Mechanism of Interferon-γ Action

CHARACTERIZATION OF INDOLEAMINE 2,3-DIOXYGENASE IN CULTURED HUMAN CELLS INDUCED BY INTERFERON-γ AND EVALUATION OF THE ENZYME-MEDIATED Tryptophan DEGRADATION IN ITS ANTICELLULAR ACTIVITY*

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Induction by interferon-γ of indoleamine 2,3-dioxygenase (a tryptophan degradation enzyme) was examined with 11 human cell lines. The enzyme induction was demonstrated in 7 of the 11 cell lines. The induced enzyme in each of the 7 cell lines was identical to the enzyme purified from human placenta, as evidenced by immunoblot analysis with a monoclonal antibody specific to the placental one. The extent of the induction varied largely with the cell line; a relatively high induction was observed with HEL (lung fibroblasts), NY (osteosarcoma), and A-431 (epidermoid carcinoma). The enzyme induction was dependent on the concentration of interferon-γ and occurred 12–18 h after addition of interferon-γ to the cultures. Interferon-α or -β was completely ineffective in this induction.

Interferon-γ inhibited the growth of the 7 cell lines observed with the enzyme induction, and this growth inhibition was accompanied with a complete deletion of tryptophan (<1 μM) in the culture medium by the induction of the enzyme. For two of these cell lines, the inhibition was partially reversed by an addition of exogenous tryptophan to the medium not to be depleted. These findings indicated that the growth inhibition by interferon-γ was in part explained by the tryptophan depletion in the medium caused by the enzyme induction.

Interferon-γ (IFN-γ) produced by activated lymphocytes is a plurifunctional protein with antiviral, antitumor (reviewed in Refs. 1 and 2), antiparasitic (3–8), and immunoregulatory (9) activities. It differs from IFN-α and -β in its structure, cellular origin (reviewed in Ref. 10), and some of its functional properties (6, 8, 11). IFN-γ exerts these biological activities through a binding of the specific receptor in the target cells (12–19). However, the molecular mechanisms which couple the receptor binding with the subsequent functional changes remain obscure, except for implications of the 2′,5′-oligoadenylate synthetase-RNase L system and a protein kinase in the antiviral activity (1, 2). Recently, IFN-γ has been shown to induce a tryptophan degradation enzyme, tryptophan pyrrolase, in some cultured human cells (20–24). This enzyme induction appeared to block the growth of intracellular parasites, Toxoplasma gondii (20) and Chlamydia psittaci (21), by depleting tryptophan in the infected cells.

Now two types of tryptophan pyrrolase are known to be present in mammals, i.e. L-tryptophan 2,3-dioxygenase (EC 1.13.11.11) and indoleamine 2,3-dioxygenase (25). These are distinguishable in organ distribution (25–28), molecular properties (29, 30), and antigenicity (31). The reported properties of the tryptophan pyrrolase induced by IFN-γ in a human cell line were similar to those of indoleamine 2,3-dioxygenase (24). In the present work, we have confirmed that the tryptophan pyrrolase was identical to indoleamine 2,3-dioxygenase purified from the human placenta by immunoblot analysis using a monoclonal antibody specific to the enzyme. Furthermore, we examined whether the enzyme induction by IFN-γ is a general phenomenon observed with various types of human cultured cells and how the enzyme induction, that is the tryptophan degradation, relates to the antitumor activity of IFN-γ. Here, we demonstrate that IFN-γ induces indoleamine 2,3-dioxygenase in 7 of 11 human cultured cell lines and that the enzyme-catalyzed tryptophan degradation facilitates the growth inhibition of some cultured tumor cells by depleting tryptophan in the culture medium. We also describe a procedure of purification of human indoleamine 2,3-dioxygenase from the placenta and its properties in a miniprint following this paper.

