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Effect of *Bacillus subtilis* spore administration on activation of macrophages and natural killer cells in mice

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Abstract

The effect of *Bacillus subtilis* (strain A102) spores on the activation of murine macrophages and natural killer cells (NK) was examined. The macrophage activity and NK activity were enhanced by oral administration of A102 spores, and slightly enhanced by oral administration of culture supernatant. There was no difference in the results of macrophage activity and NK activity using other live or dead spores. The NK activity and macrophage activity were increased with increments of concentration up to 0.1 g per mouse, and both activities were decreased at concentration of more than 0.15 g per mouse. The NK activity was increased 1 and 2 days after oral administration of A102 spores, and the activity level 2 days after administration was about 3-fold higher than the level prior to treatment. Macrophage activity was also increased from 1 to 3 days after oral administration of A102 spores, and the activity level 3 days after administration was about 3-fold higher than the level prior to treatment. The induction of interferons at 1 day after oral administration in mouse serum was 5-fold higher than that in controls. These findings indicate that oral administration of A102 gave rise to the induction of interferons, and it is likely that macrophages and NK cells were activated by interferons. © 1998 Elsevier Science B.V.

Keywords: Bacillus subtilis; Mouse; Interferon; Macrophages; Natural killer cells

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1. Introduction

The development of vaccines and chemical drugs including antibiotics has greatly aided the control of various acute types of diseases in veterinary and human medicine. Diseases which are caused by opportunistic infections often associated with stress-mediated immunosuppression have become an increasing problem in companion animals and domestic animals. The administration of antibiotics for these diseases causes various problems such as residues and appearance of bacterial resistance. Biological response modifiers (BRM) and cytokines have been shown to be effective in several diseases in veterinary and human medicine (Bryson et al., 1989; Chiang et al., 1990; Rosenberg et al., 1986; Roth and Frank, 1989; Roth and Kaeberle, 1984; Woodard et al., 1983). Some polysaccharides of plant origin such as lentinan (Chihara et al., 1970a), pachymaran (Chihara et al., 1970b), schizophyllan (Komatsu et al., 1969), mannans (Hashimoto et al., 1983; Okawa et al., 1982), glucan (Seljelid et al., 1981; Williams et al., 1978), and hemicelluloses (Nakahara et al., 1967) have been reported to be immunopotentiators of host resistance to tumor growth and infection with pathogenic microbes. Muramyl peptide (N-acetylmuramyl-L-alanyl-D-isoglutamine), which is known to be structurally identical to a portion of the monomer of peptidglycan and a nearly ubiquitous component of bacterial cell walls, is an effective immunostimulant.

A previous clinical report showed that oral administration of *Bacillus subtilis* spores enhanced the host defense system against infectious diseases of the respiratory tract (Novelli et al., 1984), and a preliminary study demonstrated that the *B. subtilis* strain A102 also enhanced the host defense system against oral infection with *E. coli* in mice (unpublished observation). However, these observations did not discern what kind of immunological cells responded to the oral administration of *B. subtilis* spores. The phagocytic cells, such as neutrophils and macrophages, play important roles in the initial host defense system against infection with microbes, and natural killer (NK) cells are also involved in the initial host defense system against infections with virus. It was reported that *B. subtilis* peptidoglycan activated the macrophage-like cell line RAW264 in vitro (Vermeulen and Gray, 1984). Therefore, enhancement of the host defense system by *B. subtilis* spores might be related to the activation of the phagocytic cells and the NK cells.

The present study demonstrates the effect of *B. subtilis* A102 spores on the activation of macrophages, measured by a chemiluminescence technique, and on NK activity measured by a ^{51}Cr release assay in mice.

2. Materials and methods

2.1. Mice

All experiments were performed using male ddY mice from Shizuoka animal laboratory Co. Ltd. (Japan) at 5–6 week old, and each experiment used 6–8 mice.

