Elimination of Anemia-inducing Substance by Cyclic Plasma Perfusion of Tumor-bearing Rabbits

Osamu Ishiko, Kouzo Hirai, Sadako Nishimura, Toshiyuki Sumi, Ken-ichi Honda, Masaaki Deguchi, Ichiro Tatsuta, and Sachio Ogita

Department of Obstetrics and Gynecology, Osaka City University Medical School, Osaka 545-8585, Japan [O. I., K. H., S. N., T. S., K-i. H., S. O.]; Department of Obstetrics and Gynecology, Wakayama Medical College, Kihoku Hospital, Wakayama 649-7113, Japan [M. S.]; and Department of Obstetrics and Gynecology, Hanwa Sumiyoshi General Hospital, Osaka 558-0041, Japan [I. T.]

ABSTRACT

We carried out a fundamental study to search for a therapeutic modality that would remove the anemia-inducing substance (AIS) from the plasma of cancer patients because it is thought to be one of the substances responsible for anemia and immunodeficiency in advanced cancer patients. Using AIS isolated from the plasma of patients with advanced ovarian carcinoma, we confirmed that adsorption of AIS to noncoated charcoal was nonspecific and high. Moreover, it was verified that VX2 carcinoma-bearing rabbits are an optimal experimental model for plasma perfusion. The data obtained on day 40 after transplantation (hemoglobin, 9.1 ± 2.1 g/dl; osmotic pressure inducing RBC lysis, 137 ± 11 mosmol/kg; lymphocyte stimulation index, 8.8 ± 8.6; and RBC fragility-inducing activity, 40 ± 9 mosmol/kg) proved similar to the hematological findings in patients with cancer cachexia. A 1-h plasma perfusion (3 ml/min) through noncoated charcoal was performed in tumor-bearing rabbits, and it resulted in the restoration of RBC fragility-inducing activity and suppression of lymphocyte blast formation to pretransplantation values. When plasma perfusion was performed every 3 days, RBC fragility-inducing activity, which increased again 3 days after perfusion, was diminished, and RBC osmotic resistance was within the normal range from the fourth perfusion onward. These results showed that cyclic plasma perfusion is effective in sustained removal of RBC fragility-inducing factor from plasma, suggesting that it might have the potential for clinical application.

INTRODUCTION

Anemia, immunodeficiency, and weight loss developing in advanced cancer patients are referred to as cancer cachexia, and the pathogenesis of the anemia, immunodeficiency, and weight loss has been studied for a long time. There have been scattered reports of cancer cachexia-inducing substances, but no conclusive evidence has been obtained thus far. In 1987, we reported the existence of an AIS that depresses the function of erythrocytes and immunocompetent cells in the plasma of patients with an advanced malignant gynecological tumor (1). In subsequent studies, AIS was successfully isolated and purified, and it has been identified as a protein with a molecular weight of approximately 50,000 (2). We have also reported that when plasma obtained from terminal cancer patients was passed through a noncoated charcoal column, the factor depresses erythrocytes and immunocompetent cells disappeared (3).

How patients' QOL could be sustained has been emphasized as an important issue in recent opinions on the management of terminal cancer patients. However, symptoms associated with cancer cachexia greatly impair patients' QOL. Thus, alleviation of these symptoms might provide a new approach to the management of cancer patients in the future.

The purpose of the present study was to elucidate the capability of noncoated charcoal to adsorb AIS purified from the plasma of advanced gynecological patients using immunodetection and to assess the effect of cyclic plasma perfusion in vivo using a cachectic animal model to improve cancer anemia.

MATERIALS AND METHODS

Experimental Animals. Tsukuba Animal Research Laboratories Co. (Ibaragi, Japan) supplied male Japanese White rabbits transplanted with VX2 carcinoma in the muscles of their right thigh. When the VX2 carcinoma in the right thigh of the rabbits grew to more than 5 cm in diameter, it was resected, and a cell suspension (1 × 10^5 cells in 1 ml of 0.15 M NaCl) was transplanted i.m. into the right thigh of normal rabbits weighing about 3 kg. Blood sampling was performed every 10 days after transplantation. These rabbits, which were bred in our own colony, were given access to standard chip feed (CR-3; Clea, Tokyo, Japan) and water ad libitum, following the guidelines of our care and use committee.

