ABSTRACT

Effects of an antitumor antibiotic, ascofuranone (AF) on the murine immune system were studied. Unlike lectins, AF did not induce any proliferative response of splenocytes. Furthermore, AF significantly inhibited proliferative response of splenocytes in response to lectins, such as concanavalin A, lipopolysaccharide, or phytohemagglutinin above 5 μg/ml.

In concanavalin A-induced T-lymphocyte response, AF selectively inhibited the formation of interleukin 2 (IL-2) receptors, which were observed above 0.4 μg/ml. On the other hand, the inhibitory effect on the proliferative response to IL-2 of T-lymphocytes, which had already obtained IL-2 receptors, was observed above 10 μg/ml. IL-2 production of splenocytes in response to concanavalin A was also suppressed by AF above 2 μg/ml and only 3% of IL-2 was produced in the presence of AF, 10 μg/ml.

However, AF-activated macrophages and their glycosylation was significantly stimulated. Activation of macrophages by AF was also confirmed by stimulation of interleukin 1 production and tumoricidal activity. However, natural killer activity of splenocytes was suppressed at the concentration where significant activation of tumoricidal activity of macrophages was observed.

Therefore, AF had a dual effect on the immune system. Macrophages were activated to produce interleukin 1 and to kill tumor cells. On the other hand, functions of lymphocytes were suppressed.

INTRODUCTION

AF3 is an antibiotic isolated from a phytopathogenic fungus, Ascochyta visiae, as a hypolipidemic substance having a prenylphenolic structure (1). Recently, we reported its antitumor activity against experimental tumors including L1210, sarcoma 180, and Ehrlich carcinoma (2). One characteristic of the antitumor activity is that AF is effective with pretreatment as well as with posttreatment, indicating that its antitumor activity is host mediated. In fact, AF treatment of normal mice enlarges solid lymphoid organs without affecting body weight gain. However, the splenocytes derived from AF-treated mice have slightly lowered mitogenic responses to PHA, Con A, and LPS.

Another biological activity of AF is the modulation of lipid metabolism. When the antibiotic was given p.o. for 10 consecutive days to normolipidemic rats, the treatment resulted in marked reduction of serum cholesterol, triglycerides, phospholipids, and free fatty acids without affecting organ weight gain and total serum protein (3). In addition, AF tends to affect lipid levels of hyperlipidemic animals. When administered p.o. to male Wistar rats which were fed a cholesterol-rich diet, AF reduces serum lipid levels and hepatic and cardiac cholesterol contents without affecting body weight gain (4).

In the study of in vitro effects on leukemia L5178Y, AF has been found to significantly affect the synthetic profile of neutral lipids from acetate. Reduction of incorporation of acetate into squalene is notable. In addition, AF inhibits hypotonic hemolysis, whereas it stimulates hemolysis induced by deoxycholate (5). These results indicate that AF modulates membrane properties directly or indirectly via the modulation of lipid metabolism.

In this paper, we examined the effects of AF on functions of lymphocytes and macrophages in vitro, including the Con A response of splenocytes, which is a simple model system of central immunological interactions, to elucidate the mechanism of host-mediated antitumor activity of AF.

MATERIALS AND METHODS

Mice. Six-wk-old male BALB/c and C57BL/6 mice were purchased from Japan Charles River Co. (Kanagawa, Japan). Six-wk-old male C3H/HeN mice were purchased from Shizuoka Experimental Farm (Shizuoka, Japan). Commercial pellet diet (CE-2; Clea, Japan, Ltd., Tokyo, Japan) and tap water were fed ad libitum. Mice were used no later than 10 wk of age.

Ascofuranone. Ascofuranone more than 99% pure was supplied by Chugai Pharmaceutical Co. (Tokyo, Japan). AF was dissolved in methanol at 10 mg/ml and diluted to appropriate concentrations with medium. Methanol at a concentration of less than 0.5% did not have any effect on the experiments.

