Isolation of an Inhibitor of Type II Interferon Induction from Tumor Ascitic Fluids

Shuzo Matsubara, Midori Suzuki, Masataka Nakamura, Kiyoto Edo, and Nakao Ishida

Department of Bacteriology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Sendai 980, Japan

ABSTRACT

A low-molecular-weight fraction (M.W. ~700) that specifically impairs the induction of type II interferon in mice by purified protein derivative of tuberculin or OK-432 was isolated from the cell-free ascitic fluid of mice bearing Ehrlich ascites carcinoma. Purification was achieved by ultrafiltration and gel filtration. The inhibitory activity of the isolated fraction was 10 times greater than that of the unfractionated starting material in the impairment of type II interferon induction. The significant inhibition was observed even when 0.2 ml of the 10,000-fold dilution of the fraction, which was previously adjusted to 0.25 A unit at 290 nm absorption, was once treated i.p. in normal mice 48 hr before challenge of type II interferon inducers. This fraction was stable to heating at 56° for 60 min. The active component, however, did not affect the in vivo induction of type I interferon by polyriboinosinic-polyribocytidylic acid or tilorone-HCl in parallel experiments, an identical low-molecular-weight fraction that impairs the type II interferon induction in mice was isolated from the ascitic fluids of rats bearing AH-100B ascites tumor and from a human hepatoma case with advanced cancer metastatic to the peritoneal cavity. However, nontumorous ascitic fluids obtained from adjuvant-stimulated mice and a human liver cirrhosis case did not contain any such inhibitory activity.

INTRODUCTION

Type II (or immune) interferon, which differs from virus type (type I) interferon with respect to several physicochemical and biological properties, is produced by lymphocytes during the cellular immune response to specific antigens or during the cellular proliferative response to mitogens (6, 25, 27). It has been suggested that the primary biological activity of type II interferon may be to play a role in the host immunosurveillance system rather than in virus inhibition (1, 3, 6, 20). Recently, we found that the induction of type II interferon by PPD in BCG-sensitized mice or by OK-432 in nonsensitized mice, but not that of type I interferon by poly(I-C) or tilorone-HCl, was gradually impaired following tumor growth (13). In addition, we showed that a low-molecular-weight factor, isolated from the ascitic fluid of mice bearing Ehrlich ascites carcinoma cells, reduces the cellular proliferative response to mitogens (6, 25, 27). It has been shown that the biological activity of type II interferon is specific to the immunosurveillance system and not to virus infection (1, 3, 6, 20). The fraction we isolated was stable to heating at 56° for 60 min. The active component, however, did not affect the in vivo induction of type II interferon by polyriboinosinic-polyribocytidylic acid or tilorone-HCl in parallel experiments, an identical low-molecular-weight fraction that impairs the type II interferon induction in mice was isolated from the ascitic fluids of rats bearing AH-100B ascites tumor and from a human hepatoma case with advanced cancer metastatic to the peritoneal cavity. However, nontumorous ascitic fluids obtained from adjuvant-stimulated mice and a human liver cirrhosis case did not contain any such inhibitory activity.

MATERIALS AND METHODS

Animals. Inbred 6-week-old DDI mice were obtained from the Experimental Animal Center of Tohoku University, Sendai, Japan. Two weeks before use for the induction of type II interferon by PPD, one group of these mice was immunized by i.v. injection of 10⁷ viable BCG organisms (Japanese strain, Koseikai BCG Laboratory, Sendai, Japan). Six-week-old Donryu rats (Funabashi Farm, Chiba, Japan) were used to harvest tumor ascitic fluid.

Tumor Ascitic Fluids. Mouse ascitic fluid was harvested from DDI mice 11 days after i.p. implantation of 10⁵ Ehrlich carcinoma cells. Rat ascitic fluid was prepared from Donryu rats 14 days after i.p. implantation of 10⁵ AH-100B hepatoma cells. Human ascitic fluid was obtained from a 56-year-old female with hepatoma and advanced cancer metastatic to the peritoneal cavity who had not received any chemotherapy for 6 days before drawing. These ascitic fluids were immediately centrifuged at 1,700 x g for 20 min to remove any cells, and then the supernatants were centrifuged at 105,000 x g for 3 hr. The clarified fluids were stored at -20° until use.