Measurement of the Osmotic Pressure at which RBCs Started to Lyse. Heparinized blood was centrifuged at 60 × g for 10 min to prepare packed RBCs and plasma. Packed RBCs

Received 3/25/99; revised 6/7/99; accepted 6/7/99.
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1 This work was supported by the Osaka Medical Research Foundation for Incurable Diseases.

2 To whom requests for reprints should be addressed, at Department of Obstetrics and Gynecology, Osaka City University Medical School, 1-4-3, Asahimachi, Abeno-ku, Osaka 545-8585, Japan. Phone: 81-6-6645-3862; Fax: 81-6-6646-5800; E-mail: ishikoo@msic.med.osaka-cu.ac.jp.

3 The abbreviations used are: AIS, anemia-inducing substance; QOL, quality of life; PHA, phytohemagglutinin; CPC, coil planet centrifuge; Hb, hemoglobin; IL, interleukin.
(250 μl) from a healthy rabbit and plasma (500 μl) from an experimental rabbit were mixed and incubated for 30 min at 37°C. After incubation, the osmotic pressure at which RBCs started to lyse in a CPC coil (Sanki Engineering, Ltd., Kyoto, Japan) was measured as described elsewhere (4). The CPC possesses a rotating coil holder for coiled polyethylene tubes that are subjected to centrifugal force during rotation. RBCs were placed in the area with the highest osmotic pressure (150 mosmol/kg) in a gradient of NaCl in one of the tubes (inside diameter, 0.7 mm; 240 cm long), and the cells were impelled to move toward the area with the lowest osmotic pressure (30 mosmol/kg) by centrifugal force at 300 × g for 15 min.

**Measurement of RBC Fragility-inducing Activity.** The same volume of PBS was added to packed RBCs from a healthy human or rabbit. The specimen was then incubated at 37°C for 30 min, and the osmotic pressure at which the RBCs started to lyse was determined (Fig. 1A). A certain volume of the sample was then added to PBS, and the pressure at the start of hemolysis was determined by the same procedure (Fig. 1B). The value of the osmotic pressure at the start of hemolysis in PBS was subtracted from the value in PBS containing the samples and recorded as RBC fragility-inducing activity (1).

**Measurement of PHA-induced Lymphocyte Proliferation.** Heparinized blood was centrifuged in a Leukoprep tube (Becton Dickinson, Oxford, CA) to obtain lymphocytes. The lymphocytes (2 × 10^5 cells/ml) were incubated in a multiwell plastic dish with 200 μl of RPMI 1640 containing 10% fetal bovine serum and 2 μg of PHA to which plasma (20 μl) from an experimental rabbit or PBS (as a control) was added. After 72 h of incubation in a 5% CO₂ incubator at 37°C, 0.5 μCi of [³H]thymidine in 10 μl of RPMI 1640 was added, and the incubation was continued for 4 h. Uptake of [³H]thymidine into the lymphocytes was measured by harvesting the cells on a glass filter and counting the scintillation rate in scintillation liquid (Emulsifier-Safe; Packard Instruments B.V., Groningen, the Netherlands). The effect of plasma from various experimental rabbits on lymphocyte proliferation stimulated by PHA was expressed as follows: the percentage of inhibition = [(cpm after incubation with PBS − cpm after incubation with the plasma)/cpm after incubation with PBS] × 100.

**Method of Plasma Perfusion.** Under i.v. anesthesia with pentobarbital sodium (25 mg/kg), Teflon catheters (24-gauge Insight; Terumo Co., Tokyo, Japan) were inserted into the rabbit’s auricular artery and vein, and blood flowing out of the artery at about 5 ml/min was separated with a membrane-type plasma separator (pore size, 0.2 μm; membrane area, 0.1 m²; Asahi Medical, Tokyo, Japan). The separated plasma was then passed through an adsorptive resin column containing non-coated charcoal as a base material at a flow rate of about 3 ml/min, after which the cellular component and plasma component were mixed back together and returned to the rabbit’s auricular vein. The quantity of plasma perfused each time was equivalent to the rabbit’s total plasma volume (the total circulating plasma volume of each rabbit was calculated as 38.8 ml/kg of the animal’s body weight.) Plasma perfusion was performed twice a week beginning 40 days after the transplantation of VX2 carcinoma.

