Cytokine-Related Immunopotentiating Activities of Paramylon, a β-(1→3)-D-Glucan from Euglena gracilis

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Paramylon, a β-(1→3)-d-glucan, isolated from Euglena gracilis, was tested for its adjuvant activity on the antibody response to sheep red blood cell (SRBC) in mice. Paramylon markedly enhanced anti-SRBC plaque-forming cell production at a dose of 10 mg/kg. It was also found that in vitro addition of lipopolysaccharide in culture to macrophages from paramylon-treated mice produced a large amount of interleukin 1 (IL-1) and there was a significant level of interleukin 6 (IL-6) produced transiently in the blood of these mice. As IL-1 and IL-6 play crucial roles in the immune response to T cell-dependent antigens like SRBC, the immunopotentiating effect of paramylon might be expressed through the action of these cytokines.

**Keywords** — paramylon; β-(1→3)-d-glucan; plaque-forming cell; adjuvant; cytokine; interleukin 1; interleukin 6

**Introduction**

Many kinds of naturally occurring polysaccharides are known to inhibit the growth of various transplantable tumors.¹⁻⁴ These bioactive polysaccharides have been pronounced to have a stimulatory effect on the immune system, and in particular on the macrophages.⁵,⁶ The most effective antitumor polysaccharides, lentinin and sizofiran, consist of a β-(1→3)-polyglucose backbone, of which every second or third residue is 6-O-substituted by short branches. In contrast, pachymann from Poria cocos is a similar branched β-(1→3)-d-glucan showing no antitumor activity,⁷ suggesting that the structural requirement for β-(1→3)-d-glucans to give antitumor activity is rather complicated. The other investigators⁸ proposed that the ultrastructure of β-(1→3)-d-glucans with a triple-helix conformation was required for antitumor activity. These polysaccharides are thought to activate macrophages to potentiate their cytostatic and cytolytic capacity. Paramylon, a linear β-(1→3)-d-glucan isolated from Euglena gracilis, was shown to possess a similar triple-helical structure⁹ to that of lentinin and sizofiran,¹⁰ but its antitumor activity against sarcoma 180 tumor was reported to be quite weak.¹¹ However, it may be that paramylon could exert more intensely its adjuvant activity to some immunological reactions other than antitumor immunity.

In this study, the adjuvant activity of paramylon to humoral antibody production was examined in mice together with its enhancing effect on the cytokine production, and this β-(1→3)-D-glucan was shown to be potent in augmentation of the ant-SRBC plaque-forming cell (PFC) and interleukin 1 (IL-1) production.

**Materials and Methods**

Isolation of Paramylon — The cells of Euglena gracilis strain Z were grown heterotrophically in darkness at 29 °C in the Oda medium supplemented with vitamin B₁ (2.5 mg/l) and vitamin B₁₂ (10 μg/l). After cultivation with forced aeration (0.5 l/l/min) for 5 d, the cells were harvested by centrifugation and washed with distilled water. The cells were killed by an addition of a mixture of MeOH-CHCL₃-7 M formic acid (7:5:3, v/v) and washed with distilled water. Yield was ca. 30 g/l (wet weight). The cell residues were incubated with 50 mg of trypsin in 50 mM phosphate buffer (pH 7.01) containing 0.3 M sucrose and 0.38 M mannitol. The residues were collected by centrifugation, washed with distilled water several times and then dried
to yield a white crystalline powder. Yield was 50—53% of dried cell weight. Anal. Calcd for 
(C₆H₁₁O₆)₂·(C₈H₁₀O₅)₂: C, 44.44; H, 6.18. 
Found: C, 44.24; H, 6.29; N, 0.00. The content 
of endotoxin in the paramylon preparation used 
here was less than 0.03 EU/ml as judged by 
Limulus test.

**Animals** — Male 6—8 week-old CDF₁, 
C3H/HeJ and BALB/c mice were obtained from 
the Shizuoka Laboratory Animal Center, 
Shizuoka. Mice were maintained on water and 
routine mouse chow **ad libitum**.

**Culture Medium** — PRMI1640 medium 
(Nissui Seiyaku Co., Tokyo) was supplemented 
with 5% heat-inactivated fetal calf serum (FCS) 
(General Scientific Laboratories, CA), 2 mM L-
glutamine, 1 mM sodium pyruvate and 60 µg/ml 
kanamycin (culture medium).

