Antitumor Effect of Intracutaneous Injection of Bacterial Lipopolysaccharide

Den'ichi Mizuno, Osamu Yoshioka, Masako Akamatu, and Tomoko Kataoka
Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Tokyo, Japan

SUMMARY

Intracutaneous injection of the lipopolysaccharide of Proteus vulgaris was found to stimulate the reticuloendothelial system and have a marked antitumor effect in mice bearing solid-type Ehrlich carcinoma and Sarcoma 180. The stimulation of the reticuloendothelial system was tested by measuring the clearing activity of intravenously injected colloidal carbon in mice. Of the various routes of injection tested, the actions of lipopolysaccharide were observed most after intracutaneous injection. Two possible modes of action of lipopolysaccharide were indicated: one is a stimulation of the reticuloendothelial system which can be expected to provoke subsequent antibody formation, and the other action is a direct cytocidal action.

INTRODUCTION

It is well known that the administration of bacterial lipopolysaccharide stimulates the reticuloendothelial system of the host. The activity of the reticuloendothelial system in the tumor-bearing host is abnormally low. It is not clear whether reticuloendothelial system stimulation induces an antitumor effect. However, it can be expected that the reticuloendothelial system must be stimulated before tumor immunity is established. Therefore, it is very interesting to use lipopolysaccharide as an agent for stimulating the reticuloendothelial system in cancer chemotherapy, apart from the conventional direct chemotherapeutic treatment (1, 2).

There are many histiocytes in the epithelial system of animals, and stimulation of the skin is likely to induce maturation of these resulting in activation of the reticuloendothelial system.

Activity in stimulation of the reticuloendothelial system is not necessarily accompanied by toxicity, and even the lipopolysaccharides of nontoxic bacteria are effective for this. In the present work the lipopolysaccharide fraction of Proteus vulgaris OX 19 was used as a stimulant of the reticuloendothelial system.

The present paper reports studies on the possible antitumor effect of intracutaneous injection of bacterial lipopolysaccharide (endotoxin) in tumor-bearing mice and rats. Independently of this work, Lemperele (4) showed a marked antitumor effect when he used Sarcoma 180 and restim as a stimulant to the reticuloendothelial system. However, his injections were given intraperitoneally. A preliminary account of this work was announced at the 9th International Cancer Congress (Tokyo) in 1966.

MATERIALS AND METHODS

Animals and Evaluation of Antitumor Effect

Mice, dd/Y males weighing about 20 gm, were maintained by inbreeding in the National Institute of Health of Japan and supplied from an animal farm in Shizuoka prefecture. Rats, Donryu males weighing 200–250 gm, from a commercial breeder in Tokyo, were used. All animals were fed on standard laboratory diet and water ad libitum.

Sarcoma 180 and Ehrlich carcinoma cells were used for the evaluation of the antitumor effect in mice, using the method of Sugiuira (6). Yoshida ascites carcinoma cells were used for studies on the antitumor effect in rats, taking longevity as the standard.

Detailed descriptions of experimental schedules, including the injection routes, are given in the Results section.

Intracutaneous injections were made into the mid-abdomen of animals, unless otherwise stated.

Direct Cytocidal Effect

Ehrlich carcinoma cells were suspended in Eagle’s medium (containing 5% serum), and each of the test antitumor agents, including lipopolysaccharide, were added individually to samples of this. After incubation for 1 hr at 37°C with continuous gentle agitation, the cells were washed three times with an equal volume of physiologic saline by centrifugation, resuspended in a suitable volume of saline, and diluted to 3 × 10⁶ cells/0.2 ml. In this test, the longer the longevity of the mice, the stronger is the cytocidal effect of the test sample.