**Gel Electrophoresis.** Gels were prepared according to the method of Laemmli (5) and consisted of a 5% stacking gel and a 15% resolving gel (reducing conditions). Samples were loaded at 15–20 μg/lane. Bands were visualized by staining with Coomassie Brilliant Blue R-250. Samples were prepared for reducing conditions by heating for 5 min at 100°C in 0.0625 ml Tris-HCl (pH 6.8), 10% glycerol, 1% SDS, 0.01% bromphenol blue, and 5% 2-mercaptoethanol. For immunoblotting, the gels were transferred to polyvinylidene difluoride transfer mem-

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**Fig. 1** Hemolytic pattern in the measurement of osmotic resistance of RBC membranes by the CPC method. A, control; B, AIS (0.2 μg/dl). Arrows indicate the starting point of hemolysis. Elevation of RBC fragility-inducing activity is defined as the value obtained by subtracting the value of the hemolytic starting point in A from that in B (in mosmol/kg).
branes that had been blocked with 5% dried skimmed milk in 0.15% Tween 20 in PBS at 4°C overnight. The membranes were washed once for 15 min and twice for 5 min in 0.5% Tween 20 in PBS at room temperature. Immunodetection was performed using polyclonal antiserum for AIS (10 μg/ml) prepared as described previously (2) in 1.5% dried skimmed milk-0.15% Tween 20 in PBS for 1 h at room temperature. After being washed three times as described above, the filters were incubated for 1 h with a horseradish peroxidase conjugate at a 1:1000 dilution, followed by one 15-min wash and four 5-min washes with 0.5% Tween 20 in PBS. The enhanced chemiluminescence detection system was used, and the blots were suspended in equal volumes of detection reagents 1 and 2 using 0.125 ml/cm² for 1 min at room temperature and then wrapped in Saran Wrap. The blots were exposed to autoradiography film (Hyperfilm-enhanced chemiluminescence) for 30 s–1 min, depending on the amount of target protein.

Phenyl-Sepharose Column Chromatography of Plasma. Blood was centrifuged at 1000 × g for 10 min to obtain 10 ml of plasma from each rabbit. The plasma was diluted 2-fold in 10 mM phosphate buffer (pH 7.4) 1.5 M NaCl, 0.1 mM EDTA, and 0.1 mM DTT (final concentrations), and the mixture was added to a phenyl-Sepharose CL-4B column (1.5 × 5 cm; Pharmacia Fine Chemicals, Uppsala, Sweden) and equilibrated with 10 mM phosphate buffer (pH 7.4) containing 1.5 M NaCl, 0.1 mM EDTA, and 0.1 mM DTT. After the nonadsorbable fraction passed through, the NaCl concentration of the buffer was gradually lowered from 1.5 M to 0 M. Eluted fractions were dialyzed against PBS [0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4)] and tested to determine whether they could change the osmotic pressure at which RBCs started to lyse in a CPC coil with a high-to-low NaCl gradient (2).

The samples from VX2 carcinoma were prepared according to the method described previously (2) and separated using phenyl-Sepharose column chromatography under the same conditions as described above.

Data Analysis. Student’s t test was used to analyze the data for significant differences, and differences were considered statistically significant when the P < 0.05. All data are expressed as the means ± SD.

RESULTS

Influence of Human AIS on RBCs and Lymphocytes and the Effectiveness of the Noncoated Charcoal Column. Human AIS isolated from the plasma of a patient with an advanced gynecological tumor was purified by affinity chromatography using rabbit AIS antibody. AIS cytotoxicity for human RBCs and lymphocytes was confirmed with purified human AIS. Packed RBCs from a healthy subject and the same volume of PBS supplemented with 0.2 μg/dl human AIS were incubated at 37°C for 30 min, and testing for RBC fragility-inducing activity yielded a value of 7.5 ± 0.2 mosmol/kg. When the AIS solution was eluted through a noncoated charcoal column, the RBC fragility-inducing activity was lost (Fig. 2A). A similar experiment on lymphocytes increased the inhibition of lymphocyte proliferation to 24.9 ± 3.0%, and this activity was lost again when eluted through a noncoated charcoal column (Fig. 2B).

