The effect of chemotherapy combined with immunostimulants on the activities of macrophages in mice was studied. The number of macrophages and exudate cells in the peritoneal cavity increased 3 days after ip injection with mitomycin-C, cyclophosphamide, and 5-fluorouracil together with OK-432 or yeast cell wall and decreased to normal level after 9 days, while the number of the cells remained decreased in mice receiving multi-drugs alone. Acid phosphatase activity of the macrophages of mice was elevated after the simultaneous injection of yeast cell wall and OK-432, and high activity was preserved in the macrophages of mice receiving yeast cell wall even after 9 days. Spreading of these cells was also enhanced. Macrophage activities examined by these assays were maximal in every respect 6 days after combination therapy. Cytostatic activity of the cells was strengthened after 6 days by combined use of OK-432 or yeast cell wall. Role of the activated macrophages in combination therapy was discussed.

The streptococcal preparations, OK-432,24) BCG,10) and zymosan4,25) have been reported to exhibit antitumor activity. Such antitumor activity seems to be based on the activation of host response against tumor growth. However, combined use of immunostimulants with antitumor drugs to bring about more fruitful tumor-inhibitory effect has been reported3,7-9,16,20) since the effect obtained by application of drugs, or immunostimulants alone, was limited. In our previous work17) antitumor effect of combined use of OK-432 and yeast cell wall with mitomycin-C was examined. When mitomycin-C at a dose of 2 µg/mouse, ineffective by a single injection, was used in combination with OK-432 or yeast cell wall, intense tumor-inhibitory effect was observed.

In the present study, effect of cytoreductive chemotherapy with multi-drugs in combination with immunostimulants, OK-432 or yeast cell wall, on the host responses in mice was studied. Kinetics of enzyme activity and cytostatic activity of peritoneal macrophages to Ehrlich ascites tumor cells after immunotherapy was examined.

Material and Methods

Mice: Random-bred ddY mice, weighing about 22 g and 6- to 8-week-old male and female, were used.

Tumor: Ehrlich carcinoma cells in ascites form, supplied from Cancer Research Institute, Faculty of Medicine, Kyushu University, Fukuoka, were maintained in ddY mice by weekly intraperitoneal passages. Mice were injected ip with Hanks' balanced salt solution and the fluid containing tumor cells was withdrawn into a syringe.

Materials Injected: Mitomycin-C (Kyowa Hakko Kogyo Co., Tokyo) and 5-fluorouracil (Kyowa Hakko Kogyo Co., Tokyo) dissolved in phosphate-buffered saline (PBS) before injection, and cyclophosphamide (Shionogi & Co., Tokyo) were used as antitumor drugs. OK-432, a streptococcal preparation, was donated by Chugai.
Pharm. Co., Tokyo. Yeast cell wall was kindly supplied by Dr. H. Hosoi, Department of Microbiology, Daiichi College of Pharmaceutical Sciences, Fukuoka. Method for preparing the yeast cell wall was described in detail elsewhere. Mitomycin-C, 5-fluorouracil, and cyclophosphamide were injected ip with or without immunostimulants. Control mice received the same dose of PBS.

Peritoneal Exudate Cells: Control mice and mice, injected with antitumor drugs in combination with or without immunostimulants, were injected ip with 8 ml of Hanks' BSS 3 days later. The abdomen was massaged and a fluid containing peritoneal exudate cells was withdrawn into chilled centrifuge tubes. The number of peritoneal exudate cells per mouse of each group was calculated. A part of the cells was pelleted by centrifugation, smeared on slides, and stained with Giemsa solution. The percentage of macrophages in each group was determined morphologically by counting more than 200 cells per slide.

Separation of Peritoneal Macrophages: Peritoneal exudate cells suspended in medium 199 supplemented with 10% fetal calf serum were incubated in plastic dishes (No. 3002, Falcon Plastics Co., U.S.A.) at 2 × 10^6 cells/ml for 30 min at 37°C and then non-adherent cells were washed out thoroughly with Hanks' BSS. Adherent cells were recovered by adding 0.02% EDTA in PBS and by gently scraping with a rubber policeman. More than 98% of the cells remaining in the dish had morphological characteristic of macrophages when stained with Giemsa solution. Assay for Cytostatic Activity of Peritoneal Macrophages: Tumor cells, suspended in medium 199 supplemented with 10% fetal calf serum, were adjusted to the concentration of 1 × 10^5/ml and 0.1 ml of this suspension was added to each well of U-shaped microplate (Cooke Engineering Co., U.S.A.). To each plate, 0.1 ml of peritoneal macrophages from each group suspended in the same medium was added at the effector-target cell ratio of 1:1 and 5:1, and the mixture was incubated at 37°C in an atmosphere of 5% CO₂. Twenty-four hours before harvesting, the culture was labeled with 0.2 μCi of ³H-thymidine in 0.05 ml of serum-free medium. Cultures were harvested on a glass fiber filter using a multiple cell harvester, Mark II (Wakenyaku Kogyo Co., Kyoto). The samples were dried and incorporation of tritiated thymidine was determined by liquid scintillation counter.