EXPERIMENTAL PROCEDURES

Interferons—Highly pure recombinant human IFN-γ (>99% pure) and IFN-β (>99.5% pure) with specific activities of 6.0 × 10⁵ and 1.3 × 10⁶ units/mg, respectively, were generously provided by Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. Natural IFN-α (>99% pure) with specific activity of 7.4 × 10⁵ units/mg was a generous gift of Green Cross, Ltd., Osaka, Japan. The activities of IFNs were assayed with Sindbis virus and FL cells as described previously (32). All IFNs were diluted with PBS (33) containing 0.1% (w/v) BSA.

Materials—The following materials were obtained from the manufacturers indicated in parentheses: nitrocellulose membrane (Toyobo Co., Ltd., Tokyo, Japan), Eagle’s minimum essential medium, RPMI 1640, ascorbic acid, and methylene blue (Wako Pure Chemicals, Osaka, Japan), bovine liver catalase (Type I) and calibration protein II (Boehringer Mannheim), BSA (Miles Laboratories), TSK-120T (ODS type, 5 μm) (Toyo Soda, Tokyo, Japan), poly(ethylene glycol) 4,000 (Merck), Centriflow CF-25 (Amicon), Superose 12
Human Cells and Growth Conditions—HEL cells (Flow 2000) were purchased from Flow Laboratories, Inc. and used between the 28th and 35th day of growth. FL, RPMI-1788, and HeLa cells were generous gifts of Dr. Inoue of the Institute of Pharmacy, Kanebo Co., Ltd., Osaka, Japan. Raji cells were donated by Dr. Shinohara of the Department of Pediatrics in our college. OKK cells were gifts of Dr. Miyamoto of the Department of Pathology in our college. A-431 (ATCC No. CRL 1555), IMR-32 (ATCC No. CCL 127), NY, KATO-III, and HEL cells were obtained from the Japanese Cancer Research Resources Bank. KATO-III and HLEC-1 cells were established by Dr. Sekiguchi et al. (34) and Dr. Doi et al. (35), respectively. The culture mediums were: HEL, FL, HeLa, OKK, NY, KATO-III, and HLEC-1 cells (90% E-MEM and 10% FCS); A-431 cells (90% Dulbecco's modified Eagle's medium and 10% FCS); RPMI-1788 and Raji cells (90% RPMI 1640 and 10% FCS); IMR-32 (80% RPMI 1640 and 20% heat-inactivated FCS). All the culture mediums contained penicillin (100 units/ml) and streptomycin (100 μg/ml). All cells were cultured at 37 °C in humidified air containing 5% CO₂. Viable cells were counted by trypan blue exclusion with a hemocytometer.

Preparation of Cellular Extract—The cultured cells were harvested by a clarification of the supernatant, washed twice with cold PBS. The cells were suspended in 0.5 ml of cold PBS and disrupted by sonication for 15 s in an ice bath at a power of 25 watts with a UP-200P Ultra Sonic Disruptor (Tomy Seiko Co., Ltd., Tokyo, Japan). The homogenate was then centrifuged at 15,000 × g for 20 min at 5 °C. The resultant supernatant (cellular extract) was stored at −80 °C until use.

Assay of Indoleamine 2,3-Dioxygenase—The standard assay mixture (100 μl) contained 50 mM potassium phosphate buffer (pH 6.5), 20 mM ascorbate, 10 mM methylene blue, 100 μg/ml catalase, 400 μM trichloroacetic acid and further incubated at 50 °C for 30 min to hydrolyze N-formylkynurenine produced by indoleamine 2,3-dioxygenase to kynurenine. After centrifugation at 2500 × g for 15 min at 20 °C, kynurenine in the supernatant was measured by a Jasco HPLC system with a reversed phase column (4.6 × 5 mm × 15 cm) of TSK-120T. The mobile phase was 10 mM ammonium acetate containing 10% (w/v) methanol, and kynurenine was detected by absorbance at 360 nm. All determinations were carried out in duplicate. The data presented are average values. One unit of the enzyme activity was defined as the amount that produced 1 nmol of kynurenine/h. Protein was determined by the method of Lowry et al. (36) with BSA as standard.