2.2. Administration of A102

A102 was grown with nutrient broth medium (pH 7.2) at 37°C. The A102 were cultured by nutrient broth added with 0.52 mM $MgSO_4$, 13.4 Mm KCl and salt mixture (100 Mm $Ca(NO_3)_2 + 1$ Mm $MnCl_2 + 0.1$ Mm $FeSO_4$) for 24 h, and then the A102 spores were collected (Schaeffer et al., 1965). Mice were orally administer A102 spores (7×10^{11}/g). The A102 spores were identified as *B. subtilis* and are named DB9011, according to 'Bergeys Manual of Systematic Bacteriology, Vol. 2 (1986)'. Culture supernatant was collected from A102 cultured just after the plateau phase. Death spores were made by autoclave for 30 min at 121°C, and the surviving fraction of spores was less than 5% under this condition using Moller spore staining method. The sample was inoculated in nutrient broth medium and cultured for 24 h at 37°C. After incubation, the cultured was stained by Moller spore staining method, and the surviving fraction was calculated from an equation of surviving fraction = (No. of viable cell)/(No. of spores + No. viable cell) × 100.

2.3. Agents

One hundred mg of luminol and 50 μl of triethylamine (Wako Pure Chemicals, Japan) were added to 50 ml of RPMI 1640 and then allowed to dissolve. After complete

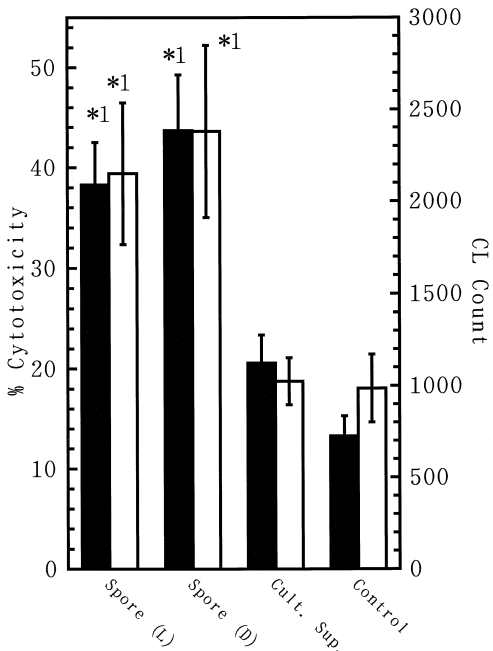

Fig. 1. Effect of oral administration with A102 spores or culture supernatant on macrophage activity and NK activity. The activity of macrophages (■) was detected by a chemiluminescence technique, and NK activity (□) was measured by ^{51}Cr release assay at 50:1 as E:T ratio. Each column and error bar represent the mean and standard error, respectively, obtained from 3 to 4 independent experiments. *1, Significantly different from the value for control, $p < 0.05$.

dissolution with sufficient agitation, the solution was passed through a 0.45 μm membrane filter (Millipore Co., USA) and stored in the dark at -20°C until use.

2.4. Target cells

YAC-1 cells were maintained in RPMI-1640 (Nissui Co., Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Co., USA), 0.3 g l^{-1} of glutamine and 60 mg l^{-1} of kanamycine at 37°C in a humidified atmosphere containing 5% CO_2.

2.5. Preparation of splenic cells

The splenic cells were collected under sterilized conditions from mouse spleen from 1 to 5 days after oral administration of A102 spores.

2.6. Preparation of lymphocytes and macrophages

The mononuclear cells were isolated from splenic cells by Lymphoprep (Nycomed Pharama AS., Norway) centrifugation at 400 $\times$ g for 10 min. Isolated mononuclear cells were washed with Dulbecco's phosphate-buffered salt solution (PBS) prior to centrifuga-

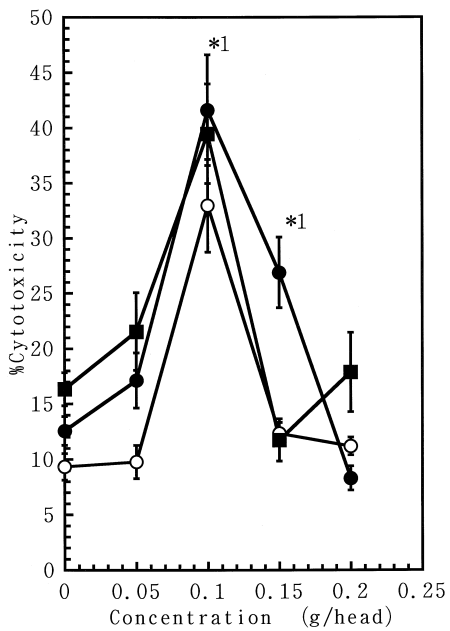

Fig. 2. Dose–response curve of NK activity induced by oral administration with A102 spores. NK activity at 25:1 (○), 50:1 (□), and 100:1 (■) as an E:T ratio were measured by ^{51}Cr release assay 2 days after oral administration with A102 spores in mice splenic cells. Each point and error bar represent the mean and standard error, respectively, obtained from 3 to 4 independent experiments. * 1, Significantly different from the value for control, $p < 0.05$.