Assay for Macrophage Spreading: Macrophage suspension (0.2 ml) was distributed into the well of Lab-Tek tissue culture chamber (Miles Lab., Inc., U.S.A.) at the final concentration of 2 × 10^5 cells/ml in Hanks' BSS and incubated at 37°C for 60 min. The chambers were washed with PBS to remove non-adherent cells, fixed, and stained with Giemsa. The spread macrophages were classified according to the staging of Rabinovitch and DeStefano. The cells in stage 2 to 3, whose veils extended beyond 1/2 of the initial cell diameter, were classified as spread cells in this study. More than 200 macrophages were scored as either rounded or spread.

Estimation of Acid Phosphatase of Macrophages: Modified azo dye coupling technique described by Sakaki was used. The degree of acid phosphatase activity of the macrophages was divided into 5 grades according to the size and number of granules per cell. Briefly, the cells containing 1 ~ 2, 3 ~ 8, 9 ~ 16, 17 ~ 32, and more than 33 moderately large granules were classified as grades I, II, III, IV, and V, respectively.

**Results**

Effect of Immunochemotherapy on the Number of Macrophages and Exudate Cells in the Peritoneal Cavity: As the number of peritoneal exudate cells increased after injection of OK-432 or yeast cell wall, as reported previously, examination was made to see whether similar effect would be observed in combination therapy with multiple antitumor drugs and immunostimulants. As shown in Fig. 1, the number of peritoneal exudate cells after 3 days did not change significantly by chemotherapy alone, but increased by simultaneous injection of OK-432 or yeast cell wall. The number of the cells after 6 days was reduced slightly in the group given chemotherapy alone. These results suggest that the suppressive effect of antitumor drugs on precursor cells of peritoneal exudate cells can be counter-acted to

688
Effect of immunochemotherapy on the number of peritoneal exudate cells and macrophages of mice

Drugs (mitomycin-C, 2 μg/mouse; cyclophosphamide, 1 mg/mouse; 5-FU, 1 mg/mouse) were injected ip at day 0. Peritoneal exudate cells and percentage of macrophages were calculated after 3 days (O, □), 6 days (○,  ●), and 9 days (●, ■). Each value represents the mean of three mice.

* Significant at 0.025 < P < 0.05. ** Significant at 0.01 < P < 0.025, compared to control.

Effect of Immunochemotherapy on Acid Phosphatase Activity of Peritoneal Macrophages:

As shown in Fig. 2, administration of multi-drugs did not affect the acid phosphatase activity of peritoneal macrophages but the activity was elevated after a simultaneous injection of yeast cell wall or OK-432, and maximal activity was observed 6 days after injection. High activity was preserved in the macrophages of mice receiving yeast cell wall, even after 9 days.

Effect of Immunochemotherapy on the Spreading of Peritoneal Macrophages:

Various days after injection of antitumor drugs, with or without immunostimulants, macrophage spreading was examined. The proportion of spread cells in the control peritoneal macrophages was usually below 20% (Table I). The macrophages of mice receiving OK-432 or yeast cell wall in combination with multi-drugs exhibited enhanced spreading.

Effect of Immunochemotherapy on Cytostatic Activity of Peritoneal Macro-
Table I. Effect of Immunochemotherapy on Macrophage Spreading in vitro

<table>
<thead>
<tr>
<th>Exp. group</th>
<th>Materials injected (ip)</th>
<th>Macrophage spreading (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td>A</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>Drugs</td>
<td>7</td>
</tr>
<tr>
<td>C</td>
<td>Drugs + OK-432</td>
<td>31</td>
</tr>
<tr>
<td>D</td>
<td>Drugs + yeast cell wall</td>
<td>35</td>
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NT = not tested

Fig. 3. Effect of immunochemotherapy on the cytostatic activity of peritoneal macrophages against Ehrlich ascites tumor cells

Drugs (mitomycin-C, cyclophosphamide, and 5-FU) combined with or without immunostimulants (OK-432 or yeast cell wall) were injected ip. The cytostatic assay was performed after 3 days. Effector–target cell ratios were 5:1 (1/1) and 1:1 (1/1). Doses of drugs and immunostimulants were the same as described in Fig. 1. Pooled macrophages from 3 mice were used as effector cells in each group.