Assay of Kynurenine Formamidase in Cultured Cells and Medium—The standard assay mixture (0.1 ml) contained 50 mM potassium phosphate buffer (pH 6.5), 1 mM N-formylkynurenine, and 50-50 μl of the cellular extract prepared as described above. The reaction, at 37 °C, was started by the addition of the substrate and terminated after 30 min by adding 20 μM of 30% (w/v) trichloroacetic acid and further incubated at 50 °C for 30 min to hydrolyze N-formylkynurenine produced by indoleamine 2,3-dioxygenase to kynurenine. After centrifugation at 2500 × g for 15 min at 20 °C, kynurenine in the supernatant was measured by a Jasco HPLC system with a reversed phase column (4.6 × 5 mm × 15 cm) of TSK-120T. The mobile phase was 10 mM ammonium acetate containing 10% (w/v) methanol, and kynurenine was detected by absorbance at 360 nm. All determinations were carried out in duplicate. The data presented are average values. One unit of the enzyme activity was defined as the amount that produced 1 nmol of kynurenine/h. Protein was determined by the method of Lowry et al. (36) with BSA as standard.

HPLC for Tryptophan and Kynurenine Metabolites—Tryptophan and kynurenine metabolites (N-formylkynurenine, 3-hydroxykynurenine, anthranilic acid, 3-hydroxanthranilic acid, kynurenic acid, xanthurenic acid, and a-aminopicuric acid) in the culture medium of human cells were determined by HPLC as described previously 20 ml of PBS containing 0.01% (w/v) hydrogen peroxide.

Preparation of Monoclonal Antibodies to Indoleamine 2,3-Dioxygenase—We prepared mAbs essentially according to the method of Kohler and Miletine (39). On day 0, three female BALB/c mice were injected subcutaneously on the back with 10 μg of indoleamine 2,3-dioxygenase purified from human placenta in 0.5 ml of complete Freund's adjuvant. On days 7 and 26, the mice were given intraperitoneally booster injections of 10 and 30 μg, respectively, of the purified enzyme in 0.1 ml of PBS, and three days after the last injection, the spleens were removed. For hybridization, 4.5 × 10⁶ spleen cells were mixed with 10 × 10⁷ mouse myeloma cells (P3-X63-Ag8-U1) and incubated with 3 ml of 50% (w/v) polyethylene glycol 4000 in PBS for 5 min at 25 °C. Then the cells were centrifuged and resuspended in 90 ml of hypoxanthine-aminopterin/thymidine medium supplemented with 10% (v/v) FCS and were distributed in 96-well trays (Falcon 3072). Two hybridomas highly producing mAbs to the enzyme were screened by a direct enzyme-linked immunosorbent assay and cloned by limiting dilution. The mAbs were produced in ascites fluid of female BALB/c mice and purified by ammonium sulfate fractionation and DEAE-cellulose (Whatman DE52) column chromatography. Both mAbs, mAb1 and mAb2, were IgG1, which was shown by Western blotting using the MonoAb-ID EIA KIT according to the manufacturer's instructions. We used mAb1 in this study because mAb1 exhibited a higher affinity to the enzyme than mAb2. mAb1 was highly specific to human indoleamine 2,3-dioxygenase and did not react with human L-tryptophan 2,3-dioxygenase in the liver. Results

