Macrophage Activation and Immunostimulating Activity of *Sphaerotilus natans* and Its Slime Fraction

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The macrophage activation and immunostimulating effects of the slime of *Sphaerotilus natans* IAM 12068, which is an aquatic sheathed bacterium, and GF-P-1, which was isolated from the slime fraction by Sepharose 4B gel filtration and has antitumor activity against Ehrlich ascites tumor in mice, were investigated. C57BL/6 mice peritoneal exudate macrophage induced by GF-P-1, which exhibited more effective antitumor activity than slime fraction, had the strongest cytostatic activity against EL-4 leukemic cells in vitro. Addition of GF-P-1 to mouse peritoneal macrophages in vitro caused marked spreading morphology, but the slime fraction did not. Acid phosphatase activity in the peritoneal cells induced by slime and GF-P-1 was also augmented as compared with that of resident macrophages. *S. natans* fractions, as well as *Salmonella typhimurium* lipopolysaccharide, were able to induce the mitogenic response in cultured spleen cells of C57BL/6 mice. Since spleen cells pretreated with rabbit anti-mouse thymocyte antiseraum showed an unaffected mitogenic response to slime, the slime may be a B-lymphocyte mitogen. When slime fraction and GF-P-1 were injected intraperitoneally into ddY mice, they exhibited an enhancing effect on antibody response in vivo. These results indicate that slime and GF-P-1 are able to activate macrophages and exhibit immunostimulating effects.

**Keywords**— *Sphaerotilus natans*; slime fraction; cytostatic activity; macrophage activation; mitogenicity; adjuvanticity

*Sphaerotilus natans*, which is a gram-negative, aquatic sheathed bacterium, belongs to the order Chlamy dobacteria. The organisms of the group are characterized by the presence of slime on the surface of the sheath.

We previously reported that slime fraction of *S. natans* has antitumor activity against Ehrlich carcinoma cells in mice. Furthermore, two fractions designated as GF-P-1 and GF-P-2, which are mainly composed of protein, carbohydrate, and lipid, were isolated from slime fraction by Sepharose 4B gel filtration. GF-P-1, which contains large amounts of fucose and unidentified sugar as neutral sugar, showed marked antitumor activity at half the dose of the slime fraction, whereas the antitumor activity of GF-P-2, which is composed mainly of protein, was weak.

Various microorganisms and their components are capable of suppressing tumor growth and have immunostimulating effects such as macrophage activation, mitogenic activity for lymphocytes or an adjuvant effect on antibody response. It is generally considered that such an immunostimulant effect of microorganisms plays some role in their antitumor activity. In this paper, we describe the in vitro cytostatic activity of C57BL/6 mice peritoneal macrophages induced by slime fraction and GF-P-1 against syngeneic EL-4 leukemic cells and the immunostimulating effects, such as mitogenic activity and an adjuvant effect.
Materials and Methods

**Strain and Medium**—*Sphaerotilus natans* IAM 12068 was provided by the Institute of Applied Microbiology, University of Tokyo, Tokyo. The organism was cultured under aeration at 30°C for 24 h in modified Stokes’ medium. The grown cells were harvested by continuous centrifugation at 4°C and were washed twice with distilled water.

**Preparation of Slime Fraction of *S. natans***—The procedure for the preparation of *S. natans* slime and its fractions, GF-P-1 and GF-P-2, was the same as described previously. In brief, washed cells were homogenized in distilled water with a Waring blender and centrifuged. The supernatant fraction was treated with 2% streptomycin sulfate and the precipitate was removed by centrifugation. The supernatant was lyophilized to provide the slime fraction. Furthermore, the slime fraction was applied to a column of Sepharose 4B (Pharmacia Fine Chemical, Uppsala, Sweden) and separated into two fractions designated as GF-P-1 and GF-P-2, respectively. Their chemical compositions were reported in the previous paper.

**Animals**—C57BL/6 mice, 5–8 weeks old, were used in the experiments. These mice were supplied by our animal colony. Male ddY mice, weighing 20–25 g, were purchased from the Shizuoka Agricultural Cooperative for Laboratory Animals, Hamamatsu.