Splenocytes and Macrophages. A single cell suspension of splenocytes, erythrocytes of which were ruptured by ammonium chloride, was prepared as described previously (2). Splenocytes were suspended in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with kanamycin, 50 μg/ml, 50 μM 2-mercaptoethanol, and 5 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 10% fetal bovine serum (Flow Laboratories, North Ryde, N.S.W., Australia).

To induce inflammatory macrophages in a peritoneal cavity, 0.5 ml of 10% thioglycollate broth (Difco, Detroit, MI) or 2 ml of 1.2% sodium caseinate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was injected i.p. 4 days before collection. Peritoneal cells were collected with MEM (Nissui Seiyaku, Tokyo, Japan) supplemented with 50 μM 2-mercaptoethanol, and 5 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid containing heparin, 50 units/ml. The cells were washed twice with MEM and suspended in RPMI 1640 medium.

Cytotoxic T-Lymphocyte Line. Five million A/J splenocytes/ml were cultured with mitomycin C-treated C57BL/6 splenocytes, 5 x 10^6 cells/ml. After 72 h incubation, cells were suspended in fresh RPMI 1640 medium containing 50% rat CM. CTLL was maintained for more than 1 yr, and the cell density was not allowed to exceed 5 x 10^5 by splitting cultures every 2–3 days and seeding cells at 5 x 10^6 cells/ml.

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2To whom requests for reprints should be addressed.

3The abbreviations used are: AF, ascofuranone; CM, conditioned medium; Con A, concanavalin A; CTLL, cytotoxic T-lymphocyte cell line; IL-1, IL-2, interleukin 1 and 2, respectively; PHA, phytohemagglutinin; UK, lymphokine; LPS, lipopolysaccharide; MEM, Eagle’s minimum essential medium; NK, natural killer.

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10⁶ splenocytes and Con A, 10 μg/ml (Sigma Chemical Co., St. Louis, MO) in RPMI 1640 medium. CM was collected after 48 h incubation at 37°C in 5% CO₂-air atmosphere. The preparation of mouse IL-2 was as described previously (6). Con A-stimulated mouse spleen cell CM, which was eluted from Blue Sepharose with 0.145 M NaCl, was used. This partially purified preparation had no detectable interferon activity.

**Mitogen Response.** One hundred splenocytes were cultured with Con A, 10 μg/ml, in RPMI 1640 medium. CM was collected after 48 h at 37°C to induce IL-2 receptors. Cells were washed twice with MEM containing α-methylmannoside, 10 mg/ml, to remove Con A and once with MEM free of α-methylmannoside to remove residual α-methylmannoside and then were suspended in RPMI 1640 medium. IL-2 receptors formed were detected by the IL-2-dependent proliferative response of the triggered lymphocytes. Forty thousand triggered cells were cultured with partially purified 5% mouse IL-2 in a 200-μl vol in a flat-bottomed microplate well (Nunc, Kamstrup, Roskilde, Denmark). After a 70-h incubation, the cells in the microplate wells were pulsed with 0.5 μCi of [³H]thymidine (102.0 Ci/mmol; New England Nuclear, Boston, MA). Cultures were harvested 4 h later and [³H]-thymidine incorporation was counted with a liquid scintillation system.

**Formation of IL-2 Receptors and Detection of Receptors Formed.** Experiments were performed as reported by Larsson (8). Splenocytes, three million cells/ml, were cultured with Con A, 5 μg/ml, for 4 h at 37°C to induce IL-2 receptors. Cells were washed twice with MEM containing α-methylmannoside, 10 mg/ml, to remove Con A and once with MEM free of α-methylmannoside to remove residual α-methylmannoside and then were suspended in RPMI 1640 medium. IL-2 receptors formed were detected by the IL-2-dependent proliferative response of the triggered lymphocytes. Forty thousand triggered cells were cultured with partially purified 5% mouse IL-2 in a 200-μl vol in a flat-bottomed microplate well for 3 or 5 days. Four h before harvesting, the microplate wells were washed with 0.5 μCi of [³H]thymidine.