Indoleamine 2,3-Dioxygenase Induction by IFN-γ in Various Human Cells—Various human cells including normal and transformed cells were cultured in medium containing IFN-γ at 10³ units/ml for 72 h, and the activity of indoleamine 2,3-dioxygenase in the cells was determined. Of 11 cell lines tested, 7 lines were responsive to IFN-γ and induced the enzyme in the cells. The extent of the induction was relatively high in HEL, NY, and A-431 cells and low in OKK, FL, KATO-III, and HeLa cells. For the negative 4 cell lines, HLEC-1, IMR-32, Raji, and RPMI1788 cells, it was further examined whether the enzyme induction occurred by culturing the cells with a 10-fold higher concentration of IFN-γ (10³ units/ml) for a longer time (up to 144 h), but no induction was observed. Next, the enzyme induction observed in the 7 cell lines was characterized in detail. The induction depended on the concentration of IFN-γ added to the medium (Fig. 1) where HEL, NY, A-431, OKK, and FL cells showed a similar dose dependence, while KATO-III and HeLa cells were less sensitive than the former 5 lines. The induction occurred 12-18 h after the addition of IFN-γ and reached a plateau at around 36-48 h (Fig. 2). However, the culture of the cells with IFN-α or IFN-β at 10-10³ units/ml for 12-144 h in place of IFN-γ was completely ineffective in the enzyme induction. This indicated that the enzyme induction was specific to IFN-γ rather than IFN-α or IFN-β.

Immunoblot Analysis—To further characterize indoleamine 2,3-dioxygenase induced by IFN-γ in the 7 cell lines (Table 1), we carried out immunoblot analysis using mAb1 specific to indoleamine 2,3-dioxygenase from human placenta. When the cellular extract containing the enzyme activity (about 10 units) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane, mAb1 bound to a protein with a Mᵦ of about 40,000 in the extract of each of the cell lines (Fig. 3, lanes 2-8). The protein corresponded to the placental enzyme (Fig. 3, lane 1). For KATO-III, although the protein to which mAb1 bound showed a slightly different migration (Fig. 3, lane 8), it comigrated with the placental enzyme when they were mixed (Fig. 3, lane 9). The different migration was, therefore, caused by...
under "Experimental Procedures." Values are means of duplicate concentrations of IFN-γ. After 72 h of culture, cells were harvested, and the enzyme activity in cellular extract was assayed as described under "Experimental Procedures." Values are means of duplicate dishes.

![FIG. 1. Effect of concentration of IFN-γ on induction of indoleamine 2,3-dioxygenase in human cultured cells.](image)

Various cells (0.5–1.0 × 10⁶ cells) were cultured in a plastic 35-mm diameter dish (Corning 25010) with 2 ml of culture medium containing IFN-γ (10³ units/ml) for 72 h. Indoleamine 2,3-dioxygenase activity in the cells was determined as described under "Experimental Procedures." Values are means of duplicate dishes.

Panel A represents the results of 3 cell lines with a high induction of the enzyme, and Panel B represents the results of 4 cell lines with a low induction of the enzyme.

![FIG. 2. Time course of induction of indoleamine 2,3-dioxygenase by IFN-γ in human cultured cells.](image)

![FIG. 3. Immunoblot analysis of indoleamine 2,3-dioxygenase induced by IFN-γ in human cultured cells.](image)

![TABLE I](image)

**Induction of indoleamine 2,3-dioxygenase by IFN-γ in various human cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Indoleamine 2,3-dioxygenase activity</th>
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<tr>
<td>Normal tissue</td>
<td>HEL</td>
<td>Embryonic lung 837</td>
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<tr>
<td></td>
<td>FL</td>
<td>Amnion 102</td>
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<tr>
<td>Carcinoma</td>
<td>A-431</td>
<td>Epidermis 496</td>
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<tr>
<td></td>
<td>OKK</td>
<td>Maxillary gland 88</td>
</tr>
<tr>
<td></td>
<td>KATO-III</td>
<td>Stomach 80</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>Uterine cervix 35</td>
</tr>
<tr>
<td></td>
<td>HLEC-1</td>
<td>Liver 0</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>NY</td>
<td>Osteosarcoma 455</td>
</tr>
<tr>
<td></td>
<td>IMR-32</td>
<td>Neuroblastoma 0</td>
</tr>
<tr>
<td></td>
<td>Raji</td>
<td>Lymphoma 0</td>
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<td></td>
<td>RPMI1788</td>
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Table 1