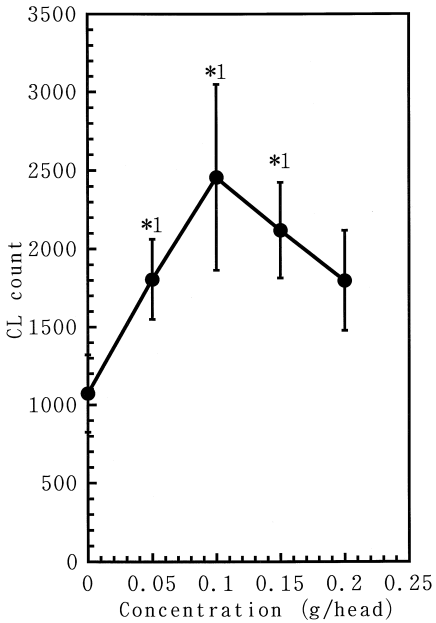

Fig. 3. Dose-response curve of chemiluminescence (CL) activity of macrophages induced by oral administration with A102 spores. The CL activity of macrophages was detected by a luminol-dependent CL technique assay 2 days after oral administration with A102 spores in mice splenic cells. Each point and error bar represent the mean and standard error, respectively, obtained from 3 to 4 independent experiments. *1, Significantly different from the value for control, $p < 0.05$.

tion at 100–150 $\times g$ for 5 min. The mononuclear cells were resuspended in 2 ml of RPMI 1640 supplemented with 10% FBS and incubated for 1 h at 37°C in a 35-mm plastic petri dish (Falcon Co., USA). After incubation, non-adherent cells in the culture were collected by centrifugation for 100 $\times g$ for 5 min for use in the NK assay, and adherent cells in the culture were collected by trypsinization and resuspended in RPMI 1640 for use in the chemiluminescence assay.

2.7. Cytotoxic assay

The cytotoxic assay was performed as described previously (Kosaka et al., 1992). Briefly, 1×10^6 target cells were labeled with 3.7 MBq (100 μCi) of sodium ^{51}chromium solution (NEN Research, USA) for 1 h at 37°C in 0.3 ml of the medium. After washing three times, 1×10^4 cells in 100 μl of the medium were placed in each well of a 96-well microplate (Falcon Co., USA). Various numbers of effector cells in 100 μl of the medium were added to the wells of the microplate in triplicate with an effector/target ratio ranging from 25:1 to 100:1. After incubation at 37°C for 4 h, each supernatant was collected and counted in a gamma scintillation counter (LB-951G, Berthold, Germany). The percent cytotoxicity (PC) was calculated as follows:

$$\mathrm{PC} = \frac{A - C}{A - B}$$

where A is the cpm for maximum release, B is the cpm in spontaneous release, and C is the cpm in released from cells during incubation with various numbers of effector cells.

2.8. Chemiluminescence (CL) assay

Oxidative burst activity was used as an indicator of neutrophils and macrophages in peripheral blood (Klebanoff, 1982; Rossi et al., 1972) and was measured by chemiluminescence (Biolumat LB-9505, Berthold, Germany) (Allen et al., 1972; Kuwabara et al., 1990). For the adaptation to the dark, 6×10^5 ml^{-1} of macrophages were incubated in a plastic vial (12×47 mm) at 37°C for 10 min. After dark adaptation, 50 μl of luminol solution was added to each vial, and the background CL was measured for 10 min. Two hundred μl of KAC-2 solution (Nihon Antibody Laboratory, Japan) as the stimulant was added to each vial. The CL count was determined for the macrophages at 60 min after the addition of luminol. Transitional CL activity was calculated by means of the following equation and compared thereafter:

$$\text{CL count} = \text{total count after stimulation} - \text{count of background}$$

where total count after stimulation was the total CL from the time of injecting the stimulant to the time when macrophages attained the maximum count.

