Potent Growth Inhibition of Human Tumor Cells in Culture by Arginine Deiminase Purified from a Culture Medium of a Mycoplasma-infected Cell Line

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ABSTRACT

Two kinds of growth-inhibitory substances were found in culture of a Rous sarcoma virus-transformed rat liver cell line, RSV-BRL. The two substances were purified from the serum-free culture medium and identified as transforming growth factor $\beta_1$ and Mycoplasma-derived arginine deiminase (EC 3.5.3.6), respectively. The arginine deiminase was an acid-labile but diithiothreitol-resistant protein with a molecular weight of 45,000 and $pI$ 4.7. Its $K_m$ value for $l$-arginine was 0.3 mM, which is about 30 times lower than that of bovine liver arginase. It was stable and active under culture conditions. When added into cultures, the arginine deiminase inhibited the growth of various human cancer cell lines at a dose of 5 ng/ml or higher by depleting $l$-arginine in the culture media. This effective dose was about 1000 times lower than that of bovine liver arginase. These results suggested the possibility of chemotherapeutic use of arginine deiminase for human cancers.

INTRODUCTION

Growth of animal cells is regulated by a variety of environmental factors such as growth factors, growth inhibitors, hormones, and nutrients. During the last few years, various types of growth-inhibitory proteins have been found in tissue extracts, body fluids, and culture media, indicating that negative growth regulators may be involved in control of cell proliferation (reviewed in Ref. 1). These studies seem to be important not only in clarifying the growth control mechanism of animal cells but also in developing new antimutual drugs.

We have been investigating growth-inhibitory substances using the nonmalignant rat liver cell line BRL and Rous sarcoma virus-transformed BRL (RSV-BRL) as the indicator cells (2). It was found previously that sera from rats and mice contain a growth inhibitor that preferentially acts on BRL cells, whereas rabbit serum has a growth inhibitor that preferentially acts on RSV-BRL cells (3). The growth inhibitor for the malignant cells has been partially purified from rabbit serum (4). Furthermore, it was recently found that the nonmalignant and malignant indicator cells (BRL and RSV-BRL) themselves secrete growth inhibitors into culture media (2, 5).

In the present study we attempted to characterize the growth-inhibitory substances present in the culture medium of RSV-BRL cells and found that one of the growth-inhibitory substances was Mycoplasma-derived arginine deiminase. This enzyme potently inhibited the growth of both BRL and RSV-BRL cells by causing arginine deficiency in their culture media. L-Arginine is a critical nutrient for the cultures of most types of mammalian cells. A similar arginine-degrading enzyme, arginase, is well known to exert growth-inhibitory activity on various cultured cells (6-8). Arginase can retard the growth of some experimental tumors in vivo (9, 10). To test the possibility of the arginine deiminase as an antitumor drug, its growth-inhibitory activity on various human cancer cells was also examined in vitro.

MATERIALS AND METHODS

Cells and Culture. The nontumorigenic epithelial cell line BRL, which had been established from liver cells of a normal adult Buffalo rat by H. Coon (11), was previously transformed by Rous sarcoma virus (12). From the culture of the transformed BRL, named RSV-BRL, five tumorigenic clones (RSV-BRL1 to RSV-BRL5) were obtained. RSV-BRL1 was used in this study. rasNH3T3 was established by transfecting Kirsten murine sarcoma virus DNA (a gift from Dr. M. Yutsudo, Osaka University) into NIH3T3 cells. The cell lines HLE, HSC-4, T98G, RPMI-8226, and VMRC were provided from the Japanese Cancer Research Resource Bank. CaSki and C4I were purchased from Dainippon Seiyaku, Osaka, Japan. NIH3T3 was a kind gift from the late Dr. T. Kakunaga, Osaka University; BRL and SCC were from Dr. K. Nishikawa, Kanazawa Medical University; YH-1 and B-32 were from Dr. S. Gotoh, University of Occupational and Environmental Health; and A549 and KB were from Dr. N. Miwa, Hiroshima Prefecture University.

These cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The basal medium (DME/F12) 4 consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) and Ham's F-12 medium (GIBCO), which was supplemented with 15 mM N-2-hydroxyethylpiperazine- N'-ethanesulfonic acid, 1.2 mg/ml of NaHCO₃, 100 units/ml of penicillin G, and 0.1 mg/ml of streptomycin sulfate. The standard culture medium was 10% FCS plus DME/F12. Plastic culture wares were obtained from Becton Dickinson Labware (Oxnard, CA).

Assay of Growth Inhibitor Activity. The activity of growth inhibitors was routinely assayed with BRL as the indicator cells as described previously (3). In some experiments, RSV-BRL and other cell lines were used as the indicator cells. Unless otherwise noted, the indicator cells (2500/well) were seeded in duplicate on 24-well plates containing 0.5 ml/well of 10% FCS plus DME/F12 and incubated for 2-4 h to allow cell attachment. The cultures were then added with a small volume (5 to 25 μl) of test samples and further incubated. After 4 days in culture, the grown cells were counted with an automatic cell counter (Sysmex microcell counter CC-108; Kagakawa). In most cases, test samples were previously dialyzed against PBS (Ca²⁺- and Mg²⁺-free phosphate-buffered saline) and sterilized by filtration. In control cultures, which were added with the same volume of PBS, the number of BRL cells increased 100- to 150-fold during the incubation. The ratio (x100) of the number of cells in a test culture to the averaged number of cells in control cultures was expressed as "relative cell number."
solution in a final volume of 0.5 ml. The mixture was incubated at 37°C for 3 h unless otherwise indicated, and the enzyme reaction was terminated by adding 0.25 ml of a mixture of H₂SO₄ and H₃PO₄ (1:3, v/v). The citrulline formed during the incubation was determined with diacetyl monoxime according to the method of Oginsky (13).

Preparation of Conditioned Medium. RSV-BRL cells were grown to confluence in 10% FCS plus DME/F12. The cultures were then rinsed twice with PBS and incubated in serum-free DME/F12 overnight. The media were discarded and replaced with fresh serum-free DME/F12, and the cultures were continued. The serum-free conditioned media were harvested three times a week and clarified by centrifugation at 800 x g for 15 min and at 20,000 x g for 30 min. The protein present in the clarified conditioned media was precipitated by 80% saturation of ammonium sulfate and collected by centrifugation at 20,000 x g for 30 min. The protein precipitates were dissolved in and dialyzed against 10 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl for the purification of growth inhibitors.

Column Chromatographies. Molecular sieve chromatography was carried out on a Cellulofine GCL-2000 column (2.6 x 98 cm; Bio-Rad Laboratories, Richmond, CA), preequilibrated with 10 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl. The concentrated conditioned medium of RSV-BRL cells was applied to the column and eluted with the same buffer at a flow rate of about 30 ml/h.

Heparin affinity chromatography was carried out on a heparinagarose column (1.5 x 8.5 cm; Bio-Rad Laboratories, Richmond, CA), preequilibrated with 10 mM Tris-HCl buffer (pH 7.5). The GI-II pool from the molecular sieve chromatography was dialyzed against the same buffer and applied to the column. The charged column was washed with the buffer and eluted with a linear gradient of NaCl from 0 to 0.1 M in 400 ml of 10 mM Tris-HCl (pH 7.5) at a flow rate of 16 ml/h.

Cibacron Blue affinity chromatography was carried out on a Blue-Cellulofine column (1.0 x 5.0 cm; Chisso Co.) preequilibrated with 10 mM Tris-HCl buffer (pH 7.5). The GI-II pool obtained by heparin-agarose chromatography was applied to the column at a flow rate of 14 ml/h, and the adsorbed material was eluted with the buffer supplemented with 2 M NaCl and 6 M urea.

