TRAXANOX AUGMENTS IMMUNE RESPONSE TO LIPOPOLYSACCHARIDE IN INBRED MICE: ROLE OF MACROPHAGES

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Abstract—Both C57BL/6 and BALB/c mice were immunized intravenously with lipopolysaccharide (LPS, 10 μg/mouse) on day 0, and hemolytic plaque forming cells (HPFC) in the spleen were assayed on day 4. Traxanox given orally at a dose of 30 mg/kg augmented the HPFC production to LPS in both mice. This agent (3–30 mg/kg) restored significantly the suppressed HPFC production to LPS in the BALB/c mice pretreated with carrageenan (0.03 mg/mouse), but did not restore it in the BALB/c mice pretreated with cyclophosphamide (25 mg/kg). The transfer of spleen adherent cells of the mice immunized with LPS and treated with traxanox to the syngeneic mice resulted in a significant increase in the HPFC production to LPS. The HPFC production to trinitrophenylated polyvinylpyrrolidone, a T cell-independent antigen, was not affected by the treatment with traxanox or carrageenan. These results suggest that traxanox has a capacity to augment the immune response by affecting macrophage function.

In the previous papers, we have reported that Y-12,141 (9-chloro-5-oxo-7-(1H-tetrazol-5-yl)-5H-[1]benzopyran[2,3-b]pyridine sodium salt pentahydrate, traxanox sodium pentahydrate**) inhibits IgE-mediated anaphylaxis in rats (1) and IgG-mediated anaphylaxis in guinea pigs (2). The anti-anaphylactic effect of this agent was suggested to be due to an inhibition of release of allergic mediators such as histamine and slow reacting substance of anaphylaxis (1–3). Traxanox sodium has been suggested to have a capacity to suppress the IgE production in the BN rat (4) and to potentiate immune responses in dd mice immunized with low doses of sheep red blood cells (SRBC) (5). In addition, this agent has an immunomodulating activity on cell-mediated immunity of mice (6) and an anti-adjuvant arthritis activity in the rat (7). The mechanisms of action of the agent, however, remain unclear.

The present study was undertaken to test the immunopotentiating activity of traxanox sodium in the immune response of mice against a thymus-derived (T) cell-independent antigen such as lipopolysaccharide (LPS) (8).

Materials and Methods

Animals: Female C57BL/6 and BALB/c mice, 4 weeks of age, were purchased from the Charles River Laboratory (Japan).
Animals were housed under ordinary conditions and allowed free access to food and water. Five- to six-week old mice were used for all studies.

**Drugs and administration:** Traxanox sodium pentahydrate (Yoshitomi Pharmaceutical Industries Ltd.), levamisole hydrochloride (LMS, Aldrich Chemical Company Ltd.), D-penicillamine (D-Pc, Sigma Chemical Company), cyclophosphamide (Endoxan®) and carrageenan (Seakem 402) were used as test compounds. Carrageenan dissolved in 0.9% (w/v) NaCl solution was injected intraperitoneally to the mice. The other test agents were dissolved or suspended in 0.5% (w/v) methylcellulose solution prior to use and given orally to the mice. Doses of traxanox sodium pentahydrate refer to the sodium salt containing no pentahydrate.

**Antigen and immunization:** The alkaline-treated LPS as the antigen was prepared as follows: LPS (E. coli 055:B5, Difco Laboratories) in an amount of 100 mg was dissolved in 6 ml of 0.25 N NaOH solution and heated for 60 min at 56°C according to the method of Neter et al. (9). Trinitrophenylated polyvinylpyrrolidone (TNP30-TVP) prepared by the method of Ishizaka et al. (10) was also used as the antigen. Immunization was performed by an intravenous injection of 1, 10 or 100 µg antigen/mouse.