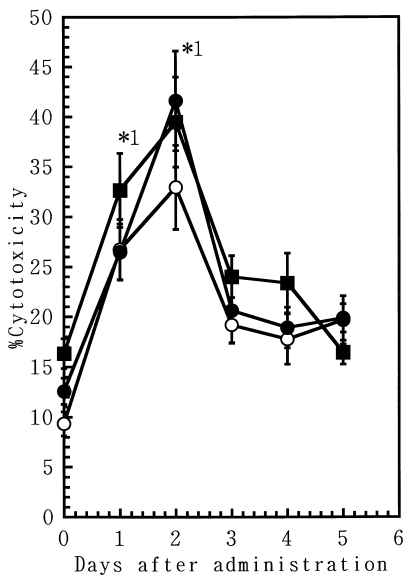

Fig. 4. Changes of NK activity after oral administration with A102 spores. NK activity at 25:1 (○), 50:1 (●), and 100:1 (■) as an E:T ratio were measured daily by ^{51}Cr release assay from a point prior to treatment, to 5 days after oral administration with A102 spores in mice splenic cells. Each point and error bar represent the mean and standard error, respectively, obtained from 3 to 4 independent experiments. *1, Significantly different from the value for control, $p < 0.05$.

2.9. Measurement of IFNs induction in mice serum

IFN in the mouse serum was measured by biological assay using mouse L929 cells as described previously (Armstrong, 1971). The mouse blood was collected from the heart using a needle, and serum was then collected by centrifugation at 800 g for 10 min. Briefly, 1×10^5 of L929 cells were plated to a 96-well flat-bottomed microplate and incubated for 17 h at 37°C. After incubation, the culture supernatant was discarded, and 100 μl of diluted mouse serum was added, followed by incubation for 3 h at 37°C. After cells were treated with mouse serum, the mouse serum was discarded and the cells were washed with PBS twice, and infected with Vesicular stomatitis virus (VSV, 1×10^4 PFU well^{-1}) for 1.5 h; the cells were then washed with PBS twice. Next, 100 μl of fresh medium was added to the cells, followed by incubation at 37°C for 24 h. The VSV was inactivated by irradiation with U.V. light. The cells were stained with 0.3% crystal violet and washed with tapped water. The CPE in the L929 cells was measured by a microplate reader (3550, BIORAD, USA) at 580 nm.

2.10. Statistical analysis

Statistical analysis of the data was performed with a software program. Each mean and standard error in the figures were calculated from 3 or 4 independent experiments,

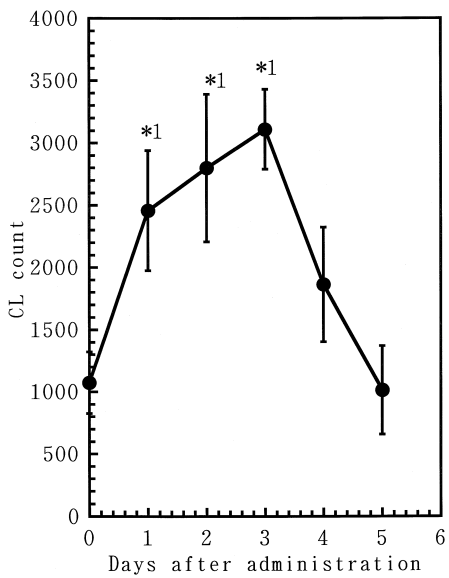

Fig. 5. Changes of macrophage activity after oral administration with A102 spores. CL activity of macrophages was detected by a luminol-dependent CL technique assay from a point prior to treatment, to 5 days after oral administration with A102 spores in mice splenic cells. Each point and error bar represent the mean and standard error, respectively, obtained from 3 to 4 independent experiments. * 1, Significantly different from the value for control, $p < 0.05$.

and differences between the control value and each point was analyzed by Student's *t*-test. Significance was defined as $p < 0.05$.

3. Results

3.1. Effect of A102 spores on NK activity and macrophage activity

NK activity and macrophage activity in mice 2 days after the administration of A102 spores were measured by ^{51}Cr release assay and CL assay, respectively, as shown in Fig. 1. NK activity and macrophage activity were significantly increased after the oral administration with life or death spores and slightly increased after the oral administration with culture supernatant of A102. These results indicated that the activation of macrophages and NK cells might be related to a component of A102 spores, but not to the secretion in the culture supernatant from A102.

3.2. Dose-response curves of NK activity and macrophage activity by A102 spores

Figs. 2 and 3 show the dose–response curves of NK activity and macrophage activity by oral administration of A102, respectively. The NK activity and macrophage activity were increased with increments of concentration up to 0.1 g per mouse, and both activities were decreased at concentrations of more than 0.15 g per mouse. These findings showed that the optimal concentration of A102 spores for the activation of NK cells and macrophages in mice was 0.1 g per mouse.