**Cell Line**—Mouse EL-4 leukemic cells were kindly provided by Dr. Kyoko Tanaka, the Aichi Cancer Center, Nagoya. The cells were maintained in tissue culture flask (Falcon, #3013) containing 10 ml of RPMI-1640 medium (Nissui Seiyaku, Co., Ltd., Tokyo) supplemented with 10% fetal calf serum (FCS: GIBCO) at 37°C in 5% CO₂/air.

**Preparation of Peritoneal Macrophages**—C57BL/6 mice were injected intraperitoneally (i.p.) with *S. natans* whole cells (1.0 mg), slime (0.5 mg), GF-P-1 (0.2 mg), or GF-P-2 (0.2 mg) on days 1, 3, 5, and 7. Three days later, the peritoneal exudate cells were collected with a syringe and washed three times with saline. The cells were disrupted by freezing and thawing in distilled water, and centrifuged at 4°C. The adherent cells were collected as peritoneal macrophages in phosphate-buffered saline containing 0.2% ethylenediaminetetraacetic acid (EDTA) with a rubber policeman.

**Assay of Cytostatic Activity of Mouse Peritoneal Macrophages**—The *in vitro* cytostatic activity against tumor cells by C57BL/6 mice peritoneal macrophages, which were induced by i.p. injection of *S. natans* or its fractions, was determined by measuring the inhibition of tumor cell deoxyribonucleic acid (DNA) synthesis using β-H-thymidine (³H-TdR) as the method of Benacerraf et al. ³H-LE-4 leukemic cells (100 μl; 2.5 x 10⁴ EL-4 cells/ml) were seeded in 96-well microplates (Falcon, #3072), and cultured at 37°C for 40 h in 5% CO₂/air. Each well was pulsed with 0.1 μCi of ³H-TdR (Radiochemical Centre, England) for the final 16 h of incubation. The cultured EL-4 leukemic cells and macrophages were harvested on a glass fiber filter (Whatman GF/A) and suspended in saline containing 0.2% ethylenediaminetetraacetic acid (EDTA) with a rubber policeman.

**Mitogen and Adjuvant**—Concanavalin A (Boehringer Mannheim, West Germany) was used as a specific T-lymphocyte mitogen. Lipopolysaccharide (LPS), which was extracted from *Salmonella typhimurium* LT-2 by the hot phenol–water method, was used as an adjuvant and a specific B-lymphocyte mitogen.

**Mitogen Studies**—Suspensions of splenic and thymic cells of C57BL/6 mice were prepared as described previously. The cells were suspended in RPMI-1640 medium supplemented with 10% FCS, and 20 μl of a sample solution and 180 μl (5 x 10⁶ cells) of the cell suspension were placed in wells of a 96-well microplate. The plate was incubated at 37°C in 5% CO₂/air. Each well was pulsed with 0.25 μCi of ³H-TdR for the final 18 h of incubation. After incubation for 48 h, the cultured spleen cells were harvested on a glass fiber filter. The radioactivity taken up by the cells was measured with a scintillation counter (Aloka, LSC-661, Aloka Co., Tokyo) in toluene scintillant containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazoly)]-benzene per liter of toluene.

**Rabbit Anti-thymocyte Serum (ATS) Treatment of Spleen Cells**—ATS was prepared according to the method of Gray et al. The prepared ATS showed cytotoxicity titers of 2³ and 2⁴ for mouse thymocytes and bone marrow cells, respectively, based on the Trypan Blue dye exclusion method.

The spleen cells from C57BL/6 mice were incubated with suitably diluted ATS at 37°C for 45 min in the presence of complement (guinea pig serum) in order to kill T-lymphocytes in the spleen cell population. As a reference, normal rabbit serum (NRS) was used.

**Hemolytic Plaque Assay**—Sheep red blood cells (SRBC) were used as the antigen. SRBC (10⁶) and/or *S. natans* and its fraction were injected i.p. into ddY mice. Spleen cells were prepared from each mouse and the number
of plaque-forming cells (PFC) in the spleen was determined by the technique of localized hemolysis in agar.\textsuperscript{12)} The results are expressed as mean PFC per five dishes, with S.D.