**Antibody Production** — Mice were in-
travenously (i.v.) immunized with 0.2 ml sheep 
red blood cells (SRBC, 2 × 10⁸ cells) into CDF₁ 
mice. Anti-SRBC PFC response was assayed 4 
d after immunization according to the modified 
method of Cunningham.¹²

**IL-1 Production** — Three days after an i.p. 
injection of 2 ml of 3% thioglycolate solution 
into CDF₁ mice, peritoneal exudate cells were 
ashed out with phosphate-buffered saline 
(PBS) (pH 7.4) through the anterior abdominal 
wall of the mice. After the peritoneal exudate 
cells were incubated for 60 min in 35 mm i.d. 
dishes, the nonadherent peritoneal cells were 
washed out with warm PBS and the adherent 
cells (1 × 10⁶ cells/ml) were incubated with or 
without 40 µg/ml lipopolysaccharide (LPS) in 
the culture medium at 37 ºC in a 5% CO₂-
humidified incubator. After 48 h, cell-free super-
натанты were collected. Aliquots (100 µl) were 
delivered into wells of a 96 well flat-bottomed 
plate and diluted three-fold successively, and IL-
1 activity was determined by the co-mitogenic as-
say using thymocytes prepared from C3H/HeJ 
mice.¹³

**Interleukin 2 (IL-2) Production** — Spleen 
cells from CDF₁ mice were adjusted to a con-
centration of 5 × 10⁶ cells/ml in the culture 
medium and 2 ml of aliquots were delivered into 
12 well flat-bottomed plates. The cells were in-
cubated with or without 5 µg/ml Con A at 37 
ºC in a 5% CO₂-humidified incubator. After 48 
h, the cell-free supernatants were collected and 
dialyzed twice against PBS and once against the 
serum-free culture medium supplemented with 
0.1 M methyl-α-D-mannopyranoside (Sigma 
Chemical Co., St. Louis, MO) and then deter-
dined for IL-2 activity using IL-2-dependent mu-
rine CTLL-2 cells by the method of Gillis et al.¹⁴

**Assay for Interleukin 6 (IL-6) Induced in Se-
rum** — Paramylon suspended in PBS contain-

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**Fig. 1.** Effect of Paramylon and Sizofiran on the Anti-SRBC PFC Response
(A) Mice were immunized i.v. with SRBC (2 × 10⁸) on day 0 and treated i.p. with paramylon for the 5 consecutive 
days (day 2 to day 7). Spleen cells prepared 4 d after immunization were assayed for anti-SRBC PFC. Data are represent-
ed as mean ± S.D. of the values from 5 mice. 
(B) Mice were immunized with SRBC and then treated with sizofiran or paramylon in the same manner as mentioned 
in (A). Data are represented as mean ± S.D. of the values from 3 to 5 mice. Significantly different from control, a)p < 0.05, 
b)p < 0.01.
ing 30 μg/ml mouse serum albumin was injected i.p. into BALB/c mice. After 3 and 6 h, blood samples were taken and centrifuged at 200 × g for 5 min. Each serum was assayed for IL-6 activity using IL-6-dependent MH60-BSF2 cells by the method of Matsuda et al. 15)

Statistics — Results were compared using Student's t-test.

Results

Adjuvant Activity of Paramylon to Antibody Responses

Figure 1A shows the adjuvant activity of paramylon to antibody responses against SRBC. A significant enhancement of IgM PFC response was observed in mice treated i.p. with 10 and 50 mg/kg paramylon for the five consecutive days (day -2 to day +2) before and after immunization (day 0), while the treatment with 1 mg/kg paramylon showed no increment in the antibody response. The immunostimulating activity was similar to that of sizofiran used as a control polysaccharide (Fig. 1B).

Serum IL-6 Level Induced with Paramylon

As IL-6 was one of the important cytokines mediating antibody responses, the serum level of IL-6 was measured after paramylon injection (Table I). Three hours after paramylon injection, IL-6 in sera was significantly increased, but reverted to the normal level in 6 h.

Effect of Paramylon on the Production of IL-1 and IL-2

Mice were i.p. treated with paramylon for the five consecutive days and peritoneal exudate cells were isolated from the mice. These cells were cultured in the presence or absence of LPS for 48 h in vitro and the culture supernatants were tested for IL-1 activity. As shown in Fig. 2, administration of paramylon in the dose range of 10—50 mg/kg showed a significant stimulating activity to IL-1 production. In order to examine paramylon effect on IL-2 production, spleen cells were prepared from the paramylon-treated mice and cultured in vitro with Con A, and the resultant

![Image of Fig. 2](https://example.com/fig2.png)

**Fig. 2. Effect of IL-1 Production by Macrophages in Paramylon-Treated Mice**

Mice were treated i.p. with paramylon for the 5 consecutive days. Peritoneal exudate cells (PEC) were recovered 24 h after the last treatment and the plastic-adherent cells (1 × 10⁶ cells/ml) were cultured with or without LPS (40 μg/kg) for 48 h. Supernatants from these cultures were assayed for IL-1 activity by the co-stimulator assay using thymocytes. Data are represented as mean±S.D. of the values obtained from groups of 3 mice. Significantly different from control, a) p<0.05, b) p<0.01. ■, without LPS; □, with LPS.