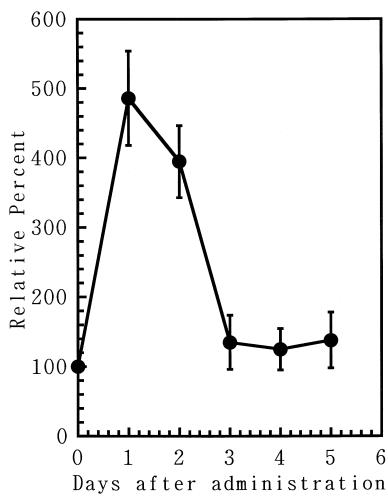

Fig. 6. Changes of IFNs induction in mice serum after oral administration with A102 spores. Relative percent (RP) was calculated as an equation of RP = (units of IFNs in mouse serum with treatment)/(units of IFNs in mouse serum without treatment)×100. Each point and error bar represent the mean and standard error, respectively, obtained from 3 to 4 independent experiments. * 1, Significantly different from the value for control, $p < 0.05$.

3.3. Changes of NK activity and macrophage activity following oral administration with A102 spores

NK activity was increased at 1 and 2 days after oral administration of A102 spores, and the activity level at 2 days was about 3-fold higher than that prior to treatment (Fig. 4). Macrophage activity was maintained at a higher level until 3 days after oral administration of A102 spores, and the activity level at 3 days was about 3-fold higher than that prior to treatment (Fig. 5). These results indicated that the activation of macrophages and NK cells was still retained for a few days after oral administration with A102, and the activation of macrophages was retained longer than that of NK cells.

3.4. Kinetics of IFNs induction in mice serum followed by oral administration with A102 spores

The induction of IFNs was rapid, and the peak time of IFNs induction was 1 day after oral administration of A102 spores (Fig. 6). The induction of IFNs at 1 day after oral administration in mouse serum was 5-fold higher than that for control. The disappearance of IFNs in mice sera occurred about 3 to 4 days after the oral administration of A102 spores. These findings demonstrated that the IFNs induction after oral administration with A102 in mice was more rapid than the enhancement of macrophage and NK activity, and the secretion of IFNs thus might be a trigger of the activation of macrophage and NK cells.

4. Discussion

Phagocytic cells, such as polymorphonuclear cells and macrophages, and NK cells in peripheral blood play important roles in the antigen-independent initial host defense system against infection with bacteria, viruses and other microbes. Our present findings demonstrated that the CL activity of macrophages and the NK activity were enhanced by oral administration with *B. subtilis* strain A102 spores, but not by culture supernatant. The optimal concentration of oral administration of A102 spores was 0.1 g per mouse for both NK and macrophage activity. These results indicated that the activation of NK cells and macrophages was caused by a component of the spores, but not by a secretion from A102 or its spore. However, A102 spores did not activate the NK cells and macrophages in vitro (data not shown). An earlier study demonstrated that an extract of *B. subtilis* cell walls, which was one of peptidoglycan, activated the macrophage-like cells in vitro and induced IL-1 from same cell line (Vermeulen and Gray, 1984). Our present results also indicated that the activation of macrophages occurred 1 day after administration of A102 spores and faster than that of NK cells. Therefore, the activation of macrophages and NK cells by oral administration of A102 spores might be related to the secretion of cytokines from mononuclear cells in peripheral blood. Our findings demonstrated that the induction of INFs occurred from 1 to 2 days after oral administration of A102 spores, and the peak induction time of INFs was 1 day after the administration. Macrophages were activated by IFN-γ (Le et al., 1983), and the

activated macrophages also secreted IL-1, IL-12, G-CSF, and TNFs (D'Andrea et al., 1992; Gery et al., 1981; Koeffler et al., 1987; Matthews, 1978). NK cells were activated by IFN-γ, IL-1, IL-2, IL-6, IL-12 and TNF (Henney et al., 1981; Kobayashi et al., 1989; Lopez-Botet and Moretta, 1985; Herman et al., 1985; Ostensen et al., 1987; Trinchieri and Santoli, 1978). These findings suggested that oral administration with A102 spores in the present study the gave rise to the induction of IFNs, and that macrophages and NK cells were activated by the IFNs and other cytokines which were secreted from leukocytes stimulated with IFNs.

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