Removal of AIS from Patients’ Serum by Adsorption with Noncoated Charcoal in Vitro. Serum samples were obtained from patients with cancer cachexia, separated by DEAE-Sepharose, and resuspended in 0.3 M NaCl solution. SDS-PAGE and Western blotting of these samples are shown in Fig. 3. Bands were detected at about M₆ 50,000 in the serum from the patients with advanced ovarian cancer, but not in the serum from patients with benign ovarian cysts, and these bands were diminished in the serum treated with noncoated charcoal (Fig. 3B). These results demonstrate that the AIS was adsorbed by noncoated charcoal.

Changes in RBCs and Lymphocytes Caused by Tumor Proliferation in VX2 Carcinoma-bearing Rabbits. Compared with the values obtained before transplantation, there were no changes in the Hb or osmotic pressure at which RBCs lysed on day 20 after transplantation. The lymphocyte stimulation index decreased by 30.4% but was within the normal range. The Hb value on day 40 was 9.1 g/dl, indicating severe anemia, whereas the mean Hb value of normal rabbits was 14.5 ± 1.4 g/dl. The osmotic pressure at which RBCs lysed was 137 mosmol/kg, and enhanced RBC fragility was observed. The lymphocyte stimulation index was 8.8, showing that immunocompetent cell function was suppressed (Table 1). Because these observations were identical to the findings in patients with cancer cachexia, VX2 carcinoma-bearing rabbits were used in the subsequent experiments on day 40 after transplantation.
RBC Fragility-inducing Activity in VX2 Carcinoma Extract and Removal of the Activity with Noncoated Charcoal in Vitro. When samples obtained from VX2 carcinoma homogenate were separated using phenyl-Sepharose column chromatography, the fraction eluted at the concentration of 0.5 M NaCl both in human ovarian cancer and in VX2 carcinoma-bearing rabbits’ plasma had RBC fragility-inducing activity. The activity of this fraction was diminished after samples were applied to the noncoated charcoal column before separation (Fig. 4, A and B).

Changes in RBC Fragility-inducing Activity and Suppression of Lymphocyte Blast Formation in Plasma during Plasma Perfusion in VX2 Carcinoma-bearing Rabbits. Plasma perfusion was performed under conditions that allowed 300 ml of rabbit blood to be treated per hour. RBC fragility-inducing activity and suppression of lymphocyte blast formation were used to assess the results. Measurements were made with blood sampled every 10 min, and the results showed that RBC fragility-inducing activity decreased slowly in a time-dependent manner until 30 min after the start of the experiment and then fell sharply to 9 ± 5 mosmol/kg and was lost at 50 min. Suppression of lymphocyte blast formation was restored to within the normal range 30 min after the start of the experiments (Table 2). These results showed that the optimal duration of plasma perfusion is 60 min. When phenyl-Sepharose column chromatography of rabbit plasma was performed before and after plasma perfusion by the same procedure used to purify human AIS, AIS activity was detected in the fractions corresponding to those in which human AIS was eluted. It was confirmed that the AIS activity in these fractions was lost as a result of plasma perfusion (Fig. 4, C and D).

Changes in AIS Activity Using Changes in RBC Fragility-inducing Activity by Cyclic Plasma Perfusion as an Index. Plasma AIS activity kinetics in response to cyclic plasma perfusion were investigated. As shown in Table 2, AIS

Table 1  Changes in Hb, the osmotic pressure at which RBCs lyse, and the lymphocyte stimulation index during tumor growth in VX-2 carcinoma-bearing rabbits

<table>
<thead>
<tr>
<th>DATa (cm)</th>
<th>Hb (g/dl)</th>
<th>OP at which RBCs lyse (mosmol/kg)</th>
<th>Lymphocyte SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.8 ± 1.7</td>
<td>105 ± 5</td>
<td>24.6 ± 4.2</td>
</tr>
<tr>
<td>20</td>
<td>7.2 ± 1.8</td>
<td>137 ± 11b</td>
<td>8.8 ± 8.6b</td>
</tr>
<tr>
<td>40</td>
<td>13.9 ± 1.3</td>
<td>108 ± 6</td>
<td>17.1 ± 4.0</td>
</tr>
</tbody>
</table>

a DAT, days after transplantation; OP, osmotic pressure; SI, stimulation index.

b P < 0.01 compared with the 20 DAT group (n = 5).