**Transfer of splenic adherent cells (SAC):** To examine the effect of traxanox sodium on macrophages, SAC obtained from the mice treated with the antigen were transferred to the syngeneic mice. Donor mice were primed with 10 µg LPS on day 0 and treated with test agents or vehicle on days 0 and 1, and their spleens were obtained on day 2 or on day 4. The SAC were prepared by the method of Nomoto (11). The SAC or la− SAC (5×10⁶ cells) were transferred intravenously to the syngeneic recipients, and they were immunized with 10 µg LPS. The la− SAC were prepared by depletion of la+ SAC with anti-la serum plus complement (Cedarlane mouse la alloantiserum). The SAC were suspended in the appropriate dilution of antiserum at a concentration of 5×10⁶ cells/ml, and they were kept for 60 min in ice-water. The cells were washed and resuspended in a 1:12 dilution of rabbit complement (Cedarlane) for 60 min at 37°C. The cells were washed twice and transferred to the recipients. Anti-la plus complement treatment killed about 25% of the SAC. Cell viability was assessed with 0.1% trypan blue in a hemocytometer.

**Transfer of spleen cells, SAC, Thy 1.2 positive cells and Thy 1.2 negative cells:** To examine the target cells for carrageenan, spleen cells, SAC, Thy 1.2 positive cells or Thy 1.2 negative cells which were prepared from the syngeneic mice were transferred to the recipient mice immediately before the immunization with 10 µg LPS on day 0. The recipient mice were pretreated with carrageenan on days −3 and −1. Splenic T cells were prepared by collecting the effluents of spleen cells allowed to adhere to nylon wool columns (12). More than 95% of the cells thus prepared were susceptible to the treatment with anti-Thy 1.2 (Cedarlane) plus complement. Splenic B cells were prepared by treating non-adherent splenic cells with anti-Thy 1.2 followed by complement lysis. Thirty-eight percent of the non-adherent cells were susceptible to the treatment with anti-Thy 1.2 plus complement.

**Detection of antibody-forming cells in the spleen:** For the assay of anti-LPS hemolytic plaque-forming cells (HPFC), SRBC were coated with LPS according to the method of Andersson and Blomgren (8). TNP-SRBC was prepared according to the method of Ornellas and Scott (13). The spleen HPFC to LPS and TNP were assayed using LPS- and TNP-coated SRBC, respectively, by the method of Hashimoto and Ohshima (14). SRBC obtained from the Funabashi Farm Co. (Chiba, Japan) were stored in Alserver's
solution at 3–6°C, and they were washed three times in phosphate-buffered saline (pH 7.2) before use.

**Detection of serum antibody:** Serum antibody to LPS and TNP was determined as hemagglutination (HA) titers of both 7S and 19S antibodies by the method of Walz et al. (15). LPS- or TNP-coated SRBC were used for the detection of antibody to LPS or TNP.

**Results**

**Effects on immune response to LPS:** Figure 1 shows that traxanox sodium (30 mg/kg) given orally on days 0 and 1 increased significantly the HPFC numbers to LPS antigen (10 µg/mouse) in C57BL/6 mice assessed on day 4. The same result was obtained in the mice treated with LMS (10 mg/kg) or D-Pc (100 mg/kg). The potentiating effect of traxanox sodium on the HPFC production assessed on day 4 was more potent than that assessed on day 7.

Figure 2 indicates that the HPFC response to LPS assessed on day 4 was dependent on the dose of antigen and that the response was potentiated by the treatment with traxanox sodium (30 mg/kg) in BALB/c mice. In the mice immunized with a large dose of LPS (100 µg/mouse), both LMS (10 mg/kg) and D-Pc (100 mg/kg) failed to augment the number of the HPFC. LMS (10 mg/kg), however, potentiated the HPFC response to LPS at a dose of 10 µg/mouse. In these experiments, traxanox sodium, LMS and D-Pc had no effect on the number of spleen cells.