Results

In Vitro Cytostatic Activity of Peritoneal Exudate Macrophages from \textit{S. natans}-Injected Mice against EL-4 Leukemic Cells

\textit{In vitro} cytostatic activity against EL-4 cells by peritoneal exudate macrophages was evaluated by measuring the inhibition of tumor cell DNA synthesis in terms of the incorporation of $^3$H-TdR. Peritoneal macrophages induced by injection of slime (0.5 mg/d) or GF-P-1 (0.2 mg/d) exhibited a marked cytostatic activity at effector-to-tumor cell (E/T) ratios of 10 and 20 (Fig. 1).

On the other hand, peritoneal macrophages induced by whole cells (0.5 mg/d) of \textit{S. natans} and resident macrophages showed no inhibitory activity at the E/T ratio of 10.

Next, the cytostatic activity of peritoneal exudate macrophages induced by GF-P-1 or GF-P-2 was compared (Fig. 2). GF-P-1 exerted marked cytostatic activity at E/T ratios of 10 and 20, whereas GF-P-2 showed inhibition at the E/T ratio of 20, but exhibited no activity at the E/T ratio of 10.

Morphological Change of Macrophages Treated with Fractions of the Slime

We examined the morphological change of mouse peritoneal macrophages on addition of GF-P-1 or GF-P-2 \textit{in vitro}. As shown in Fig. 3-b, the macrophages incubated with GF-P-1 for 40 h exhibited marked spreading, but macrophages incubated with GF-P-2 (Fig. 3-c) and resident macrophages (Fig. 3-a) did not show any morphological change. The results suggest that GF-P-1 can cause the activation of peritoneal macrophages.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Cytostatic Activity of Peritoneal Macrophages Induced by Treatment with \textit{S. natans} and Its Fraction, Measured in Terms of $^3$H-TdR Incorporation of EL-4 Leukemic Cells \textit{in Vitro}}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Cytostatic Activity of Peritoneal Macrophages Induced by Treatment with GF-P-1 or GF-P-2, Measured in Terms of $^3$H-TdR Incorporation of EL-4 Leukemic Cells \textit{in Vitro}}
\end{figure}

\begin{align*}
\text{cpm (effector + tumor) - cpm effector alone} & \quad \times 100 \\
\text{cpm tumor alone}
\end{align*}
Acid Phosphatase Activity in Peritoneal Exudate Cells

Acid phosphatase activity in peritoneal exudate cells from mice that had received i.p. S. natans whole cells, slime, GF-P-1, or GF-P-2 was measured (Table I). The activity of peritoneal exudate cells as a control (saline) was 10.8 milliunits/mg protein, whereas the group of mice treated with GF-P-1 (0.2 mg) showed a high activity of 25.7 milliunits/mg protein. However, the acid phosphatase activity induced by GF-P-1 or GF-P-2 (each 0.2 mg) was considerably weak in comparison with that induced by S. natans whole cells (1.0 mg) or slime (0.5 mg).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/d)</th>
<th>Acid phosphatase activity (milliunits/mg protein)</th>
<th>Stimulation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (control)</td>
<td>0</td>
<td>10.8</td>
<td>1.0</td>
</tr>
<tr>
<td>S. natans whole cells</td>
<td>1.0</td>
<td>51.9</td>
<td>4.8</td>
</tr>
<tr>
<td>Slime</td>
<td>0.5</td>
<td>40.3</td>
<td>3.7</td>
</tr>
<tr>
<td>GF-P-1</td>
<td>0.2</td>
<td>25.7</td>
<td>2.4</td>
</tr>
<tr>
<td>GF-P-2</td>
<td>0.2</td>
<td>21.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Mice were injected i.p. with each fraction on days 1 and 3. The activity of acid phosphatase was assayed on day 5. a) One unit was defined as the enzymatic activity which liberates 1 µmol of para-nitrophenol per min at 37 °C.

Mitogenic Effect of S. natans Fraction on Cultured Spleen Cells

To evaluate the mitogenic effect of S. natans fraction, different doses (1—100 µg/ml) of each fraction were added to the cultured spleen cells of C57BL/6 mice and the incorporation of ³H-TdR into these cells in vitro was measured (Table II). S. natans slime and its fractions exhibited a strong mitogenicity, and the dose–response relationships of S. natans slime and GF-P-1 were almost the same. Although slime and GF-P-1 were most effective at a dose of 10 µg/ml, GF-P-2 was most effective at a dose of 100 µg/ml. The mitogenic activities of GF-P-1
and GF-P-2 were more potent than that of the original slime fraction.