Hydrogen-bond chromatography was carried out on a Sepharose CL-6B column (1.0 x 2.5 cm; Pharmacia LKB Biotechnology, Uppsala, Sweden), preequilibrated with 25 mM Tris-HCl (pH 7.5) containing 2 M ammonium sulfate, according to the method of Fujita et al. (14). The GI-II pool obtained by Blue-Cellulofine chromatography was supplemented with solid ammonium sulfate to 2 M and applied to the Sepharose column. The charged column was washed with the equilibration buffer and eluted with a linear gradient of ammonium sulfate from 2 M to 0 M in 140 ml of 25 mM Tris-HCl (pH 7.5) at a flow rate of 14 ml/h.

Determination of Protein Concentrations. Protein concentrations were determined by the dye method with a Bio-Rad Protein Assay Kit, using bovine serum albumin as the standard. Protein contents of purified growth inhibitors were estimated from the intensities of silver-stained protein bands on SDS-PAGE using the same standard protein as above.

Detection and Elimination of Mycoplasma. Mycoplasma contamination was tested with the bisbenzimidazole fluorochrome Hoechst 33258. Contaminating Mycoplasma was eliminated by treating the host cells twice with 1 μg/ml of an antibiotic, MC-210 (Dainippon Seiyaku) for 7 days.

RESULTS

Growth-inhibitory Substances Present in Conditioned Medium of RSV-BRL Cells. RSV-BRL cells were grown in serum-containing medium to reach confluence and then incubated in serum-free basal medium (DME/F12) for 2 days. The culture medium was collected and concentrated by ammonium sulfate precipitation. When the concentrated conditioned medium was added into cultures of BRL and RSV-BRL cells, their growth was inhibited dose dependently. To characterize the growth-inhibitory factors present in the conditioned medium, the conditioned medium was fractionated by molecular sieve chromatography. The resultant fractions were dialyzed against PBS and assayed for growth-inhibitory activity with BRL and RSV-BRL cells (Fig. 1A). The growth-inhibitory activity was separated into a major peak at an apparent molecular weight of 45,000 (fractions 52–59) and a minor peak at the void volume (fractions 32–34). Two additional activities were detected as shoulders in molecular weight ranges of 300,000–700,000 (fractions 42–50) and 10,000–40,000 (fractions 60–68). The large and small peak fractions respectively inhibited the growth of BRL and RSV-BRL cells to similar extents, while the two shoulder fractions were more inhibitory for BRL than RSV-BRL cells.

When small portions of the column fractions were incubated

![Fig. 1. Molecular sieve chromatography of concentrated conditioned medium of RSV-BRL cells on a Cellulofine GCL-2000 column. A, relative number of BRL cells; F, relative number of RSV-BRL cells; C, A280. The elution positions of thyroglobulin (M, 670,000), γ-globulin (M, 158,000), ovalbumin (M, 43,000), myoglobin (M, 17,000), and vitamin B₁₂ (M, 1,350) are indicated by arrows. (A) Growth-inhibitory activity was assayed at a dose of 100 μl/35-mm dish (total, 2 ml) after dialysis against PBS at 4°C overnight. (B) Growth-inhibitory activity was assayed at a dose of 5 μl after dialysis against 1 M acetic acid (pH 2.3) at 4°C overnight. Fractions 42–50 (GI-I) and fractions 52–59 (GI-II) were respectively pooled and used for further purification. Other experimental conditions are given in the text.](https://cancerres.aacrjournals.org)
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with 1 M acetic acid and then assayed, the growth-inhibitory activity at $M_\text{r}$ 300,000–700,000 increased about 100-fold to be the major activity, indicating that this growth inhibitor had been secreted as a latent or less active form into the culture medium (Fig. 1B). The major growth inhibitors in fractions 42–50 and 52–59 were tentatively designated as GI-I and GI-II, respectively.