**Effects on suppressed immune response to LPS:** Treatment of BALB/c mice with cyclophosphamide resulted in a decrease in the HPFC numbers and 19S HA titers to LPS (10 µg/mouse). Traxanox sodium (100 mg/kg) increased slightly the HPFC numbers to LPS, but did not restore it to the normal range in the mice treated with cyclophosphamide (Table 1).

Carrageenan (0.03 mg/mouse) also suppressed the production of the HPFC and 19S HA (Fig. 3). The treatment with traxanox and LMS resulted in a dose-dependent increase in the HPFC numbers and 19S HA titers to LPS in the mice pretreated with carrageenan. The mice treated with traxanox sodium (10 and 30 mg/kg) or LMS (10 mg/
kg) showed the normal immune response to LPS even in the carrageenan-treated mice (Fig. 3). On the other hand, traxanox sodium, LMS and D-Pc failed to affect the HPFC production to LPS suppressed by the pretreatment with a large dose (0.5 mg/mouse) of carrageenan (Fig. 4). In these experiments, 7S HA titer to LPS was not detected in sera.

Effects on immune response to TNP-PVP:
Figure 5 shows that the production of the HPFC and 19S HA titer to TNP-PVP was not affected by treatment with traxanox sodium (30 mg/kg), LMS (10 mg/kg), D-Pc (100 mg/kg) or carrageenan (0.03 mg/mouse) in C57BL/6 mice.

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### Table 1. Effect of traxanox sodium and D-penicillamine on the development of HPFC and 19S HA titer to LPS in female BALB/c mice treated with cyclophosphamide

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Spleen cells (×10⁶)</th>
<th>HPFC</th>
<th>19S HA titer (log₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>119±3</td>
<td>1.53±0.14</td>
<td>1.81±0.16</td>
</tr>
<tr>
<td>Cyclophosphamide (CY)</td>
<td>25</td>
<td>67±3**</td>
<td>0.59±0.09**</td>
<td>0.40±0.06**</td>
</tr>
<tr>
<td>CY+Traxanox sodium</td>
<td>10</td>
<td>68±3**</td>
<td>0.65±0.15**</td>
<td>0.45±0.11**</td>
</tr>
<tr>
<td>CY+Traxanox sodium</td>
<td>30</td>
<td>70±8**</td>
<td>0.81±0.08**</td>
<td>0.54±0.05**</td>
</tr>
<tr>
<td>CY+Traxanox sodium</td>
<td>100</td>
<td>67±6**</td>
<td>0.94±0.08**</td>
<td>0.62±0.07**</td>
</tr>
<tr>
<td>CY+D-Penicillamine</td>
<td>100</td>
<td>66±9**</td>
<td>0.48±0.06**</td>
<td>0.31±0.04**</td>
</tr>
</tbody>
</table>

Each group of animals was immunized intravenously with LPS at a dose of 10 μg/mouse on day 0 and treated orally with test drugs on days −1 and 0. Cyclophosphamide was given orally to animals on days −1 and 0. Spleen cells, HPFC and 19S HA titer in serum were assessed on day 4. Results are shown as the mean±S.E. (n=7). **P<0.01 (significantly different from the vehicle treatment).

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Fig. 2. Effects of traxanox sodium, levamisole and D-penicillamine on the development of HPFC to LPS in female BALB/c mice. Each group of animals (n=6) was intravenously immunized with LPS at a dose of 10 μg/mouse (■) or 100 μg/mouse (▲) on day 0 and was treated orally with test drugs on days 0 and 1. The HPFC in the spleen were assessed on day 4. **P<0.01 (significantly different from the vehicle treatment).
Fig. 3. Effects of traxanox sodium and levamisole on the development of HPFC and 19S HA to LPS in female BALB/c mice pretreated with carrageenan. Each group of animals (n=6) was intravenously immunized with LPS at a dose of 10 μg/mouse on day 0 and was treated orally with test drugs on days -2, -1, 0 and 1. Carrageenan (0.03 mg/mouse) was intraperitoneally given to the animals on days -3 and -1. The HPFC in the spleen and 19S HA titer were assessed on day 4. **P<0.01 (significantly different from the vehicle treatment).