Carbon Clearance Activity

The activity of the reticuloendothelial system was tested by the clearance of colloidal carbon (Pelikan ink, from Günther Wagner, Hanover, Germany). Pelikan ink was diluted 4 times with 1% gelatin and 0.2 ml of this carbon solution was injected into the tail veins of mice. At intervals up to 30 min, mice were sacrificed and blood samples were obtained from the axillae. Each point on the curves represents the average of the values of 3–5 mice. Blood samples of 0.1 ml were diluted with 2.5 ml of 0.1 M Na₂CO₃ solution, and the concentration of colloidal carbon was determined photometrically at a wavelength of 650 nm (Hitachi-Perkin Elmer 139) (3). The stand-

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ard assay was done 4 minutes after injection of colloidal carbon, and the clearing activity is expressed as shown in the Results section.

Preparation of Lipopolysaccharide from *P. vulgaris*, OX19

*Proteus vulgaris*, strain OX 19, was grown in a conventional nutrient broth. The lipopolysaccharide of the cells was extracted by the method of Westphal (7). The acute LD<sub>50</sub> value of this lipopolysaccharide is 1.6 mg/mouse (80 mg/kg) when given by intraperitoneal injection.

Chemicals

Mitomycin, bleomycin, 6-mercaptopurine, and 8-azaguanine were gifts from Kyowa Hakko Co., Nippon Kayaku Co., Takeda Chemical Industries, and Tanabe Pharmaceutical Co. respectively.

RESULTS

Activation of the Reticuloendothelial System by Intracutaneous Injection of Lipopolysaccharide

A suspension of 100 µg of lipopolysaccharide in 0.1 ml saline was administered intracutaneously or intraperitoneally, and two days later the carbon clearance activity was measured (Chart 1). The half time of intravascular removal of colloidal carbon was as follows: untreated mice, 16.5 min; intraperitoneally treated mice, 2.8 min; intracutaneously treated mice, 70 sec.

A nonlethal dose of lipopolysaccharide was administered intracutaneously. Two days later colloidal carbon was injected intravenously. Four minutes after injection of carbon blood samples were taken for measurement of carbon clearance. The percentage activation of the reticuloendothelial system was calculated by the following formula:

\[
\text{Percentage activation of the reticuloendothelial system} = \frac{\text{O.D. normal} - \text{O.D. treated}}{\text{O.D. normal}},
\]

where O.D. normal is the O.D. of blood after injection of colloidal carbon only, and O.D. treated is the O.D. of blood from the mice which had been treated with lipopolysaccharide 2 days before the colloidal carbon. The minimum effective dose in this experiment was found to be 100 µg lipopolysaccharide/mouse (Chart 2). The time required to obtain the maximum response after injection of lipopolysaccharide was studied. Lipopolysaccharide (100 µg/mouse in 0.1 ml of saline) was injected intracutaneously into mice on Day 0 (Chart 3). The

![Chart 2](image)

Chart 2. Dose-response curve to lipopolysaccharide (LPS) of *Proteus vulgaris*. A nonlethal dose of LPS was administered intracutaneously. Two days later, colloidal carbon was injected intravenously, and 4 min after carbon injection, blood samples were taken for measurement of carbon clearance. Each point shows the average of the values of 3-5 mice.

![Chart 3](image)

Chart 3. Carbon-clearing activity in mice after intracutaneous injection of lipopolysaccharide (LPS) of *Proteus vulgaris*. LPS (100 µg/mouse in 0.1 ml of saline) was injected at Day 0. Points are the averages of the values of 3-5 mice.
maximum clearance response appeared from one to two days after lipopolysaccharide injection.

Next, the response of tumor-bearing mice was tested. Ehrlich carcinoma cells (3 × 10⁶ cells in 0.2 ml) were injected into animals subcutaneously, and then 100 μg of lipopolysaccharide were administered intracutaneously 3 times, 2, 4, and 6 days after tumor inoculation. Carbon clearance was measured 1, 2, 3, 5, 7, and 9 days after inoculation of cells. The activity is expressed as the percentage activation on the basis of the 4-minute values (Chart 4). There was no significant difference between tumor-bearing mice and normal mice in the response to intracutaneous lipopolysaccharide injection.

