, it appears that direct or indirect activation of phagocytes or lymphocytes by the adjuvant may result in enhancement of resistance of animals to microbial infections.

The increase in susceptibility of the irradiated animals to bacterial infections is correlated not only with granulocytopenia, but also with the alteration of the phagocytic and bactericidal activities of PMNs (6, 8, 16). When mice were irradiated with 450 rads, more than 20 days were required for the restoration of these depressed

activities to their normal levels (6). We have also found that the peripheral blood leukocyte count is improved from its reduced level by treatment with L18-MDP(Ala), but not to the normal level by day 7 (data not shown). The depression of PMN function by exposure of mice to 400 rads may neutralize the stimulating effect of the adjuvant on restoration of the function from depressed levels.

CY has also been shown to reduce the number of various types of free cells, including PMNs, blood monocytes, and some subpopulations of lymphocytes (1). L18-MDP(Ala) stimulated the restoration of the resistance, previously reduced, to infection with *E. coli* or with *C. albicans*. Buhles and Shifrine (1) found a rapid restoration of the number of peripheral blood granulocytes in CY-treated mice receiving *Mycobacterium bovis* BCG or Freund complete adjuvant and suggested that the adjuvant protection against bacterial infection in CY-treated mice may be due in part to the stimulation of the production of progenitor cells of granulocytes by the adjuvants. CY has been used widely as an anticancer drug (4, 7, 18) and is known to reduce ultimately the host defense to microbial infections. L18-MDP(Ala) may be useful for treating infections occurring in such cancer patients.

The results of the present study suggest that the clinical application of L18-MDP(Ala) for therapy of patients with some degree of immunosuppression caused by various agents is worthy of study.

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