Fig. 3 SDS-PAGE (A) and Western blots (B) after a 15% SDS-PAGE using a polyclonal antibody to human AIS. Samples were obtained from a precipitation of the patients’ serum with DEAE-Sepharose. Lane 0, molecular weight markers; Lanes 1 and 2, advanced ovarian carcinoma; Lane 3, benign ovarian cyst; Lanes 4 and 5, samples (same as those in Lanes 1 and 2, respectively) treated with noncoated charcoal.

Fig. 4 Phenyl-Sepharose column chromatography of VX2 carcinoma extract and plasma from rabbits with VX2 carcinoma. A, untreated tumor extract. B, tumor extract treated using a noncoated charcoal column. C, plasma from the untreated rabbits. D, plasma from rabbits treated by plasma perfusion. The fractions indicated by arrows were assayed for their effect on AIS activity (expressed in the top graphs). In the bottom graphs, protein concentrations are represented by the curved line, and NaCl concentrations are represented by the straight line.
activity was lost as a result of a 60-min plasma perfusion. However, it rose to 23.8 ± 3.6 mosmol/kg on day 2 after perfusion and returned to active preperfusion levels on day 3. Cyclic plasma perfusion was therefore performed on days 3, 6, and 9, resulting in a peak value of AIS activity of 12.0 ± 2.8 mosmol/kg on day 6 and 2.1 ± 0.4 mosmol/kg on day 9. Finally, no AIS activity was detected on or after the twelfth day or the fourth cyclic plasma perfusion (Fig. 5). The Hb concentration of the rabbits undergoing cyclic plasma perfusion started to increase on day 9 after the start of perfusion, increased to 12.1 ± 0.9 g/dl on day 15, and showed no remarkable change afterward.

**DISCUSSION**

There have been many reports on studies to clarify the pathogenesis of cancer cachexia. In particular, vigorous attempts have been made to identify the substance responsible for cancer cachexia, and the topic is as an attractive subject for investigators dealing with cancer. Nakahara and Fukuoka (6) proposed the toxohormone secreted by cancer tissue as the responsible substance. Similarly, studies focusing on cytokines, tumor necrosis factor/cachectin, IL-1, and IL-6 have been performed (7–15). Meanwhile, Todorov et al. (16) and Hirai et al. (17) discovered lipid mobilizing factor in the urine of cancer patients, which has the ability to stimulate glycerol release from freshly isolated murine epididymal adipocytes, and successfully purified it. However, the substance responsible for cancer cachexia has not been identified to date (18–20).

In 1987, we reported the existence of AIS, a substance that made RBCs osmotically fragile and decreased RBC deformability, in the plasma of a patient with terminal ovarian carcinoma. Subsequent studies showed that AIS binds to the cell membrane of RBCs and lowers glucose influx and pyruvate kinase activity, thereby causing RBC dysfunction and ultimately leading to RBC lysis and the development of cancer anemia (1). Anti-AIS antibody was prepared by immunizing rabbits with AIS, and plasma AIS was obtained from patients with various cancers. The results showed that AIS is present only in advanced cancer patients and that it is secreted by tumor tissue. It was also found to suppress cellular immunity. These observations led us to conclude that AIS is one of the substances responsible for cancer cachexia (1). We subsequently succeeded in isolating AIS from the plasma of an advanced cancer patient (2).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>RBC fragility-inducing activity (mosmol/kg)</th>
<th>Suppression of blast formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40 ± 9</td>
<td>51.4 ± 3.3</td>
</tr>
<tr>
<td>10</td>
<td>36 ± 8</td>
<td>49.8 ± 2.9</td>
</tr>
<tr>
<td>20</td>
<td>22 ± 4a</td>
<td>38.1 ± 4.0a</td>
</tr>
<tr>
<td>30</td>
<td>9 ± 5b</td>
<td>22.5 ± 5.2b</td>
</tr>
<tr>
<td>40</td>
<td>5 ± 3b</td>
<td>21.8 ± 4.7b</td>
</tr>
<tr>
<td>50</td>
<td>0 ± 1b</td>
<td>18.2 ± 6.3b</td>
</tr>
<tr>
<td>60</td>
<td>0 ± 1b</td>
<td>15.2 ± 5.9b</td>
</tr>
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</table>

*P < 0.05 compared with the Time 0 group.