The various possible routes of injection of lipopolysaccharide were compared giving a single injection of 100 μg lipopolysaccharide/mouse. Two days after the injection, the carbon clearance activity was measured. This is expressed as the half-time of clearance in Chart 5. Intracutaneous injection into the abdomen gave the most stimulation on the reticuloendothelial system in mice. The intravenous route had a very similar effect, followed by the intracutaneous route in the back, the subcutaneous, and the intraperitoneal route in this order. As shown later, the antitumor effect of lipopolysaccharide by the intravenous route was less than that by the intracutaneous route.

Antitumor Effect of Lipopolysaccharide Injected Intracutaneously on Solid-Type Ehrlich Carcinoma and Sarcoma 180 Carcinoma Cells

To test whether the antitumor effect of lipopolysaccharide, if present, can be observed before or after tumor cell transplantation, the following experiment was carried out. Six groups of 10 mice were treated with lipopolysaccharide before cell inoculation while 7 groups of 10 mice were treated with lipopolysaccharide after cell inoculation. The injection schedules were as follows. The groups were intracutaneously injected with lipopolysaccharide (100 μg/mouse in 0.1 ml) once on the following days: pretreated groups, 10, 8, 6, 4, 3, and 2 days before cell inoculation; posttreated groups, 1, 2, 3, 4, 5, 6, and 8 days after cell inoculation. All mice were killed on the 14th day after cell inoculation, and the solid tumors were weighed. The effect is expressed as the ratio of the tumor weight in the treated and control animal.

Chart 4. Response of the reticuloendothelial system of mice to injection of lipopolysaccharide (LPS) of Proteus vulgaris and Ehrlich ascites carcinoma cells. Ehrlich carcinoma cells (3 × 10⁶ cells/mouse) were injected subcutaneously, and 100 μg of LPS were administered intracutaneously (i.e.) 3 times, 2, 4, and 6 days after cell inoculation. Carbon clearance was measured 1, 2, 3, 5, and 9 days after cell inoculation.

Chart 5. Comparison of injection routes as expressed by the half-time of clearance. The various possible routes for injection of lipopolysaccharide were compared using single injection of 100 μg lipopolysaccharide/mouse. Two days after injection carbon clearance was measured. i.e., intracutaneous.

Chart 6. Antitumor effect of lipopolysaccharide of Proteus vulgaris injected intracutaneously (100 μg/mouse) once 10, 8, 6, 4, and 2 days before and 1, 2, 3, 4, 5, 6, and 8 days after cell inoculation. All mice were killed on the 14th day after cell inoculation, and the solid tumors were weighed. The effect is expressed as the ratio of tumor weight in the treated and control animal.
weight of the treated animals to that of the controls. Post-
treatment after 2 days was the most effective. Therefore, in
the following studies on the antitumor effect, lipopolysaccharide
was injected 3 times, 2, 4, and 6 days after cell inoculation.

Generally, the antitumor effect of lipopolysaccharide (400
\( \mu g \)/mouse/day for 7 days) injected intraperitoneally on the
solid type of Ehrlich carcinoma was 35% of the control, whereas
the toxicity (Table 1) appeared as a lethal effect of 2/9. Mitomycin C
was quite effective for positive control, but it was toxic. Its injection (50
\( \mu g \)/mouse/day for 7 days) gave a marked decrease of body weight and a lethal effect of 4/9.

The intracutaneous injection of lipopolysaccharide (100 \( \mu g \)/
mouse/day, every other day on the 2nd, 4th and 6th days)
had an effect of 15–19%. No toxicity was observed (Tables 2, 3).

Intravenous injection had less effect than intracutaneous
injection (Table 2). A significant antitumor effect was ob-
tained in a group of mice injected three times intracutaneously
with lipopolysaccharide even at a dose of 1–10 \( \mu g \)/mouse/day.

A similar antitumor effect was obtained in an experiment
using the solid type of Sarcoma 180 carcinoma cells (Table 4).
Intracutaneous injection had more effect than intraperi-
toneal injection (Table 4).

Effect on Solid-type Ehrlich Carcinoma of Lipopolysaccharide
Injection

The appearance of the tumor after injection of lipopoly-
saccharide is shown in Figs. 1 and 2. Fibrous tissues can be
seen to surround or penetrate into the tumor cells (Figs. 1, 2).