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**Contaminated proteins and/or substances in the cellular extract.** The faint bands other than the band with a Mₐ of about 40,000 appearing in lanes 2–9 in Fig. 3 were due to nonspecific bindings of the anti-(mouse IgG) antibody to some proteins in the cellular extracts because they were still seen when the mAb was omitted from the procedure of immunoblot analysis (not shown). These results demonstrated that the enzymes induced by IFN-γ in the 7 cell lines were identical to the placental enzyme.

**Catalytic Properties**—Previous studies (40, 41) have characterized indoleamine 2,3-dioxygenase purified from rabbit intestine as follows: (i) the enzyme is a hemoprotein; (ii) the ferrous (Fe²⁺) enzyme is active for the dioxygenation reaction; (iii) in vitro the ferrous enzyme is rapidly oxidized to the ferric (Fe³⁺) enzyme, which has no enzyme activity; and (iv) the enzyme, therefore, requires a reducing system composed of ascorbic acid and methylene blue to maintain the active ferrous enzyme during catalysis in vitro, where the role of methylene blue is considered to be an electron carrier from ascorbic acid to the ferric enzyme. Similarly, the human
enzyme was a hemoprotein as shown in the Miniprint and required absolutely both ascorbic acid and methylene blue for the enzyme activity in vitro (Fig. 4). Essentially, the same requirement was observed for the enzyme induced by IFN-γ in the human cells, as exemplified with the enzyme in HEL cells (Fig. 4). In addition, the Kₘ values (17.5–24.0 μM) for L-tryptophan of the enzyme in the cells were almost equal to that (21.0 μM) of the placental enzyme; the example observed with the enzyme in HEL cells was shown in Fig. 5. Thus, no significant difference in these catalytic properties was present between the induced enzyme and the placental enzyme.

Effect of the Induction of Indoleamine 2,3-Dioxygenase on Tryptophan Metabolism in Cultured Cells—With an increase in the enzyme activity in the cultured cells, tryptophan in the medium decreased concomitantly with accumulation of its metabolite, kynurenine, and finally tryptophan was depleted (<1 μM) in the medium, and other various metabolites on the kynurenine pathway such as 3-hydroxykynurenine, anthranilic acid, 3-hydroxyanthranilic acid, o-aminophippuric acid, kynurenic acid, and xanthurenic acid were also undetected (<1 μM for each metabolite) in the medium. These results indicated that under the culture condition tryptophan in the medium was taken up into the cells and oxidized by the induced enzyme to N-formylkynurenine, which was finally excreted into the medium as kynurenine after hydrolysis by a kynurenine formamidase. To know in detail the conversion of N-formylkynurenine to kynurenine under the culture condition, we measured kynurenine formamidase activity in the 7 cell lines where enzyme induction was observed. The results were summarized as follows: (i) all of the 7 lines contained the enzyme activity ranging from 96 to 221 nmol of kynurenine/h/mg of protein under the standard assay conditions as described under "Experimental Procedures"; (ii) the activity had no relation to the indoleamine 2,3-dioxygenase activity induced in the cells; and (iii) the activity was not significantly changed by culture with IFN-γ (10³ units/ml). At the indicated time after the addition of IFN-γ, the levels of tryptophan (Panel A) and kynurenine (Panel B) in the culture medium were determined by HPLC, and the enzyme activity in cellular extract was assayed as described under “Experimental Procedures.” Values are means of duplicate dishes. Control represents the result of the culture without IFN-γ. +IFN-γ, experiment; O, control.