**Proliferative Response of CTLL Cells in Response to IL-2.** CTLL cells were cultured in a 200-μl vol in flat-bottomed microplate wells. Each well contained 1 x 10⁴ CTLL cells together with the putative IL-2-containing sample. After a 24-h incubation, the microplate wells were pulsed with 0.5 μCi of [³H]thymidine. Cultures were harvested 4 h later.

**Production of IL-2.** Five million splenocytes in a 1-ml vol in a flat-bottomed 16-mm diameter plastic plate well (Nunc) were cultured in the presence of Con A, 5 μg/ml. After a 24-h culture at 37°C, the supernatants were collected and centrifuged to remove contaminated cells. They were stored at −20°C until used. IL-2 activity in the supernatants was measured by proliferative response of CTLL cells in response to IL-2 (9).

**Assessment of Glycolysis of Macrophages.** Two million peritoneal cells in a 1-ml vol in a flat-bottomed plastic plate well were incubated at 37°C. After a 2-h culture, nonadherent cells were removed by three vigorous washes with MEM. Residual adherent cells were cultured for 20 h in a 1-ml vol. The concentration of glucose in the supernatant at the termination of the culture was measured by a blood analyzing system (Chugai Pharmaceutical Co., Tokyo, Japan) using an enzymatic method. Glucose consumption was computed using the formula

\[
\frac{(1 - \text{concentration of glucose at the termination of culture})}{\text{concentration of glucose in medium}} = 100 \times \frac{\text{test cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}}
\]

**Cytotoxic Assay of Macrophages.** Four hundred thousand peritoneal cells in a 100-μl vol were cultured at 37°C in a flat-bottomed microplate well. After a 2-h incubation, nonadherent cells were removed by three vigorous washes with MEM. Residual adherent cells were cultured with 10,000 ¹⁰⁶Cr-labeled YAC-1 cells in a 200-μl vol for 20 h. At the termination of culture, 100 μl of the supernatant were removed and the radioactivity was assessed. The spontaneous release was determined by incubating ¹⁰⁵Cr-labeled YAC-1 cells alone and the maximum release was determined by incubating ¹⁰⁵Cr-labeled YAC-1 cells with 0.5% sodium dodecyl sulfate. The following formula was used to compute percent lysis

\[
\% \text{ lysis} = 100 \times \frac{\text{test cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}}
\]

**Preparation of Target Cells.** YAC-1, a T-cell lymphoma induced by Moloney leukemia virus in A/Sn mice, was maintained in RPMI 1640 medium.

**Cytotoxic Assay of Natural Killer Activity.** One million splenocytes in a 200-μl vol in a flat-bottomed microplate well were cultured with 10,000 ¹⁰⁶Cr-labeled YAC-1 cells for 4 h. The radioactivity in 100 μl of the supernatant at the termination of the culture was assessed. Determination of spontaneous and maximum release and computation of the percentage of lysis were performed in the same manner as the cytotoxic assay of macrophages.

**RESULTS**

**Effects of Ascofuranone on Lectin-induced Proliferative Response of Splenocytes.** AF significantly inhibited lectin-induced proliferative response of splenocytes. Two typical results of ten experiments are shown in Tables 1 and 2. More extensive inhibition was observed in LPS-induced proliferative response where the response was completely suppressed by AF, 5 μg/ml, whereas Con A- and PHA-induced proliferations were completely suppressed at 10 μg/ml. Unlike lectins, AF showed no mitogenic activity (Table 1).

In Con A-induced proliferative response, AF suppressed the proliferative response in the presence of a suboptimal concentration of Con A (0.4 μg/ml) more extensively than in its optimal concentration (2 μg/ml). A supraoptimal concentration of Con A (10 μg/ml) suppressed the proliferative response even without AF, and AF showed no effect on the suppression at a high concentration of Con A (Table 2).