GI-I present in fractions 42–50 was extracted with 1 M acetic acid and further purified by preparative SDS-PAGE under nonreducing conditions and then by isoelectric electrophoresis on a thin-layer polyacrylamide gel. When analyzed by SDS-PAGE, the purified inhibitor showed a homogeneous band with a molecular weight of 25,000 under nonreducing conditions or 13,000 under reducing conditions (data not shown). Its $pI$ was about 9.2 as determined by polyacrylamide gel isoelectric electrophoresis. About 11 μg of GI-I were obtained from 6 liters of the conditioned medium. Analysis of the partial NH$_2$-terminal amino acid sequence has shown that GI-I must be TGF-β1.

Purification of GI-II. The GI-II fractions obtained by the molecular sieve chromatography (fractions 52–59 in Fig. 1A) were pooled, dialyzed against 10 mM Tris-HCl (pH 7.5), and subjected to affinity chromatography on a heparin-agarose column. The growth-inhibitory activity was weakly adsorbed to the column and eluted at about 0.06 M NaCl. The active fractions from the heparin-agarose column were pooled and then applied to a Cibacron blue-conjugated column (Blue-Cel lulofine column). GI-II activity passed through the column without adsorption, although about 70% of the total protein was adsorbed to the column (data not shown). The nonadsorbed fractions were pooled, added with solid ammonium sulfate to make a final concentration of 2 M and then subjected to hydrogen bond chromatography on a Sepharose CL-6B column (Fig. 2). GI-II was adsorbed to the column at 2 M ammonium sulfate and eluted from the column by decreasing the ammonium sulfate concentration to about 1.4 M. The active fractions were pooled, dialyzed against 10 mM Tris-HCl (pH 7.5), and subjected to isoelectric electrophoresis in a sucrose gradient column (Fig. 3). The growth inhibitor was focused at pH 4.7. GI-II thus purified was analyzed by SDS-PAGE under nonreducing conditions (Fig. 4). The GI-II preparation showed a nearly homogeneous band with a molecular weight of 45,000. The electrophoretic mobility of the $M_\text{r}$ 45,000 protein was hardly affected by treatment with 2-mercaptoethanol, showing that it was a single-chain peptide. In this purification procedure about 1 μg of GI-II was purified from 6 liters of the conditioned medium with an activity yield of 8% and with a 1200-fold enrichment.

In another experiment, 100 liters of the serum-free conditioned medium of RSV-BRL cells were used as the starting material to prepare the GI-II protein for structural analysis. In this purification the isoelectric electrophoresis was replaced with anion-exchange chromatography on a DEAE-Sepharose column. The active GI-II fraction obtained from the chromatography was finally subjected to reverse-phase HPLC on an Altex Ultrapore C3 column. Although the acidic conditions of the reverse-phase HPLC inactivated GI-II, about 30 μg of the GI-II $M_\text{r}$ 45,000 protein were purified to homogeneity. The 18 NH$_2$-terminal amino acids of the $M_\text{r}$ 45,000 protein were determined with an automatic gas-phase protein sequencer (Fig. 5). A computer search indicated that the NH$_2$-terminal sequence had no significant homology to any known protein in the National Biomedical Research Foundation protein data
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bank (Release No. 18) and in the Swiss-Pro data bank (Release No. 9.0).

Characterization of GI-II. The growth-inhibitory activities of the purified GI-I (TGF-β 1) and GI-II (fraction 28 in Fig. 3) on BRL and RSV-BRL are shown in Fig. 6. GI-I inhibited the growth of BRL much more potently than RSV-BRL. In most cases the extent of the maximal growth inhibition ranged from 75 to 85% for BRL and from 20 to 40% for RSV-BRL. The dose required for 50% growth inhibition was about 1 ng/ml for both kinds of cells. The effect of GI-II on amino acid composition was about 4 ng/ml for both kinds of cells. The effect of GI-II on these cells appeared cytostatic during the initial 3 days in incubation, and the majority of the cells were alive and could grow normally if the medium was replaced with a fresh one without GI-II. However, prolonged incubation with an excess amount of GI-II increased the number of dead cells. The unique dose-response curve of GI-II suggested some special growth-inhibitory mechanism of this protein.