Significance of Indoleamine 2,3-Dioxygenase-mediated Tryptophan Degradation on Anticellular Activity of IFN-γ—When the 7 cell lines, which were positive for the enzyme induction, were cultured with IFN-γ (10³ units/ml), the growth of all the cell lines was effectively inhibited as exemplified with OKK and HeLa cells (see lines with open circles in Fig. 7B). This growth inhibition by IFN-γ was accompanied by a complete depletion of tryptophan and an accumulation of kynurenine in the medium (Fig. 7A), as presented in Fig. 6. It was possible that the growth inhibition was caused by the newly synthesized metabolite, kynurenine or N-formylkynurenine; in other words, the metabolite might behave as a growth inhibitor. However, this possibility seemed to be excluded by the fact that no growth inhibition of these lines was observed when they were cultured in medium containing the metabolite at 100 or 500 μM (not shown). A more plausible mechanism of the growth inhibition was the tryptophan de-
pletion in the culture medium, because tryptophan is one of the essential amino acids for growth of mammalian cells. To examine this possibility, we added exogenous tryptophan to the medium not to be depleted at the points indicated by the arrows in Fig. 7B (50 μM per one addition). As a result, the growth inhibitions were partially reversed by the addition of tryptophan (see lines with closed triangles in Fig. 7B). In these experiments, 60–80% of tryptophan added was oxidized and accumulated as kynurenine in the medium. These experiments indicated that the growth inhibitions observed for OKK and HeLa cells were in part caused by the tryptophan depletion. However, for the other 5 cells, such a reversal was not demonstrated (data not shown).

**DISCUSSION**

In this study, we demonstrated that the tryptophan pyrrolase induced by IFN-γ in cultured human cells was identical to indoleamine 2,3-dioxygenase but not to L-tryptophan 2,3-dioxygenase by immunoblot analysis using a monoclonal antibody specific to the former enzyme (Fig. 3). This conclusion was further supported by the absolute requirement of both ascorbic acid and methylene blue for the dioxygenase activity (Fig. 4) and the high affinity (Kₐ ≈ 20 μM) for L-tryptophan (Fig. 5). In contrast, L-tryptophan 2,3-dioxygenase in human liver required neither ascorbic acid nor methylene blue for the activity (30) and exhibited a low affinity (Kₐ ≈ 400 μM) for L-tryptophan.⁴

We also showed that the extent of induction of indoleamine 2,3-dioxygenase by IFN-γ varied largely with the type of human cell lines (0–837 units/mg of protein) (Table I). Since IFN-γ exerts the biological activity through a binding of the specific receptor in the surface of the cell (12–19), it is possible that the number of the receptor determines the degree of the biological responses to the ligand, as recently demonstrated for the tumor necrosis factor receptor system (42). We are, therefore, currently attempting to clarify the relationship of the number of the receptor in the surface of cells with the extent of the enzyme induction in the 11 cells.

Although all three IFNs, IFN-α, IFN-β, and IFN-γ, exhibit apparently similar antigenic and antiviral activities against many cultured cell lines (1, 2), it has been suggested that the detailed molecular mechanism of action of IFN-γ differs from those of the other IFNs. This suggestion is based on the following observations: (i) IFN-γ compared with the other IFNs had a potent antiviral activity (32, 43–49); (ii) IFN-γ showed a different kinetics of induction of antiviral state from those of the other IFNs (47, 50, 51); and (iii) the receptor of IFN-γ on the cell surface differed from those of the other IFNs (12, 14–18). Indeed, Rubin and Gupta (47) and Weil et al. (52) have found that IFN-γ stimulated the synthesis of some proteins which were either not all or only weakly inducible by IFN-α or IFN-β in some human cell lines. In this context, of interest is that the induction of indoleamine 2,3-dioxygenase in all 7 cell lines (Table I) was specific to IFN-γ but not to the other IFNs. Pfefferkorn (20) and Byrne et al. (23) have also observed a similar specificity in the induction of tryptophan oxidizing activity in some human cell lines. Thus, the dioxygenase may be one of the unique cellular proteins induced by only IFN-γ in some cultured cell lines, and this enzyme induction may explain partly the reported differences between IFN-γ and the other IFNs.