In order to determine whether the slime fraction is a T or B-lymphocyte mitogen, T-lymphocytes in a spleen cell population were killed by ATS in the presence of complement. After the treatment with ATS, the mitogenic response of cultured cells to concanavalin A, a T-lymphocyte mitogen, was markedly reduced, whereas the incorporation of $^3$H-TdR into ATS-treated spleen cells was unaffected. The slime fraction was also capable of increasing the incorporation of $^3$H-TdR into ATS-treated spleen cells (Table III).

On the other hand, $^3$H-TdR incorporation of thymocytes stimulated by the slime fraction was weak (data not shown). These findings suggest that the slime acts on a population of B-lymphocytes.

**Adjuvant Effect of S. natans Slime and Its Fraction on Antibody Response**

The adjuvant effect of S. natans whole cells, slime, and its fractions on the antibody response to xenogeneic erythrocytes in ddY mice was determined (Table IV). The whole cells of S. natans and its fraction exhibited an adjuvant effect on day 2 after immunization. Slime fraction (0.5 mg) and GF-P-1 (0.25 mg) showed stimulation index values of 6.7 and 7.6.
respectively. In contrast, the activity of *S. natans* whole cells (1.0 mg) and GF-P-2 (0.25 mg) was weak.

**Discussion**

Previously, we examined the antitumor activity of *S. natans* and found that the slime, which is formed on the outer sheath of the organism, and GF-P-1, which was obtained from the slime fraction by Sepharose 4B gel filtration, have remarkable antitumor activity against Ehrlich ascites tumor cells in mice. Since the antitumor activity was abrogated by treatment of mice with i.p. injection of silica, the activity seemed to be mediated by macrophages activated by *S. natans* fractions.

Various immunomodulators, including LPS or lipid A, can induce spreading morphology of peritoneal macrophages in vitro,13 this being one of the criteria for macrophage activation.14 Activation of macrophages by GF-P-1 may be necessary for expression of antitumor activity in vivo and in vitro. In this paper, we have shown that GF-P-1 is able to activate mouse peritoneal macrophages, as judged by the induction of morphological change, enhancement of acid phosphatase activity, and exhibition of cytostatic activity against tumor cells in vitro. Peritoneal macrophages incubated with GF-P-1 induced marked spreading morphology (Fig. 3-b). On the other hand, macrophages incubated with GF-P-2 and resident macrophages showed spherical morphology (Fig. 3-a, -c). Moreover, cytostatic activity by *S. natans* whole cells and GF-P-2 was weak. Our results indicate that spreading morphology of macrophages may result in an enhancement of the potency of cytostatic activity.

*S. natans* fractions exhibited strong mitogenic activity. In particular, 10 µg/ml of GF-P-1 was more effective than the same dose of *S. typhimurium* LPS. However, 100 µg/ml of GF-P-2, which possesses weak antitumor activity, exhibited the most effective mitogenic activity in this experiment, except for concanavalin A (Table II). The potency of the mitogenic activity did not coincide with that of the antitumor activity.

Slime and GF-P-1 had effective adjuvant activity 2 d after SRBC injection. After 4 d, there was no difference in the number of plaque-forming cells among the groups (data not shown). This result suggests that slime and GF-P-1 as well as bacterial LPS15 are capable of initiating nonspecifically the maturation of antibody-producing cell precursors and converting them to antibody-producing cells at an early stage of antibody production. When various immunomodulators stimulate macrophages, they produce many kinds of cytokine.16 To elucidate the mechanism of immunological activity of GF-P-1, production of cytokines should be investigated, for instance, macrophage-arming factor, interferon and so on.

It is well known that bacterial LPSs show mitogenicity and adjuvanticity.17 *S. natans*
belongs to the category of gram-negative bacteria and may possess LPS in the outer membrane. Previously, we carried out isolation of LPS from the organism by the hot phenol–water technique, but the yield and the biological activity were very low in comparison with those of LPS from other gram-negative bacteria. The immunostimulating effect of GF-P-1 does not seem to be due to LPS, since the content of LPS in GF-P-1 detected by the Limulus test was only trace (data not shown). To elucidate the mode of action of antitumor activity of GF-P-1, further purification of the substance will be necessary.

References