There was a obvious influence on cell viability when determined by dye exclusion test, even by AF, 10 μg/ml, suggesting that the inhibitory effect was not due to a decrease in cell viability. Although there were small differences among the inhibition rates of each experiment, probably because of minor differences in the condition of the culture or cell preparation, the same tendency was always observed in all other experiments.

**Effect on IL-2 Receptor Formation.** The proliferative response of lymphocytes in response to Con A is separated into two distinctive steps (8, 11, 12). At first, IL-2 receptor formation was induced in lymphocytes in response to Con A and then
**EFFECTS OF AF ON THE IMMUNE SYSTEM**

**Table 1**

Inhibition of lectin-induced proliferative response of splenocytes

<table>
<thead>
<tr>
<th></th>
<th>Without lectin</th>
<th>With Con A (2 µg/ml)</th>
<th>With LPS (10 µg/ml)</th>
<th>With PHA (2 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3,783 ± 5199</td>
<td>39,261 ± 4,142</td>
<td>18,006 ± 1,647</td>
<td>18,802 ± 666</td>
</tr>
<tr>
<td>AF 0.3 µg/ml</td>
<td>3,609 ± 424 (95)</td>
<td>35,633 ± 1,062 (91)</td>
<td>22,479 ± 2,988 (125)</td>
<td>18,823 ± 708 (100)</td>
</tr>
<tr>
<td>0.6 µg/ml</td>
<td>2,061 ± 650 (54)</td>
<td>34,029 ± 2,536 (87)</td>
<td>16,325 ± 1,209 (87)</td>
<td>19,480 ± 772 (104)</td>
</tr>
<tr>
<td>1.3 µg/ml</td>
<td>1,874 ± 152 (50)</td>
<td>34,470 ± 4,580 (88)</td>
<td>24,758 ± 2,585 (137)</td>
<td>19,873 ± 855 (106)</td>
</tr>
<tr>
<td>2.5 µg/ml</td>
<td>1,330 ± 386 (35)</td>
<td>28,860 ± 966 (74)</td>
<td>17,805 ± 382 (99)</td>
<td>18,122 ± 2,818 (96)</td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>322 ± 94 (9)</td>
<td>18,111 ± 2,006 (46)</td>
<td>4,895 ± 2,581 (26)</td>
<td>13,198 ± 1,516 (70)</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>183 ± 53 (4)</td>
<td>2,245 ± 382 (6)</td>
<td>162 ± 46 (1)</td>
<td>3,101 ± 20 (16)</td>
</tr>
</tbody>
</table>

* Mean ± SD of triplicate cultures.
+ Numbers in parentheses, percentage of control.
° Statistically significant at P < 0.01.

**Table 2**

Inhibition by AF of splenocyte Con A-induced proliferative response

<table>
<thead>
<tr>
<th></th>
<th>Without Con A</th>
<th>0.4 µg/ml</th>
<th>2 µg/ml</th>
<th>10 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,233 ± 1859</td>
<td>42,459 ± 8,026</td>
<td>53,641 ± 3,045</td>
<td>522 ± 314</td>
</tr>
<tr>
<td>AF 0.3 µg/ml</td>
<td>1,023 ± 245 (83)</td>
<td>47,678 ± 3,568 (112)</td>
<td>53,047 ± 3,045 (99)</td>
<td>427 ± 125 (81)</td>
</tr>
<tr>
<td>0.6 µg/ml</td>
<td>844 ± 113 (68)</td>
<td>47,310 ± 1,520 (111)</td>
<td>52,802 ± 3,121 (98)</td>
<td>376 ± 59 (72)</td>
</tr>
<tr>
<td>1.3 µg/ml</td>
<td>788 ± 171 (64)</td>
<td>41,538 ± 9,559 (98)</td>
<td>50,424 ± 4,096 (94)</td>
<td>436 ± 116 (84)</td>
</tr>
<tr>
<td>2.5 µg/ml</td>
<td>575 ± 155 (47)</td>
<td>42,447 ± 6,209 (100)</td>
<td>52,034 ± 2,856 (97)</td>
<td>389 ± 62 (75)</td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>1,453 ± 101 (37)</td>
<td>42,717 ± 6,209 (100)</td>
<td>52,034 ± 2,856 (97)</td>
<td>389 ± 62 (75)</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>523 ± 77 (42)</td>
<td>807 ± 545 (2)</td>
<td>13,684 ± 2,602 (25)</td>
<td>340 ± 70 (65)</td>
</tr>
</tbody>
</table>