* Significant at 0.01 < P < 0.025 compared to control (A).

Cytochalasin: Cytostatic activity of peritoneal macrophages after injection of multi-drugs, with or without immunostimulants, is shown in Figs. 3~5. Peritoneal macrophages from control mice hardly exhibited cytostatic activity against DNA synthesis of tumor cells. The macrophages obtained from mice receiving antitumor drugs with or without immunostimulants 3 days before, did not show cytostatic activity at effector–target cell ratio of 1:1, except those from the group receiving OK-432 simultaneously (Fig. 3). However, the activity was augmented by the combined use of yeast cell wall at effector–target cell ratio of 5:1. After 6 days, cytostatic activity of each group was not observed at effector–target cell ratio of 1:1 although a low degree of the activity was observed in the group that received additional injection of OK-432 (Fig. 4). However, the activity estimated at effector–target cell ratio of 5:1 was much more pronounced in both groups that received immunostimulants. After 9 days, cytostatic activity of the macrophages from each group receiving

Fig. 4. Effect of immunochemotherapy on the cytostatic activity of peritoneal macrophages against Ehrlich ascites tumor cells

Drugs combined with or without immunostimulants were injected ip. The cytostatic assay was performed after 6 days. Effector–target cell ratios were 5:1 (1/1) and 1:1 (1/1). * Significant at 0.001 < P < 0.005 (C) and P < 0.001 (D), compared to the control (A). ** Significant at P < 0.001 (A, B, C, and D at effector–target cell ratio of 5:1) compared to tumor cell control.
COMBINATION THERAPY AND MACROPHAGES

Fig. 5. Effect of immunochemotherapy on the cytostatic activity of peritoneal macrophages against Ehrlich ascites tumor cells

Drugs combined with or without immunostimulants were injected ip. The cytostatic assay was performed after 9 days. Effector-target cell ratios were 5:1 (/////) and 1:1 (///).  
- Significant at P<0.001 compared to control (A).  
- Significant at 0.01<P<0.025 (A) and 0.025<P<0.05 (B), compared to tumor cell control.

Chemotherapy in combination with or without immunostimulants was almost the same as that of the cells from control mice. These results suggest that the tumor-inhibitory effect produced by cyto-reductive chemotherapy could be augmented further by activated peritoneal macrophages exhibiting cytostatic activity, following the simultaneous injection of immunostimulants.

Discussion

Activation of reticuloendothelial system and increase in the number of phagocytes after the administration of various immunostimulants have been reported.2,18) Bruley-Rosset et al.9) reported that acid phosphatase activity of peritoneal macrophages increased by incubation with living BCG, water-soluble extract from Mycobacterium smegmatis, and lipopolysaccharide in vitro, and the activated cells were cytotoxic against tumor cells. Activated peritoneal macrophages rapidly spread when plated on glass or plastic surface.6,18) Activated mononuclear phagocytes are the effector cells which mediate cytostatic13,14) and cytocidal effects.1,11) Peritoneal exudate cells activated with OK-432 or yeast cell wall were not only cytostatic (Fig. 4) but also cytocidal17) against Ehrlich ascites tumor cells, although cytostatic effect of activated macrophages has been reported to differ markedly according to the target cells used.15)

In the present study, the number of peritoneal macrophages and exudate cells increased after 3 days by simultaneous injection of immunostimulants. This increase of the cells observed shortly after immunochemotherapy seems to counteract the suppressive effect on bone marrow observed later as shown in Fig. 1. Acid phosphatase activity and spreading of peritoneal macrophages were augmented by combined use of immunostimulants. The cytostatic activity of the macrophages was markedly augmented after 6 days by combined use of OK-432 or yeast cell wall, while the activity did not decrease after 3, 6, and 9 days by administration of multi-drugs alone. If resident peritoneal macrophages have fewer precursor cells, and newly immigrated mononuclear phagocytes after the administration of immunostimulants are precursors of activated macrophages, as suggested by Ruco and Meltzer,22) it could be expected that the prominent tumor-inhibitory effect might be brought about as the result of increased number and augmented cytostatic activity of the peritoneal macrophages after immunochemotherapy.

Besides the elimination of tumor cells by the direct effect of chemotherapeutic drugs, combined immunochemotherapy is considered to be beneficial in that the bone marrow suppression by cyto-reductive chemotherapy can be contradicted to a certain extent and elimination of residual tumor cells can well be anticipated by activated macrophages by a combined use of immunostimulants. Further studies are necessary to clarify the rela-
tion between antitumor effect and host responses after combination therapy, especially in connection with specific immune response.

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REFERENCES