There are some reports indicating that arginase exerts a growth-inhibitory effect on cultured mammalian cells by converting L-arginine present in the culture media to ornithine (6-8). In addition, Mycoplasma-derived arginine deiminase, which converts L-arginine to citrulline, has been suggested to show a similar growth-inhibitory activity (17, 18). Therefore, the effect of GI-II on amino acid composition was examined under the conditions for the assay of growth-inhibitory activity (Table 1). When GI-II was incubated with BRL cells at 37°C, the arginine content in the medium gradually decreased and became undetectable after 4 days. The decrease of arginine content was accompanied with a reciprocal increase of citrulline content, indicating that the culture contained arginine deiminase. The changes for other amino acids, if any, were far less than that for arginine. As expected, the addition of excess amounts of L-arginine into the culture of BRL cells reversed the growth inhibition caused by GI-II in a dose-dependent manner (Fig. 7). This indicated that the growth inhibition by GI-II was due to the depletion of L-arginine in culture medium. The addition of 1 mM citrulline into culture medium gave no growth-inhibitory effect on the indicator cells (data not shown).

The incubation of GI-II with L-arginine in a cell-free reaction mixture directly proved that this protein had arginine deiminase activity (Table 2). The K_m value of the arginine deiminase for L-arginine was determined to be about 0.3 mM (data not shown). The stability of the arginine deiminase under various conditions is summarized in Table 3. This enzyme is stable at neutral pH but unstable in acid. About 50% of the activity remained after 55% (w/v) trichloroacetic acid at 1 ml of the media, followed by centrifugation. Amino acids in the resultant supernatants were analyzed by a Bio-Rad IR-120 column (0.5 x 5 cm) and then eluted with 3 M NaOH. The eluted amino acid samples were lyophilized and subjected to a PICO-TAG amino acid analyzer (Milipore, Milford, MA). Cit and Orn indicate citrulline and ornithine, respectively.

![Fig. 7. Effect of addition of extra L-arginine on growth-inhibitory activity of purified GI-II on BRL cells. The indicated amounts of L-arginine were added into 0.5 ml of culture medium containing BRL cells and GI-II (2.5 ng). Each point represents the average for duplicate wells; bars, range of values.](image)

### Table 1 Effect of purified GI-II on amino acid composition in culture medium

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Control (None)</th>
<th>GI-II (5 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>0.045 ± 0.020</td>
<td>0.043 ± 0.020</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.035 ± 0.015</td>
<td>0.035 ± 0.015</td>
</tr>
<tr>
<td>Serine</td>
<td>0.020 ± 0.010</td>
<td>0.020 ± 0.010</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.015 ± 0.005</td>
<td>0.015 ± 0.005</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.010 ± 0.005</td>
<td>0.010 ± 0.005</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.005 ± 0.005</td>
<td>0.005 ± 0.005</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.005 ± 0.005</td>
<td>0.005 ± 0.005</td>
</tr>
<tr>
<td>Serine</td>
<td>0.005 ± 0.005</td>
<td>0.005 ± 0.005</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.005 ± 0.005</td>
<td>0.005 ± 0.005</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.005 ± 0.005</td>
<td>0.005 ± 0.005</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.005 ± 0.005</td>
<td>0.005 ± 0.005</td>
</tr>
</tbody>
</table>

### Table 2 Arginine deiminase activity of purified GI-II

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Citrulline formed (nmol/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>260</td>
</tr>
<tr>
<td>20</td>
<td>520</td>
</tr>
</tbody>
</table>