Nontumorous Ascitic Fluids. Mouse ascitic fluid was prepared from adjuvant-stimulated mice according to the method of Elleman and Eidinger (5). Human ascitic fluid was also obtained from a 48-year-old female with chronic liver cirrhosis who had not received any chemotherapy for 4 days before drawing.

Ultrafiltration and Gel Filtration. All ascitic fluids were first passed through a UM-10 membrane (Amicon, Lexington, Mass.) that excluded ascites constituents with molecular weights greater than 10,000, and then the filtrates were lyophilized. Each white powder was redissolved in 6 ml of PB and applied to a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column (2.3 x 65 cm). The resulting fractions (3 ml) eluted with PB were measured for UV absorption at 280 and 290 nm and were tested for their inhibitory activity on interferon induction in mice. Selected fractions were desalted through a Sephadex G-10 column (2.2 x 45 cm) with distilled water. This and all the purification procedures were carried out at 4°.

Interferon Inducers. For the in vivo induction of type II interferon, PPD (Parke-Davis Laboratories, Detroit, Mich.) and OK-432 (Research Laboratories, Chugai Pharmaceutical Co., Tokyo, Japan) were used. OK-432, a widely used immunopotentiating agent prescribed for the immunotherapy of cancer.
patients (11), is a whole-cell preparation of an avirulent strain of group A Streptococcus pyogenes (18). This agent was previously demonstrated to induce a high-titered immune type interferon involving the cooperation of T-cells and Mφ in mice (12). Poly(l-C) (P-L Biochemicals, Milwaukee, Wis.) and tilorone-HCl (Merrel-National Laboratories, Cincinnati, Ohio) were used as type I interferon inducers.

**Assay of Interferon Induction-Inhibiting Activity.** Eluates fractionated by gel filtration of each ascitic fluid were first adjusted to 0.25 A$_{290}$ nm unit by diluting with PB, and then serially diluted 10 times. Two-tenth ml of each dilution was injected i.p. into normal or BCG-sensitized mice. In our previous paper (13), significant inhibition of type II interferon induction in mice was demonstrated to persist for 2 to 7 days after i.p. injection of cell-free tumor ascitic fluid. Thus, interferon inducers were challenged 48 hr after treatment with the fractions. Pooled sera were obtained from groups of 4 mice at a time which produces maximum interferon activity as follows (14): 4 hr after injection of PPD (500 µg/kg i.v.) in BCG-sensitized mice; 24 hr after injection of OK-432 (5 mg/kg i.p.); 2 hr after injection of poly(I-C) (2.5 mg/kg i.v.); and 24 hr after administration of tilorone-HCl (200 mg/kg P.O.). The serum specimens were assayed for antiviral activity by the cytopathic effect inhibition technique on L-1D cell monolayers using the Indiana strain of vesicular stomatitis virus, as described elsewhere (12).

The interferon titer was expressed in units/ml as the reciprocal of the highest dilution of test serum that reduced the cytopathic effect by 50%. In this assay, 1 interferon unit almost equals 2.0 reference units of standard mouse fibroblast interferon (NIH, Bethesda, Md.).

**RESULTS**

**Isolation of an Inhibitor of Type II Interferon Induction from Ehrlich Ascitic Fluid.** Cell-free Ehrlich ascitic fluid (about 600 ml) was first subjected to pressure ultrafiltration through a UM-10 membrane (m.w. >10,000 exclusion). The in vivo analysis for inhibitory activity of type II interferon induction demonstrated that the activity was recovered in the filtrate without appreciable loss. The lyophilized filtrate (about 4.3 g) was dissolved in 6 ml of 10 mM PB and purified by gel filtration through Sephadex G-25. Two fractions with different 290:260 nm absorption ratios, EP-I and EP-II, were obtained (Chart 1). When the inhibitory activity on type II interferon induction by OK-432 in mice was quantitated with 0.2 ml of the serial 10-fold dilutions of each pooled fraction, it was essentially found to be concentrated in EP-II (Table 1). Significant inhibition occurred even when 0.2 ml of the 10,000-fold dilution of EP-II, which was previously adjusted to 0.25 A$_{290}$ nm unit, was given. This treatment dose corresponded to 10$^{-5}$ to 10$^{-6}$ ml of the original ascitic fluid. EP-I was less active than the unfractionated starting filtrate. On the other hand, EP-II did not affect the in vivo induction of type I interferon by poly(l-C) or tilorone-HCl (Table 2). Furthermore, adjuvant-induced ascitic fluid did not impair the interferon induction of any of the inducers used (Table 2). These results indicate that EP-II specifically inhibits the in vivo induction only of type II interferon.