The Effect of Intracutaneously Injected Lipopolysaccharide
on the Ascites Form of Yoshida Carcinoma Cells

The effect of intracutaneous injection of lipopolysaccharide
on Yoshida carcinoma cells was studied by the survival curve

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**Table 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose of LPS i.p. 7 times (( \mu g ))</th>
<th>Wt. difference*</th>
<th>Survivors</th>
<th>Tumor wt. (mg)</th>
<th>Percent (treated/control x 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>9/9</td>
<td>998</td>
<td>100</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>60</td>
<td>-7.2</td>
<td>5/9</td>
<td>176</td>
<td>17.7</td>
</tr>
<tr>
<td>LPS</td>
<td>400</td>
<td>-2.5</td>
<td>7/9</td>
<td>355</td>
<td>35.6</td>
</tr>
<tr>
<td>LPS</td>
<td>100</td>
<td>-0.6</td>
<td>9/9</td>
<td>846</td>
<td>34.7</td>
</tr>
<tr>
<td>LPS</td>
<td>25</td>
<td>-1.7</td>
<td>9/9</td>
<td>848</td>
<td>35.0</td>
</tr>
</tbody>
</table>

*Antitumor effect of lipopolysaccharide (LPS) of *Proteus vulgaris* injected i.p. on the solid-type of Ehrlich carcinoma cells.

*Animal weight difference: average weight change of treated host minus average weight change of control host.

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**Table 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (( \mu g )) and route of LPS</th>
<th>Wt. difference*</th>
<th>Survivors</th>
<th>Tumor wt. (mg)</th>
<th>Percent (treated/control x 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>5/5</td>
<td>1566</td>
<td>100</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>50, 7 times, i.p.</td>
<td>-4.2</td>
<td>5/5</td>
<td>654</td>
<td>28.3</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>50, 3 times, i.c. (Days 2, 4, 6)</td>
<td>-1.2</td>
<td>5/5</td>
<td>846</td>
<td>43.3</td>
</tr>
<tr>
<td>LPS</td>
<td>100, 3 times, i.v. (Days 2, 4, 6)</td>
<td>0</td>
<td>5/5</td>
<td>565</td>
<td>28.9</td>
</tr>
<tr>
<td>LPS</td>
<td>100, 3 times, i.c. (Days 2, 4, 6)</td>
<td>1.1</td>
<td>5/5</td>
<td>284</td>
<td>14.6</td>
</tr>
</tbody>
</table>

*Antitumor effect of lipopolysaccharide (LPS) of *Proteus vulgaris* injected intracutaneously (i.c.) on the solid-type of Ehrlich carcinoma cells.

*Animal weight difference: average weight change of treated host minus average weight change of control host.
Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (µg) and route of LPS</th>
<th>Wt. difference</th>
<th>Survivors</th>
<th>Tumor wt. (mg)</th>
<th>Percent (treated/control X 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>9/9</td>
<td>763</td>
<td>100</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>50, 7 times, i.p.</td>
<td>-8.0</td>
<td>7/10</td>
<td>225</td>
<td>29.5</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>100, 3 times, i.e. (Days 2, 4, 6)</td>
<td>-7.0</td>
<td>10/10</td>
<td>168</td>
<td>22.0</td>
</tr>
<tr>
<td>LPS</td>
<td>100, 3 times, i.c. (Days 2, 4, 6)</td>
<td>0.3</td>
<td>9/9</td>
<td>143</td>
<td>18.7</td>
</tr>
<tr>
<td>LPS</td>
<td>10, 3 times, i.e.</td>
<td>-4.4</td>
<td>9/9</td>
<td>159</td>
<td>20.8</td>
</tr>
<tr>
<td>LPS</td>
<td>1, 3 times, i.c.</td>
<td>-2.3</td>
<td>10/10</td>
<td>303</td>
<td>39.7</td>
</tr>
</tbody>
</table>

Antitumor effect of lipopolysaccharide (LPS) of Proteus vulgaris injected intracutaneously (i.e.) on solid-type of Ehrlich carcinoma cells.