Fig. 4. Effects of traxanox sodium, levamisole and D-penicillamine on the development of HPFC to LPS in female BALB/c mice pretreated with carrageenan. Each group of animals (n=6) was intravenously immunized with LPS at a dose of 10 μg/mouse on day 0 and was treated orally with test drugs on days -2, -1, 0 and 1. Carrageenan (0.5 mg/mouse) was intraperitoneally given to the animals on days -3 and -1. The HPFC in the spleen were assessed on day 4. **P<0.01 (significantly different from the vehicle treatment).

Effects on splenic adherent cells (SAC) in vivo: The transfer of SAC obtained on day 2 from the donor mice treated with traxanox sodium augmented the HPFC production to LPS in C57BL/6 mice (Fig. 6) and BALB/c mice (Figs. 7 and 8). The SAC obtained on day 2 from the donor mice treated with LMS or D-Pc also showed the same effect. When
the Ia− SAC were transferred to the recipients, traxanox sodium had no effect at all (Fig. 8). In addition, the SAC obtained on day 4 from the donors treated with traxanox sodium (100 mg/kg) or D-Pc (100 mg/kg) augmented the HPFC production of the recipients (Fig. 9).

Effects of non-immune spleen cells on HPFC production to LPS in mice pretreated with carrageenan: Carrageenan (0.03 mg/mouse) suppressed significantly (P<0.01) the HPFC production to LPS in BALB/c mice. This suppression was not modified by the transfer of Thy 1.2 negative cells (B cells) and Thy 1.2 positive cells (T cells) which
were prepared from the intact BALB/c mice. The transfer of SAC or spleen cells (5x10^6), however, resulted in an increase in the HPFC numbers (Fig. 10). The increase in the HPFC numbers of the mice pretreated with carrageenan depended on the numbers of

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### Table: Effects of Drugs on SAC and la SAC

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Immune SAC transferred (5x10^6 cells)</th>
<th>x10^3 HPFC/x10^7 spleen cells ([])</th>
<th>x10^4 HPFC/spleen ([]), mean±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traxanox sodium</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Penicillamine</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 7.** Effects of traxanox sodium, levamisole and D-penicillamine on splenic adherent cells (SAC) of female BALB/c mice. Each group of donor mice (n=6–8) was intravenously immunized with LPS at a dose of 10 μg/mouse on day 0 and was treated orally with test drugs on days 0 and 1. The SAC prepared from the donors on day 2 were transferred to the syngeneic recipient mice (n=6) immediately before the immunization with 10 μg LPS/mouse. The HPFC in the spleen were assessed on day 4. **P<0.01 (significantly different from the vehicle treatment).**

**Fig. 8.** Effects of traxanox sodium on la^+^ splenic adherent cells (la^+^ SAC) of female BALB/c mice. Each group of donor mice (n=6–8) was intravenously immunized with LPS at a dose of 10 μg/mouse on day 0 and was treated orally with traxanox sodium on days 0 and 1. The SAC or la^+^ SAC prepared from the donors on day 2 were transferred to the syngeneic recipient mice (n=6) immediately before the immunization with 10 μg LPS/mouse. The HPFC in the spleen were assessed on day 4. *P<0.05, **P<0.01 (significantly different from the vehicle treatment).
Fig. 9. Effects of traxanox sodium and levamisole on splenic adherent cells (SAC) of female BALB/c mice. Each group of donor mice (n=6-8) was intravenously immunized with LPS at a dose of 10 μg/mouse on day 0 and was treated orally with test drugs on days 0 and 1. The SAC prepared from the donors on day 4 were transferred to the syngeneic mice (n=6) immediately before the immunization with 10 μg LPS/mouse. The HPFC in the spleen were assessed on day 4. *P<0.05, **P<0.01 (significantly different from the vehicle treatment).