**Table I. IL-6 Induction in Mouse Serum by Paramylon Treatment**

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Time after injection</th>
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<tbody>
<tr>
<td></td>
<td>3 h</td>
</tr>
<tr>
<td></td>
<td>(units/ml)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>466 ± 29</td>
</tr>
<tr>
<td>Paramylon 10</td>
<td>607 ± 39⁵⁺</td>
</tr>
<tr>
<td>Paramylon 50</td>
<td>1709 ± 44¹⁵</td>
</tr>
</tbody>
</table>

Mice were injected i.p. with paramylon suspended in PBS containing 30 μg/ml mouse serum albumin. After 3 and 6 h, each serum of mice was assayed for IL-6 activity using MH60-BSF2 cells. Data are represented as mean ± S.D. Significantly different from control, a) p<0.05, b) p<0.01.

**Table II. IL-2 Production by Con A-Stimulated Spleen Cells from Paramylon-Treated Mice**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>IL-2 activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Con A</td>
</tr>
<tr>
<td>None</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>10</td>
<td>9.4 ± 1.1</td>
</tr>
<tr>
<td>50</td>
<td>13.8 ± 1.8⁵⁺</td>
</tr>
</tbody>
</table>

Mice were treated i.p. for 5 consecutive days. Spleen cells prepared from mice 24 h after the last treatment were cultured for 48 h with or without Con A (5 μg/ml). Supernatants from these cultures were assayed for IL-2 activity using CTL-2 cells. Data are represented as mean ± S.D. Significantly different from control, a) p<0.05.
culture supernatants were tested for IL-2 activity. As shown in Table II, a significant but low enhancement in IL-2 production was observed only upon the administration of a higher dose of paramylon.

**Discussion**

The present study showed that paramylon, a linear β-(1→3)-D-glucan isolated form *Euglena gracilis*, has adjuvant activity for the induction of anti-SRBC PFC and for the IL-1 production. We also confirmed that its adjuvant activity was comparable to that of sizofiran.

The adjuvant activity of paramylon to antibody response has not been documented. The reason for this might in part be due to its obscure antitumor activity observed in the early study, *i.e.*, it caused a partial tumor growth inhibition without complete regression at the low dose (1 mg/kg/d × 10) but not at the high dose (5 mg/kg/d × 10). The present study demonstrated that the adjuvant activity of paramylon for anti-SRBC PFC was markedly exerted when mice were i.p. injected with paramylon at the rather higher dose, 10 and 50 mg/kg and not at the lower dose of 1 mg/kg. These data prove that paramylon gives different immunopotentiating actions to antitumor immunity and antibody production.

Paramylon administration caused a transient increase in IL-6 in blood. It was comparable to the amount of IL-6 induced by 0.1 μg of LPS in mice (data not shown). As IL-6 differentiates activated B cells to terminally matured, Ig-secreting plasma cells, in addition to play an accessory role in T cell activation synergistically with IL-1 and induction of hematopoiesis, paramylon-induced IL-6, even though a low level, might effectively enhance the antibody response to T cell-dependent antigens like SRBC.

It is well known that some polysaccharides are readily ingested by macrophages after injection, and then activate macrophages. For instance, sizofiran was reported to stimulate the reticuloendothelial system (RES) function, judged by the carbon clearance activity, and lentin was to activate macrophages to induce IL-1 without modifying the RES function. In the present study, we observed that macrophages from paramylon-treated mice were also activated to produce a large amount of IL-1 in the presence of LPS (Fig. 2). IL-1 has been shown to have multiple biological actions in immunological and inflammatory reactions such as enhancing antigen-induced T cell activation and triggering IL-2 and IL-4 production as well as IL-2 receptor expression. Furthermore, IL-1 plays a role in B cell activation and proliferation and has hematopoietic activity for the earliest precursors in the bone marrow. In addition, it was found that the *in vivo* administration of IL-1 enhanced the immune response.

On the contrary to macrophage activation, evidence for lymphocyte activations was barely obtained in the paramylon-treated mice, *i.e.*, no increase in mitogen responsiveness (data not shown) and a faint increase in IL-2 production in spleen cells (Table II).

Altogether with the present data, it is suggested that the adjuvant activity of paramylon to anti-SRBC PFC production might mainly depend on the augmented production of cytokines, especially IL-1, in consequence of macrophage activation.

**References**