To remove the large quantity of salts contained in EP-II, further purification was conducted through a Sephadex G-10 column by eluting with distilled water. When the resulting fractions were measured for UV absorption at 290 nm, 2 major peaks, EG10-I and EG10-II, were detected (Chart 2). The inhibitory activity was specifically recovered in EG10-I which was eluted immediately after the void volume, and the weight yield was finally about 10 mg as lyophilized powder. Since Sephadex G-10 gel fractionates components with molecular weights of less than 700, that of the active component appears to be around 700. EG10-I showed major absorption at 235 and 290 nm and less absorption at 260 nm (Chart 3). The active component was stable to heating at 56° for 60 min but not for

![Chart 1](image1)

**Table 1**

<table>
<thead>
<tr>
<th>Interferon titer (units/ml)</th>
<th>Dilution</th>
<th>Filtered fluid</th>
<th>EP-I</th>
<th>EP-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original solution</td>
<td>&lt;20</td>
<td>80</td>
<td>&lt;20</td>
<td></td>
</tr>
<tr>
<td>1:10</td>
<td>&lt;20</td>
<td>1,280</td>
<td>&lt;20</td>
<td>&lt;20</td>
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<tr>
<td>1:100</td>
<td>&lt;20</td>
<td>5,120</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>1:1,000</td>
<td>160</td>
<td>2,560</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>1:10,000</td>
<td>2,560</td>
<td>5,120</td>
<td>1,500</td>
<td></td>
</tr>
<tr>
<td>1:100,000</td>
<td>2,560</td>
<td>5,120</td>
<td>5,120</td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>5,120</td>
<td></td>
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<td></td>
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</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Interferon titer (units/ml)</th>
<th>Fluid</th>
<th>PPD</th>
<th>OK-432</th>
<th>Poly (I-C)</th>
<th>Tilorone-HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP-II</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>6400</td>
<td>3200</td>
<td></td>
</tr>
<tr>
<td>Adjuvant-induced ascitic fluid</td>
<td>1600</td>
<td>2560</td>
<td>6400</td>
<td>3200</td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>800</td>
<td>2560</td>
<td>6400</td>
<td>6400</td>
<td></td>
</tr>
</tbody>
</table>
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180 min (Table 3). Further purification and chemical characterization are under way.

Isolation of an Inhibitor of Type II Interferon Induction from Human- and Rat-derived Ascitic Fluids. To investigate whether the inhibitory activity of type II interferon induction is found in other species, ascitic fluids obtained from rats with AH-100B ascites tumor and a human hepatoma case were subjected to identical purification techniques as described above. Sephadex

Chart 2. Sephadex G-10 column chromatography of EP-II. The lyophilized powder of EP-II (about 800 mg) was dissolved in 4 ml of distilled water and then eluted through a 2.2- x 45-cm column equilibrated with distilled water. The absorbance at 290 nm was determined for each fraction. The fractions designated as EG10-I and EG10-II were pooled and lyophilized. The total weight of EG10-I was about 10 mg, and that of EG10-II was about 350 mg.

Chart 3. Absorption spectrum of the active component of EG10-I.

Table 3
Heat stability of the inhibitory activity of EG10-I on type II interferon induction in mice by OK-432

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time of treatment</th>
<th>Interferon titer (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>45 days</td>
<td>&lt;20</td>
</tr>
<tr>
<td>37</td>
<td>24 hr</td>
<td>&lt;20</td>
</tr>
<tr>
<td>56</td>
<td>72 hr</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>60 min</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>180 min</td>
<td>1280</td>
</tr>
</tbody>
</table>

G-25 column chromatography of these tumor ascitic fluids revealed elution profiles which resembled that of Ehrlich ascitic fluid (Chart 4). The analysis for the inhibitory activity of type II interferon induction by OK-432 in mice demonstrated that the activity was specifically recovered in the second peak fractions (Peak II) as was the case with mouse ascitic fluid (Table 4). In other words, the inhibitory effect was not species specific. Nontumorous ascitic fluid from a patient with chronic liver cirrhosis had the same chromatographic pattern as did the tumor origins, but no activity was detected in any fractions (Table 4).