Fig. 10. Effects of non-immune spleen cells transferred on the development of HPFC to LPS in female BALB/c mice. Each group of animals (n=6) was intravenously immunized with LPS at a dose of 10 μg/mouse on day 0. The Thy 1.2 negative cells, Thy 1.2 positive cells, SAC and spleen cells (5×10⁶) prepared from the syngeneic mice were transferred to the mice immediately before the immunization. The HPFC in the spleen were assessed on day 4. *P<0.05 (significantly different from the carrageenan-treated mice).

SAC transferred immediately before the immunization (Fig. 11).

Discussion
In the present study, traxanox sodium was found to augment the HPFC response to LPS and not to augment the response to TNP-PVP in C57BL/6 or BALB/c mice. Since LPS has been reported to be a T cell-independent antigen (8), the potentiation of
the HPFC production to LPS by traxanox sodium, LMS and D-Pc (Figs. 1 and 2) may be due to the enhancement of the function of B cells and/or macrophages. There is some evidence that LMS may serve to improve antigen processing and presentation by macrophages (16) and not to stimulate B cell-enriched spleen cells (17).

Recently, we found that traxanox sodium has a capacity to restore the cell-mediated immunity of the mice suppressed by the pretreatment with cyclophosphamide or carrageenan (6). The HPFC production to LPS, a T cell-independent antigen, was also suppressed by the pretreatment with cyclophosphamide and carrageenan (Table 1 and Fig. 3). Traxanox sodium as well as LMS, however, did not affect the HPFC response to LPS in the mice pretreated with cyclophosphamide and a large dose of carrageenan (0.5 mg/mouse, Fig. 4). In contrast, the suppression of the HPFC response to LPS by carrageenan at a dose of 0.03 mg/mouse was found to be restored by traxanox sodium and LMS (Fig. 3). Therefore, these drugs may improve functions of the carrageenan-sensitive cells.

Carrageenan was shown to be cytotoxic for adherent mononuclear cells or macrophages in vitro and not for lymphocytes (18) and was shown to impair activity of mononuclear phagocytes in vivo (19). In addition, there are some papers which report that carrageenan suppresses the immune response to a T cell-dependent antigen such as SRBC (10, 20), but does not affect the response to a T cell-independent antigen (10, 21). These observations suggest that carrageenan fails to impair B cell response directly, and at the same time, they provide evidence that T cell-independent responses are also macrophage-independent. In contrast to the response to LPS, the immune responses to TNP-PVP, a T cell- and macrophage-independent antigen (10), are not augmented by traxanox sodium, LMS and D-Pc, and they are not inhibited by carrageenan (Fig. 5). Therefore, it is concluded from the above data that traxanox sodium, LMS and D-Pc have no effect on B cells in vivo.

If the immune responses to LPS, which is a T cell-independent antigen and a poly-
clonal B cell mitogen, could be induced in vivo without affecting macrophage functions, the effect of traxanox sodium, LMS and D-Pc or carrageenan on the response to LPS would be inexplicable. Our preliminary experiments show that the HPFC response to LPS is about eight-times higher than that to SRBC in the mice immunized with LPS (100 μg/mouse, i.v.). This finding suggests that the mechanisms of the immune response to LPS are different from the activity of LPS as a polyclonal B cell mitogen. Ishizaka et al. (10) reported that the antibody response to TNP-LPS, a T cell-independent antigen, was inhibited by carrageenan treatment, suggesting that the response is macrophage-dependent. In addition, Janezie et al. (22) found that the humoral response to LPS in carrageenan-treated rats was reduced to approximately 20% of that in control animals. Their conclusion was that LPS, although a T cell-independent antigen, was macrophage-dependent. In accordance with these findings, we also confirmed that the HPFC response to LPS was suppressed by carrageenan. Consequently, LPS may be a macrophage-dependent antigen in vivo. From these findings, we proposed that if traxanox sodium could activate the macrophage functions, the effect of the drug might be detected by cell transfer experiments.