# Animal weight difference: average weight change of treated host minus average weight change of control host.

Table 4

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (µg) and route of LPS</th>
<th>Wt. difference</th>
<th>Survivors</th>
<th>Tumor wt. (mg)</th>
<th>Percent (treated/control X 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>10/10</td>
<td>1510</td>
<td>100</td>
</tr>
<tr>
<td>LPS</td>
<td>100, i.p. (Days 2, 4, 6)</td>
<td>-4.8</td>
<td>9/10</td>
<td>864</td>
<td>57.2</td>
</tr>
<tr>
<td>LPS</td>
<td>10, i.p.</td>
<td>-0.9</td>
<td>10/10</td>
<td>868</td>
<td>57.5</td>
</tr>
<tr>
<td>LPS</td>
<td>1, i.p.</td>
<td>-2.4</td>
<td>10/10</td>
<td>903</td>
<td>59.8</td>
</tr>
<tr>
<td>LPS</td>
<td>100, i.e. (Days 2, 4, 6)</td>
<td>-2.6</td>
<td>10/10</td>
<td>383</td>
<td>25.3</td>
</tr>
<tr>
<td>LPS</td>
<td>10, i.e.</td>
<td>-1.1</td>
<td>10/10</td>
<td>831</td>
<td>54.9</td>
</tr>
<tr>
<td>LPS</td>
<td>1, i.e.</td>
<td>-2.3</td>
<td>9/9</td>
<td>712</td>
<td>47.3</td>
</tr>
</tbody>
</table>

Antitumor effect of lipopolysaccharide (LPS) of Proteus vulgaris injected intracutaneously (i.e.) on the solid-type of Sarcoma 180 carcinoma cells.

# Animal weight difference: average weight change of treated host minus average weight change of control host.

Intracutaneous injection of lipopolysaccharide (5 mg/kg, 3 times, 2, 4 and 6 days after cell inoculation) had more effect than intraperitoneal injection with a fixed dose and injection schedule. Sixty percent of the rats survived on the 30th day after cell transplantation.

Cytocidal Action of Lipopolysaccharide

The experiments described above suggest that the lipopolysaccharide of *P. vulgaris* has two actions as an antitumor agent. One is to stimulate the reticuloendothelial system (Charts 1, 4), and the other is a cytocidal action. The direct cytocidal effect of the lipopolysaccharide of *P. vulgaris* was tested following the procedures described in the Methods.

The effects of mitomycin C, 8-azaguanine, bleomycin, and lipopolysaccharide were compared (Chart 8). As expected, the effects decreased in the following order: mitomycin C, bleomycin, lipopolysaccharide, and 8-azaguanine. Thus the effect of...
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showed neither necrosis nor hemorrhage (Figs. 1, 2). We feel that our results cannot be compared with those of Shear, since his injection was so toxic. However, it cannot be concluded that the lipopolysaccharide of P. vulgaris has no direct cytocidal activity against tumor cells. It was shown that lipopolysaccharide has a slight direct cytocidal effect (Chart 8).

Therefore, we should like to conclude that two factors are involved in the antitumor effect of lipopolysaccharide: one is intracutaneous injection, which causes marked activation of the reticuloendothelial system, and the other is a slight cytocidal effect of lipopolysaccharide itself.

It is possible that lipopolysaccharide of other nonpathogenic Gram-negative bacteria can induce the same action. In fact, we observed that the lipopolysaccharide of E. coli has the same activity as that of P. vulgaris, although the former is more toxic than the latter.

The possible requisites of lipopolysaccharide for its antitumor effect can be distinguished as two characters: one is a nontoxic chemical which can stimulate the reticuloendothelial system (e.g., a nontoxic polysaccharide of plant origin) and the other is a cytotoxic agent against tumor cells. The combined use of these two agents will give a better effect. However, we have not yet succeeded in this type of therapy because most cytocidal antitumor agents inhibit the activity of the reticuloendothelial system.

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Antitumor Effect of Intracutaneous Injection of Bacterial Lipopolysaccharide

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