Combination therapy by intralesional injection of OK432 followed by intraperitoneal administration of lentinan and bacterial lipopolysaccharide caused almost complete regression of solid-type tumor MH134. All three components were needed for maximal antitumor activity. Mice in which MH134-tumor had regressed due to this combination therapy showed an augmented antitumor delayed hypersensitivity reaction (measured by the footpad test) and resistance to rechallenge with MH134, but they had no cytolytic antibodies in their serum detectable by the complement cytotoxicity test. The possible importance of local inflammation induced by OK432 in this combination therapy is discussed.

Key words: Lentinan — Lipopolysaccharide — OK432 — Combination therapy — MH134 hepatoma

We are studying combination therapy with immunomodulators to obtain higher antitumor activities against murine transplantable tumors. A combination therapy with lentinan and bacterial lipopolysaccharide (LPS), named LL therapy, was very effective against some immunogenic tumors, such as MM46 mammary carcinoma and allogeneic Ehrlich carcinoma,4,19) but this therapy was not effective against some weakly immunogenic tumors, such as MH134 hepatoma.1)

For the development of a more useful combination therapy, the effective tumor spectrum must be expanded. Recently, we reported that growth of tumors that are insensitive to LL therapy is inhibited by LL therapy plus treatment with cyclophosphamide, which may function as an inhibitor of suppressor cell activity.3) As another possible approach, we tried to make the LL therapy effective on MH134 hepatoma by an addition of a third component that induces local inflammation7,9) with accumulation of effector cells in tumor lesions. Here, we report that LL therapy caused regression of MH134 tumors when given after intraperitoneal administration of OK432, prepared from Streptococcus haemolyticus by Okamoto et al.13,14) The possible role of OK432 is discussed in relation to the importance of local inflammation in this combination therapy.

**MATERIALS AND METHODS**

**Mice and Tumor** Inbred male C3H/He mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, and they were 2 months old at the beginning of experiments. MH134, a transplantable ascites hepatoma, was passaged weekly in the peritoneal cavity of C3H/He mice. This tumor easily metastasizes, mainly to lymphoid organs: for example, metastases in inguinal lymph nodes were palpable in all mice 2 weeks after injection of $3 \times 10^5$ MH134 cells into their footpads.
Agents OK432 was kindly provided by Chugai Seiyaku Co., Tokyo, and lentinan from Ajinomoto Co., Kawasaki. Lipopolysaccharide (LPS) from E. coli 0127, B8 was purchased from Difco Lab. (Detroit, Mich.).

Antitumor Test MH134 cells were washed and inocula of 2.0 × 10^5 or 1.0 × 10^6 cells were implanted intradermally in the abdomen of C3H/He mice. Tumors developed within a few days after inoculation. The largest and smallest diameters of the tumors were measured with vernier calipers and the geometrical mean was calculated. OK432 in 0.1 ml of saline was injected into 5 sites in a tumor lesion. Lentinan or LPS in 0.2 ml of saline was administered ip. The significance of differences in value was tested by means of the χ^2-test or Student's t-test.

Assay of the Delayed Hypersensitivity Reaction against Tumor Cells (T-DHR) T-DHR was determined by the modified footpad test described previously. Briefly, the antigen fraction in Hanks' balanced salt solution (HBSS) was prepared by sonication of 8-day-old ascitic tumor cells. Mixtures of the antigen fraction (600 μg of protein) and 20 μg of alum in 0.03 ml were injected into the footpad of one hind leg and the same volume of a solution of 20 μg of alum in HBSS as a control was injected into the footpad of the other hind leg. Footpad swelling was calculated from the following formula:

Footpad swelling = (FT24hAI − FT0hAI) − (FT24hHI − FT0hHI) where FT is the foot thickness, 0h indicates the time just before the injection, and 24h indicates 24 hr after the injection, AI indicates injection of antigen fraction, and HI indicates injection of HBSS. Each mouse was used only once for a footpad test and tests were performed in a blind way by making measurements without knowing which treatment the mice had received. In previous work, the tumor specificity of the footpad swelling was confirmed by cross examination of MH134 and MM46.

Complement-dependent Cytolytic Activity of Sera The in vitro lysis of tumor cells was determined by measuring the release of ^51Cr, as described previously. Briefly, ^51Cr-labeled MH134 cells (5.0 × 10^3/well) and test antiserum (final 1/20) were incubated at 37°C in 100 μl of RPMI-1640 medium containing 10% fetal calf serum for 1 hr. Then guinea pig serum (final 1/4) was added as a source of complement (Toshiba Kagaku Co., Tokyo). After incubation for 1 hr at 37°C, the mixture was centrifuged and the released ^51Cr was counted. In control experiments, MM46 tumor cells were completely lysed by serum from MM46-immunized mice under the conditions described above.