* Mean ± SD of triplicate cultures.
° Numbers in parentheses, percentage of control.
 Statistically significant at P < 0.01.

**Table 3**

Inhibition of IL-2 receptor formation by AF

<table>
<thead>
<tr>
<th></th>
<th>Radioactivity incorporated (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td>Control</td>
<td>7,538 ± 833</td>
</tr>
<tr>
<td>AF 0.4 µg/ml</td>
<td>2,356 ± 880 (31)</td>
</tr>
<tr>
<td>2 µg/ml</td>
<td>2,588 ± 649 (34)</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>1,870 ± 648 (22)</td>
</tr>
</tbody>
</table>

* Mean ± SD of triplicate cultures.
° Statistically significant at P < 0.01.

**Table 4**

Inhibitory effect of AF on reactivity of Con A-triggered splenocytes to IL-2

<table>
<thead>
<tr>
<th></th>
<th>Radioactivity incorporated (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td>Control</td>
<td>21,810 ± 6,768</td>
</tr>
<tr>
<td>AF 0.4 µg/ml</td>
<td>21,229 ± 5,211 (97)</td>
</tr>
<tr>
<td>2 µg/ml</td>
<td>22,976 ± 3,217 (105)</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>6,097 ± 2,849 (23)</td>
</tr>
</tbody>
</table>

* Mean ± SD of triplicate cultures.
° Numbers in parentheses, percentage of control value.
° Statistically significant at P < 0.01.

trigged lymphocytes proliferated in response to IL-2 in the medium. As shown in Table 3, splenocytes cultured with Con A for 4 h incorporated [³H]thymidine, whereas those without Con A did not respond to IL-2. When AF was added during the induction period of IL-2 receptors, a significant inhibitory effect was observed above 0.4 µg/ml. This inhibition was diminished as the responding period was prolonged. Inhibition on day 3 was 70% while on day 5 was only 20%. **Effect on the Reactivity of Lymphocytes to IL-2.** While the inhibitory effect on the IL-2 receptor formation was evident at 0.4 µg/ml, that on the proliferative response of Con A-primed splenocytes to IL-2 was not observed at this concentration (Table 4). Approximately 70% inhibition was observed with AF at 10 µg/ml on both days 3 and 5. The inhibitory effect of AF on the reactivity to IL 2 was also investigated using CTLL cells. In this case, statistically significant inhibition was observed above 2.5 µg/ml in the case of 1% rat CM, and above 5 µg/ml in the case of 5% CM. Therefore, it could be concluded that the inhibitory effect was dependent on the concentration of IL-2 in the medium. However, no effect was observed at less than 0.6 µg/ml (Table 5) although IL-2 receptor formation was significantly inhibited at this concentration. There-
of IL-2 by splenocytes (Table 6). The inhibition was relatively extensive; with AF at 10 μg/ml, IL-2 production was reduced to less than 10% of control. However, AF, 0.4 μg/ml, did not affect the production. Unlike Con A, AF did not show any inductive activity to produce IL-2.

Effect on Glycolysis of Macrophages. Macrophages as well as lymphocytes play an important role in the immune system. Since AF suppressed functions of lymphocytes, we also examined its effect on glycolysis of macrophages, because glycolysis is one of the indicators of activated macrophages. As shown in Table 7, AF significantly stimulated glycolysis of macrophages at a relatively higher concentration, 10 μg/ml, with which it almost completely inhibited functions of lymphocytes. In fact, maximum stimulation was marked at 20 μg/ml. Similar results were obtained in five other experiments including those using casein-induced inflammatory macrophages or resident macrophages.