DISCUSSION

The present study demonstrated that cell-free ascitic fluids obtained from mice with Ehrlich ascites carcinoma contain a factor (m.w. ~700) which can impair type II interferon induction
in mice by PPD or OK-432. This factor, however, did not affect the in vivo induction of type I interferon by poly(I:C) or tilorone-HCl. Such low-molecular-weight active fractions were also found to be present in ascitic fluids obtained from rats with AH-100B ascites tumor and from a human hepatoma case. It can be said that the factor can act beyond its species, since the inhibitory activity was detected using the mouse interferon production system. In contrast, ascitic fluids prepared from mice and a human without tumor contained components which were chromatographed in a position identical with that of tumor ascitic fluids on Sephadex G-25, but with no activity. In other words, the inhibitory factor of type II interferon induction was detected only from tumor ascitic fluids. However, it is still uncertain whether the inhibitor originates from tumor cells. To determine the origin, the isolation of such a inhibitor has been examined using the culture fluids of tumor cells.

The exact mechanism of action of the inhibitor in the in vivo induction of type II interferon by PPD or OK-432 is still unknown. It has been noted that interferon production by cells in vitro is impaired by exogenous treatment with prostaglandins (22), corticosteroids (8), nucleotides (9), and Vinca alkaloids (7). These agents, which affect the physiological movements of cytoplasmic membrane and the intracellular skeletal system of viable cells, however, impair the production of both types of interferons. Although further purification is required to characterize the chemical nature of our inhibitor, as shown in the present study, its several physicochemical properties obtained by Sephadex gel chromatography, heat stability test, and UV scanning may suggest that the inhibitor differs from the pharmacological agents. On the other hand, the neutralizing antagonists of the interferon action have been detected in a great variety not only of the culture fluids and the extracts of malignant cells (24) but also in nonmalignant tissues and cells (2). However, the cell-free Ehrlich ascitic fluid used in this study, as described previously (13), did not neutralize the antiviral activity of PPD- or OK-432-induced interferons in our assay system. Another possibility that the producing time of serum interferon in tumor-bearing mice may differ from that in nontumor bearers was also denied by a comparative experiment of the kinetics of type II interferon production in mice of both groups, in which observations were made over a 48-hr period after injection of PPD or OK-432. This circumstantial evidence indicates that the inhibitor may impair the production mechanism of type II interferon in mice.

Hyporeactivity to interferon induction is induced by repeated administrations of the same interferon inducer or by persistent infections of viruses and microorganisms (8, 23). Minagawa and Ho (16) recently reported that acid-labile and acid-stable HF were produced in addition to type II interferon when BCG-sensitized mice were challenged with BCG. The acid-labile HF produced 2 hr after challenge was indistinguishable from type II interferon in some physicochemical and biological characteristics, and the acid-stable HF produced 4 to 8 hr after challenge antigenically resembled type I interferon. These HF were suggested to be interferons with molecular weights of approximately 56,000 and 92,000. Stringfellow (21) also isolated a high-molecular-weight hyporeactive factor, which is physicochemically similar to type I interferon, from the sera of mice infected with encephalomyocarditis virus. In addition, these HF were shown to be heat-labile proteins. Therefore, our inhibitor of type II interferon induction differs from such HF.

Another explanation for the hyporeactivity produced by our inhibitor is the association with the suppressed function of immunocompetent cells, which is generally observed in tumor bearers (4, 10). We previously demonstrated that OK-432 may involve both T-cells and MΦ for the induction of type II interferon in healthy mice without tumors (12). Besides, PPD has been shown to elicit type II interferon together with other lymphokines from T-cells of BCG-sensitized animals (25). In contrast, it has been suggested that both poly(I:C)- and tilorone-HCl-induced interferons originate in radioresistant cell populations derived from hemopoietic stem cells, probably MΦ, in mice (19, 26). It has been shown that in tumor bearers the immune responsibility of lymphocytes is primarily impaired rather than MΦ (15). This observation, therefore, may support the possibility that the inhibitor mainly suppresses T-cell functions.

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