RESULTS

Growth Inhibition of MH134 Hepatoma by OK432 The antitumor activity of intratumoral administration of OK432 against MH134 hepatoma was tested to determine the effective doses and times of administration of OK432. Solid tumors grew to 4–5 mm in diameter 4 days after intradermal inoculation of 2.0 × 10^5 MH134 cells, and then treatment with OK432 was started.

Table I shows that a single injection of 10 KE of OK432, but not of 1 or 3 KE, on day 4 strongly inhibited the growth of MH134, and 3 injections of 1 KE of OK432 on days 4, 7 and 10 were also very effective.

Combination Antitumor Therapy with OK432, Lentinan and/or LPS against MH134 Hepatoma Previous reports showed that LL therapy, that is administration of lentinan (6.25 mg/kg) plus LPS (0.5 mg/kg), on day 12 after tumor implantation caused regression of MM46 and Ehrlich carcinoma, but not of MH134 hepatoma. Thus, we tested the anti-MH134 activity of LL therapy with a single intratumoral injection of OK432 (1 KE) on day 5. Treatment with OK432 alone under these conditions was slightly effective.

Table I. Antitumor Activity of OK432 against MH134

<table>
<thead>
<tr>
<th>Treatment(a)</th>
<th>Tumor diameter(b) (mm)</th>
<th>Regressed(c) tested(d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.8±2.7</td>
<td>0/6</td>
</tr>
<tr>
<td>OK432 (1KE-d4)</td>
<td>12.2±6.5</td>
<td>0/5</td>
</tr>
<tr>
<td>OK432 (1KE-d4, 7 and 10)</td>
<td>3.3±2.2**</td>
<td>3/4*</td>
</tr>
<tr>
<td>OK432 (3KE-d4)</td>
<td>10.5±4.7*</td>
<td>1/4</td>
</tr>
<tr>
<td>OK432 (10KE-d4)</td>
<td>4.0±6.9**</td>
<td>3/4*</td>
</tr>
<tr>
<td>OK432 (1KE-d4) + lentinan + LPS</td>
<td>4.4±3.7**</td>
<td>5/6**</td>
</tr>
</tbody>
</table>

(a) OK432 (1KE-d4) indicates that OK432 (1KE/mouse) was administered intratumorally on day 4 after inoculation of MH134. Lentinan (6.25 mg/kg) and LPS (0.5 mg/kg) were administered ip on day 12.
(b) Mean tumor diameter ± SD on day 25.
(c) Number of tumor regressors/Number of mice tested (day 25).
(d) Significant difference from the control (*P<0.05, **P<0.01).
Table II. Antitumor Activity of Combination Therapy against MH134

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor diameter&lt;sup&gt;a)&lt;/sup&gt; (mm)</th>
<th>Regression ratio (%)&lt;sup&gt;b)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
</tr>
<tr>
<td>Control</td>
<td>18.6±2.5</td>
<td>0/8&lt;sup&gt;c)&lt;/sup&gt;</td>
</tr>
<tr>
<td>OK432</td>
<td>14.1±6.4</td>
<td>1/8</td>
</tr>
<tr>
<td>OK432+LPS</td>
<td>14.0±5.3</td>
<td>1/6</td>
</tr>
<tr>
<td>OK432+lentinan</td>
<td>12.0±6.9</td>
<td>2/7</td>
</tr>
<tr>
<td>OK432+lentinan+LPS</td>
<td>3.8±4.4**</td>
<td>4/6*</td>
</tr>
<tr>
<td>Lentinan+LPS</td>
<td>—</td>
<td>14.6±3.9</td>
</tr>
</tbody>
</table>

<sup>a)</sup> OK432 (1 KE/mouse) was administered intratumorally on day 5 after id inoculation of 2.0×10⁶ cells of MH134. Treatment with lentinan and LPS was as described in footnote a) of Table I.

<sup>b)</sup> Mean tumor diameter±SD on day 27.

<sup>c)</sup> Number of tumor regressors/Number of mice tested (day 27). Significant difference from OK432-treated group (*P<0.05, **P<0.01).

Table II and Fig. 1 show that either OK432 or LL therapy alone partially retarded tumor growth, but caused almost no tumor regression. On the other hand, a combination of OK432 and LL caused rapid and progressive regression of tumors (Fig. 1), the tumor regression ratio being 11/14 (79%). Combinations of OK432 plus LPS or OK432 plus lentinan seemed to be more effective than OK432 alone but less effective than a combination of OK432 plus LPS and lentinan (LL). These results indicate that the three agents in combination have synergistic antitumor activities.

In these experiments, we observed that OK432-treated tumors showed strong necrosis and retarded growth shortly after administration of LPS or LPS plus lentinan.