In order to demonstrate the above hypothesis, we carried out cell transfer experiments using the immune SAC obtained from the syngeneic donor mice as macrophages. As shown in Figs. 6 to 9, the HPFC response is higher in the immune SAC-transferred recipients than in the SAC-non-transferred animals, suggesting that the HPFC response to LPS in vivo may be affected by the SAC in the mice. Figures 6, 7 and 8 also suggest that the SAC obtained on day 2 after the immunization from the donor mice treated with traxanox sodium (100 mg/kg), LMS (10 mg/kg) and D-Pc (100 mg/kg) are capable of augmenting the HPFC production to LPS. About 92% of the traxanox sodium given orally to mice was excreted during 24 hr after the administration (unpublished data). The transferred SAC were obtained from the mice 24 hr after the administration of the drug. Therefore, the drug within the cells transferred to the recipients appears to contribute little to the HPFC production to LPS. The data in Fig. 9 support this consideration. Namely, the SAC obtained on day 4 showed the same effect as those obtained on day 2. Compared with the effective doses of traxanox sodium (10 and 30 mg/kg) in Fig. 3, the high effective dose (100 mg/kg) in Fig. 6 may be due to the low number (5×10⁶) of immune SAC transferred. On the other hand, the activity of the SAC of the mice treated with traxanox sodium was found to be abolished by depletion of la⁺ cells from the SAC by treatment with anti-la⁺ alloantiserum plus complement (Fig. 8). These results suggest that traxanox sodium augments the immune response to LPS by affecting la⁺ macrophage functions in vivo.

The above finding on D-Pc is consistent with the reports showing that the drug enhances the functional activity of the macrophages in the regulation of the lymphocyte response to mitogen (23–25). Otomo et al. (26) have also shown that D-Pc may act as an immunomodulating agent and have suggested that the effect of the drug may be mediated through functions of macrophages.

Although it has been reported that carrageenan is selectively cytotoxic for macrophages (18, 19), additional experiments were carried out in order to confirm the role of macrophages in the immune response to LPS in vivo. The data in Figs. 10 and 11 suggest that both the Thy 1.2 positive and the Thy 1.2 negative lymphocytes transferred have no ability to restore the suppressed HPFC production to LPS in the mice pre-
treated with carrageenan. In contrast to these cells, the transfer of the SAC or spleen cells to the carrageenan-treated mice resulted in an increase in the HPFC numbers, suggesting that the SAC transferred are capable of potentiating the HPFC production in carrageenan-treated mice. The necessity for a large number of SAC (1 x 10^8) to restore the suppressed HPFC production to LPS in the carrageenan-treated mice may be due to the cytotoxic effect of carrageenan on the SAC transferred. These findings confirm that LPS is a macrophage-dependent antigen, and carrageenan is cytotoxic for macrophages in vivo. Therefore, it is concluded from the present studies that traxanox sodium augments the immune responses to LPS via the activation of macrophage functions.

On the effect of traxanox sodium on the immune responses, our recent studies have suggested that the drug augments the immune response of dd mice to low doses of SRBC and restores the decrease of immune response caused by immunosuppressive drugs (5). These data are consistent with those of the present studies. In contrast, the results showing that traxanox sodium suppresses the IgE production in the BN rat (4) and the cell-mediated immunity in higher responder mice (5) are not in accord with the present data. However, it is shown recently that traxanox sodium suppresses the HPFC production in higher responder mice to SRBC by inducing suppressor T cells which may be mediated by activation of macrophage functions (27).

The conclusion drawn from the present experiments is that traxanox sodium augments the immune responses to LPS, but not to TNP-PVP in vivo. The mode of potentiating action by this drug on the immune response to LPS may be due to an activation of macrophage functions.

References
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