**DISCUSSION**

AF inhibited the lectin-induced proliferative response of splenocytes. Since the inhibition was observed in both the LPS-
induced proliferative response and PHA- and Con A-induced proliferative responses, it could be concluded that AF prevents both T- and B-lymphocytes from proliferating in response to lectins. In addition, AF inhibited both IL-2 receptor formation and IL-2 production in Con A-induced T-cell response, as shown in Tables 3 and 6. Moreover, AF suppressed NK activity of splenocytes during a cytotoxic assay. Because NK cells are members of the large granular lymphocytes (19), this suggests that AF inhibits functions of lymphocytes including cytotoxic activity in vitro.

In Con A-induced proliferative response, AF at lower concentrations, less than 1 μg/ml, preferentially prevented IL-2 receptor formation. Since AF inhibited the growth and protein synthesis of cultured leukemia at 10–20 μg/ml (5), the inhibitory effect of AF on IL-2 production or proliferative response observed at 5–20 μg/ml would be mainly caused by those effects. However, in regard to the inhibition of IL-2 receptor formation, it is not possible that AF inhibits the cellular macromolecular syntheses because AF at 0.4 μg/ml has only negligible influence on macromolecular syntheses of leukemia cells. Therefore, the mechanism of inhibition of IL-2 receptor formation may be different from that of IL-2 production and the proliferative response. As mentioned above, AF modulates lipid metabolism and affects membrane properties. Because the signal of Con A is transmitted through the membrane and IL-2 receptors are induced on the plasma membrane, modulation of membrane properties might greatly affect these events. Therefore, the selective inhibition of IL-2 receptor formation by AF could be mediated through the altered properties of plasma membrane.

Although AF inhibited functions of splenocytes, the antibiotic activated macrophages as assessed by stimulation of glycolysis. Furthermore, functions of macrophages such as production of IL-1 and tumoricidal activity were also stimulated by AF. These effects were similar to those of LPS. AF, however, is different from LPS in that LPS also induces the proliferation of B-cells while AF inhibits the proliferation.

Maximum activation of macrophages was observed with AF at 20–40 μg/ml which at the same time inhibited functions of splenocytes and the growth and macromolecular syntheses of cultured leukemia, L5178Y (5). Since AF is a low molecular substance, its antigenicity should be low. AF therefore seems to affect the cellular metabolism of macrophages directly, unlike polysaccharides and peptides which are assumed to function like foreign antigens. Since AF modulates cellular lipid metabolism (5), one possible explanation of its macrophage activation is that the modulation of lipid metabolism of macrophages causes their functional activation. Interaction of the immune network is performed on the plasma membrane. Since the properties of the membrane are greatly affected by its lipid composition (20, 21), modulation of lipid metabolism by AF would lead to modulation of immune function. In addition, alteration of phospholipid metabolism has been reported in various immunological phenomena (22–24). AF may activate macrophages specifically by affecting the lipid metabolism characteristic of macrophages. Studies in regard to this possibility are now in progress.

The results obtained in this paper suggest two possible mechanisms of host-mediated antitumor activity of AF: (a) inhibition of the suppressive mechanism mediated by lymphocytes and (b) activation of macrophages that participate in tumor killing. The former possibility is less plausible because AF rather enhanced induction of suppressor T-cells which prevent the production of IL-2.4 Pretreatment by AF induces the resistance against tumors. The prophylactic antitumor activity needs no vaccination, suggesting that the immune system that the host animal originally has as a defense mechanism against tumor is activated by AF (2). Macrophages as well as NK cells are now considered to be such defense populations (17, 18). *In vitro* activation of macrophages reported in this paper therefore is consistent with the prophylactic antitumor activity of AF.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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In Vitro Effects of an Antitumor Antibiotic, Ascofuranone, on the Murine Immune System


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