**Antitumor Activity and Effects on Host Immunity of Combination Therapy against MH134 Hepatoma Inoculated at a Large Dose**

When MH134 hepatoma was inoculated at a dose of 1.0×10⁶ cells, tumors grew rapidly. With such large inocula, even 3 or 6 treatments with OK432 (1 KE) were only partially effective and did not cause complete tumor regression. Table III shows that under these conditions, a single treatment with LL increased the antitumor activity of OK432 and caused almost complete tumor regression. These results, which were confirmed in another independent experiment (data not shown), indicate that combination therapy with the three components increased the limited effectiveness of a relatively small dose of OK432 alone. Table III also shows that sc injection of OK432 in a different site, away from the tumor tissues, had no antitumor activity. Thus, contact between the tumor and OK-
In this experiment, the anti-MH134 footpad response was examined on day 21, sera of test mice were obtained on day 22 for antibody titration, and mice in which tumors had regressed were rechallenged intradermally with $2.0 \times 10^5$ MH134 cells on day 35. Table III shows that mice cured by therapy with OK432 plus LL were resistant to challenge with further MH134 cells. The footpad response to the MH134 antigen fraction, which shows the MH134-specific delayed hypersensitivity reaction, was augmented by this combination therapy. On the other hand, no cytolytic antibody activity dependent on guinea pig complement was detected in any sera tested (data not shown). These results indicate that a kind of systemic antitumor immunity was potentiated by this combination therapy.

**DISCUSSION**

Previous reports showed that on ip administration, lentinan and LPS have synergistic antitumor activities against MM46 and Ehrlich carcinoma, but not MH134 hepatoma. This paper shows that prior intralesional administration of OK432 made the LL therapy effective on MH134 hepatoma. This combination therapy with OK432 plus LL gave a tumor regression ratio of about 80% and provided the cured mice with resistance to rechallenge. In this respect, OK432 plus LL was superior to LL plus cyclophosphamide as an antitumor therapy. In the combination therapy, all three components, OK432, lentinan and LPS, were necessary, because no combination of two of the three components was as effective as OK432 plus LL (Table II).

We think that these three components each have a different role. OK432 seemed to have an important role, because a large dose or frequent administration of OK432 had strong antitumor activity, especially...
against MH134 inoculated at a small cell number (Table I). The initial effect of OK432 is thought to be local, because intrallesional administration of OK432 was needed for its action. Intrallesional administration of OK432 is reported to have antitumor activity against various tumors.6, 13, 16) This antitumor action of OK432 was explained in terms of direct cytotoxicity16) and immunological activity; that is, its effect in causing local inflammation with accumulation of leukocytes, macrophages and lymphocytes, and activation of their functions.7, 8, 15) In this combination therapy, the immunological activity of OK432 is thought to be important, because OK432 could be replaced by Propionibacterium avidum, which has similar immunological activity21) to OK432 (unpublished data). Thus, we think that the inflammation elicited by OK432 makes LL therapy effective.

The antitumor action of lentinan and/or LPS against OK432 - treated MH134 is similar to that against untreated MM46 or Ehrlich carcinoma: LPS inhibited tumor growth rapidly whereas lentinan inhibited it slowly, and thus LL caused rapid and long-lasting regression of tumors4) (Fig. 1). The different susceptibilities of different tumors to LL therapy is thought to result from differences in immunogenicity of the tumors.3) According to this hypothesis, the intrallesional administration of OK432 is thought to compensate for the low immunogenicity of MH134. It is noteworthy in this connection that North reported the synergistic antitumor activity of intrallesional administration of Corynebacterium parvum and LPS, and postulated that the delayed hypersensitivity reaction to C. parvum in the tumor lesion may compensate for the low immunogenicity of tumors.12)

Furthermore, Watanabe et al. recently found that OK432, like BCG and C. parvum, has priming effects on production of tumor necrosis factor (TNF) by LPS.20) Thus, LPS administration after intrallesional injection of OK432 may trigger local and enhanced production of TNF, perhaps by causing accumulation of macrophages in the tumor lesion. TNF may participate as effector molecules independently of tumor antigens in this combination therapy. The detailed mechanisms of the therapy are under investigation.

In this combination therapy, lentinan is necessary. Lentinan has various immunological activities.5, 10, 11) We think that it potentiates systemic immunity with the help of other agents, because the augmented systemic immunity induced by OK432 plus LL in the MH134 system was similar to that induced by lentinan alone in the highly antigenic MM46 system11); that is, an augmented antitumor delayed hypersensitivity reaction and antitumor resistance. The role of humoral antibodies as a systemic immune response, which was recently reported in the MH134 system,17) is not clear because these antibodies could not be detected in our complement-dependent cytotoxicity test. This report showed that a combination of immunomodulators can be used as a new therapy effective on tumors with relatively low antigenicity; however a preliminary antitumor test against EL-4 indicated that OK432 plus LL was not effective on some tumors (unpublished data).

Finally, it should be mentioned that clinical testing of combination therapy with bacterial products should proceed with caution, because the sensitivity of the host to bacterial products, such as LPS, could be enhanced by prior treatment with bacterial bodies.

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