**Fig. 5** Changes in RBC fragility-inducing activity in plasma by plasma perfusion. ○, RBC fragility-inducing activity after a single plasma perfusion (n = 3). ●, RBC fragility-inducing activity after cyclic plasma perfusion (n = 5). Each arrow indicates a single plasma perfusion. Values are expressed as the means ± SD. a, P < 0.01 compared with the value before plasma perfusion. b, P < 0.001 compared with the value before plasma perfusion.

Based on the results of this series of investigations, a fundamental study was carried out to find a way to remove AIS from tumor-bearing subjects. The previously reported results (3) of *in vitro* experiments on plasma from advanced cancer patients showed that the increased RBC fragility-inducing activity and elevated suppression of lymphocyte blast formation in plasma returned to levels within the normal range after plasma perfusion through noncoated charcoal. However, because AIS had not yet been successfully isolated, there was no confirmation that the normalization of RBC and lymphocyte functions was due to the adsorption of AIS by noncoated charcoal.

In the first study to demonstrate high but nonspecific adsorption of AIS by noncoated charcoal using isolated human AIS (Fig. 2) and serum from patients with cancer cachexia (Fig. 3), we examined the effects of plasma perfusion with noncoated charcoal and the optimal conditions for performing it using VX2 carcinoma-bearing rabbits as an animal model of cachexia. A single 60-min plasma perfusion was shown to be fully effective in removing AIS (Table 2). Although no AIS activity was detected at 24 h after a single plasma perfusion, AIS activity began increasing again on day 2, reaching 23.8 ± 3.6 mosmol/kg, and returned to the preperfusion level on day 3.

This was expected because AIS is produced by tumor tissue. We therefore repeated the perfusion on day 3, which decreased the peak AIS activity to 12.0 ± 2.8 mosmol/kg. Perfusion again on day 6 produced a greater reduction in the AIS peak to 2.1 ± 0.4 mosmol/kg, and, ultimately, no AIS activity was detected from the fourth perfusion onward (Fig. 5). These results revealed that a 60-min perfusion every 3 days in a tumor-bearing rabbit model is the optimal condition for effective cyclic plasma perfusion.

Although it is certain that human AIS has a strong affinity for noncoated charcoal, the question arises as to whether noncoated charcoal nonspecifically absorbs other similar biological active substances in the experimental system using VX2 carci-

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Table 2: Changes in AIS activity for RBCs and lymphocytes during plasma perfusion of VX2 carcinoma-bearing rabbits

<table>
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</table>

* P < 0.05 compared with the Time 0 group.

b P < 0.01 compared with the Time 0 group (n = 5).
noma-bearing rabbits. When purifying AIS from rabbit plasma by phenyl-Sepharose column chromatography using the same procedure as described for human AIS purification, AIS activity was detected in fractions corresponding to those in which human AIS was found, and it was no longer detected after plasma perfusion (Fig. 4). We previously measured the rate of plasma protein adsorption by noncoated charcoal and found a relatively high adsorption rate for $a_1$-macroglobulin; however, the rate was in proportion to the adsorption rate for total protein (12%). Cytokines, such as tumor necrosis factor, IL-1, and IL-6, showed very little adsorption (5.5%, 7.45%, and 6.8%, respectively). Although these observations are insufficient to answer this question, the biological activity of rabbit AIS was clearly eliminated by cyclic plasma perfusion, as seen for human AIS.

In a previous study concerning the effects of plasma perfusion on VX2 carcinooma-bearing rabbits, cyclic plasma perfusion prevented body weight loss immediately after the start of treatment, and body weight started to increase after 3 weeks of treatment. Cyclic plasma perfusion also improved immune dysfunction and prolonged survival without significant side effects (21). In another study, AIS was found to have the ability to induce lipolysis, and cyclic plasma perfusion decreased this activity of plasma in VX2 carcinooma-bearing rabbits (22). These findings suggest that cyclic plasma perfusion could improve the poor condition of advanced tumor-bearing subjects.

Although advances have been made in studies on the origin of cancer cachexia, investigations on therapeutic modalities based on etiology are almost nonexistent. Recently, management aimed at improving the condition of terminal cancer patients has been directed toward en-

ACKNOWLEDGMENTS

We acknowledge the technical aid of Drs. Shinichi Nakata, Tomoyo Yasui, and Hiroyuki Yoshida.

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