### Table 3 Stability of GI-II (arginine deiminase)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Arginine deiminase activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(100)</td>
</tr>
<tr>
<td>4°C for 1 wk</td>
<td>94</td>
</tr>
<tr>
<td>37°C for 24 h</td>
<td>51</td>
</tr>
<tr>
<td>60°C for 30 min</td>
<td>10</td>
</tr>
<tr>
<td>100°C for 5 min</td>
<td>0</td>
</tr>
<tr>
<td>0.1 M acetate acid (pH 2.8) (4°C for 1 h)</td>
<td>11</td>
</tr>
<tr>
<td>PBS + 50 mM dithiothreitol (4°C for 24 h)</td>
<td>97</td>
</tr>
<tr>
<td>PBS + 6 M urea (4°C for 24 h)</td>
<td>58</td>
</tr>
</tbody>
</table>

* GI-II was dialyzed against 0.1 M acetate acid at 4°C for 24 h.
incubation at 37°C for 24 h, although its activity was mostly lost after incubation at 60°C for 30 min or at 100°C for 5 min. The arginine deiminase was resistant to 50 mM dithiothreitol but partially inactivated by 6 M urea.

The test for Mycoplasma infection revealed the presence of Mycoplasma in the culture of RSV-BRL cells. When the contaminating Mycoplasma organisms were removed from the culture of RSV-BRL cells by the use of an antibiotic MC210, the growth-inhibitory activity in the conditioned medium markedly decreased and the arginine deiminase activity disappeared (Table 4). These results demonstrated that GI-II is Mycoplasma-derived arginine deiminase.

Growth-inhibitory Activity of GI-II (Arginine Deiminase) on Various Cell Lines. The results described above clearly show that the arginine deiminase derived from mycoplasmas contaminating RSV-BRL cells inhibits cell growth by consuming L-arginine in culture medium. To compare its growth-inhibitory activity with that of a similar arginine-degrading enzyme, arginase, commercial bovine liver arginase with a specific activity of 150–250 units/mg protein (Sigma Chemical Company, St. Louis, MO) was tested for growth-inhibitory activity on BRL cells. The arginase inhibited the growth of BRL at doses higher than 5 μg/ml, about 1000 times the effective doses of the arginine deiminase (data not shown). Bovine liver arginase has a $K_m$ of 10.5 mM, about 30 times that of the arginine deiminase, and an optimum pH at 9.3 (19). The marked difference in their effective doses appears to come largely from the difference in their $K_m$ values.

The in vitro growth-inhibitory activity of GI-II was tested with 3 pairs of nontransformed and transformed cell lines and with 11 kinds of human cancer cell lines (Table 5). GI-II nonspecifically inhibited the growth of the nontransformed and transformed BRL and NIH3T3, but it was more inhibitory for SV40-transformed human fibroblast line than its nontransformed counterpart. GI-II more or less inhibited the growth of all human cancer cell lines tested. Among them, HLE (hepatoma), CaSki (cervix squamous cell carcinoma), and VMRC (melanoma) were especially sensitive to the arginine-degrading enzyme.

**DISCUSSION**

In the present study two kinds of growth-inhibitory substances (GI-I and GI-II) were purified from the conditioned medium of RSV-BRL cells and identified as TGF-β and arginine deiminase, respectively. TGF-β is a family of structurally and functionally related growth regulators (reviewed in Refs. 1, 20, and 21). TGF-β is secreted from many types of cultured cells in a latent form (22, 23). RSV-BRL also secreted TGF-β as a latent protein complex with molecular weights of 300,000 to 700,000 into culture medium. The protein complex showed only a slight growth-inhibitory activity, but the treatment with acid increased its activity about 100-fold by liberating the active $M_r$, 25,000 TGF-β, from the complex. The active TGF-β potently inhibited the growth of BRL cells but hardly that of RSV-BRL cells, from which TGF-β had been secreted. We have previously reported similar selective growth inhibition of BRL cells by growth inhibitors prepared from rat serum and human platelets (3, 24).

There is no doubt that TGF-β plays fundamental roles in growth control of cell growth and other various cellular functions. However, it seems hopeless to use TGF-β as an antitumor agent because of its relatively low activity on malignant cells.

Arginine deiminase (EC 3.5.3.6) is abundant in microorganisms such as Mycoplasma, bacteria, and yeast. The arginine deiminases purified from these sources are composed of two identical subunits with a molecular weight of approximately 50,000 (25, 26). The arginine deiminase (GI-II) found in the conditioned medium of RSV-BRL cells was derived from the Mycoplasma contaminating the malignant rat liver cells. The arginine deiminase purified from the culture of RSV-BRL cells appeared to be a single peptide ($M_r$, 43,000), but this should be further confirmed because abnormal elution of proteins is often observed in molecular sieve chromatography.

**Mycoplasma** infection is one of the most important problems in studies on cell culture. Mycoplasmas often affect the metabolism and function of the host cells. It has been reported that the infection of arginine-utilizing mycoplasmas or the addition of their extracts inhibits the growth of mammalian cells in culture (17, 18). These previous studies suggested that arginine deiminase might be the growth-inhibitory principle present in mycoplasmas inasmuch as the growth inhibition by mycoplasmas or their extracts was prevented by adding an excess amount of L-arginine into culture medium. However, growth-inhibitory activity of purified arginine deiminase has not been reported before. The arginine deiminase purified in this study inhibited growth in the presence of 0.5 ml/well of 10% PCS plus DME/F12, and incubated with two different concentrations of GI-II. BRL and RSV-BRL were seeded at a density of 2500 cells/well. Other experimental conditions are given in "Materials and Methods."
the growth of various murine and human cell lines as little as 5 ng/ml.

The use of amino acid-degrading enzymes as antitumor agents is one of the approaches for the treatment of human cancer. The most representative example is asparaginase (27). It has been successfully used for the treatment of lymphoblastic leukemia, leukemic lymphosarcoma, and lymphosarcoma. Arginase is another enzyme that exerts growth-inhibitory or cytotoxic effect on various cultured cells (6–8). This enzyme inhibits cell growth by the same mechanism as arginine deiminase. Bach and Swaine (9) showed that arginase could retard the growth of Walker carcinoma in vivo. Savoca et al. (10) reported that arginase covalently bound with polyethylene glycol, but not native enzyme, effectively extended the survival times of mice given injections of Taper liver tumor cells. The effectiveness of such amino acid-degrading enzymes as antitumor agents depends on their enzymatic properties (Km, optimum pH, specific activity, stability at neutral pH), selectivity on tumor cells, stability in blood circulation, and immunogenicity. The attachment of polyethylene glycol to proteins can reduce their immunogenicity in animals and increase their stability to various hydrolytic enzymes, resulting in the increase of their blood circulatory lives (10, 28).

Our study demonstrated that the in vitro growth-inhibitory dose of the arginine deiminase (GI-II) was about 1000 times lower than that of bovine liver arginase. It is very likely that the great difference in their minimum effective doses derives largely from the difference in their Km values for L-arginine: 0.3 mM with the arginine deiminase and 10.5 mM with the arginase. As shown in Table 5, the growth-inhibitory effect of the arginine deiminase appears specific for neither transformed nor tumorous cells. Although it was more or less growth-inhibitory for all of 11 human cancer cell lines tested, three of them, HLE, CaSki, and VMRC, were specially sensitive to this enzyme. This suggests the chemotherapeutic value of arginine deiminase for some specific kinds of human cancers. We did not identify the type of Mycoplasma which had contaminated RSV-BRL cells. Preliminary studies have shown that the arginine deiminase purified from Mycoplasma arginini has a similar growth-inhibitory activity. The in vitro and in vivo antitumor activities of various kinds of arginine deiminases are currently under investigation.

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