Our results indicated that under the culture conditions indoleamine 2,3-dioxygenase induced by IFN-γ actively oxidized tryptophan into N-formylkynurenine, which was rapidly converted to kynurenine by an action of a constitutive kynurenine formamidase in the cells (Fig. 6). Kynurenine thus formed was not metabolized further into various forms along the well known kynurenine pathway in the cells and accumulated in culture medium (Fig. 6). This tryptophan oxidation finally resulted in a complete depletion of this amino acid in the medium (Figs. 6 and 7). Of particular importance is that this tryptophan depletion facilitated the growth inhibition of some cells, OKK and HeLa cells (Fig. 7), although for the other 5 cells we could not demonstrate the positive role of the amino acid depletion in the growth inhibition. Perhaps other unrevealed anticular mechanisms, which are more crucial than the tryptophan depletion, are operative in the 5 cells.

These in vitro studies with cultured human cells strongly suggest that the in vivo cellular level of indoleamine 2,3-dioxygenase is under the control of IFN-γ. It is generally accepted that IFN-γ is produced by lymphocytes, and the production may be enhanced as a result of an immune reaction against various pathogens, e.g., virus and tumor. Interestingly, Yoshida et al. (54) have observed that a marked induction of this enzyme occurred in the mouse lung infected with influenza virus, and more recently, Yasui et al. (50) have found that the enzyme level was significantly higher (up to 20-fold) in the human lung tissues bearing tumor than that in the normal tissue. This increase suggests an elevated production of IFN-γ in the tissues as a result of a host-tumor reaction, and probably by the enzyme induction, i.e., the tryptophan depletion, the host may be attempting to suppress the growth of tumor, as observed with the two cultured tumor cells (Fig. 7).

REFERENCES
MATERIALS AND METHODS

The following materials were purchased from the suppliers indicated in brackets: D-
Tryptophan, 5-hydroxy-L-tryptophan, and 5-hydroxy-L-kynurenine (Nakajima Chemicals,
Kyoto, Japan), kynurenamine dihydrobromide, 5-hydroxytryptamine hydrochloride, and 5-hydroxy-
L-kynurenine (Sigma Chemicals, St. Louis, Mo., U.S.A.). 5-Hydroxy-L-tryptophan, kynurenamine,
and the enzyme. The reaction, at 37°C, was started by the addition of the substrate and terminated
after 30 min by adding 0.2 ml of 30% (w/v) trichloroacetic acid, and further incubated for 50°C
for 30 min to hydrolyze N-formylkynurenine produced by indoleamine 2-
dioxygenase to kynurenine. After centrifugation at 2,500 × g for 15 min at 20°C, the supernatant
(0.5 ml) was mixed with an Ehrlich reagent (0.8 ml) containing 0.3% (v/v) N-dimethylaminobenze-
aldoxime in acetic acid. The yellow pigment derived from kynurenine was determined at 499 nm
by a Hitachi 300-20S spectrophotometer.

Purification of ILO from Human Plasma

Unless otherwise stated, all the procedures were carried out at 0°C and potassium
phosphate buffer, pH 6.5, was used throughout.

Step 1: Crude Extracts

Five normal plasma samples, which were stored 1 to 3 days after delivery at
−70°C in a refrigerated, were obtained from an obstetric clinic. Cells (about 10
milliliters) were homogenized with dithiothreitol (100 mM) and a small amount of
Tween 20 and 1 ml of 0.9% (v/v) dithiothreitol. After centrifugation at
4°C for 2 h at 100,000 x g, the supernatant (1 ml) was added to 1 ml of 20
mM potassium phosphate buffer (pH 7.4). The supernatant (1 ml) was added to
1 ml of 20 mM potassium phosphate buffer (pH 7.4).

Step 2 and 3: 1. Phospho-cellulose and Hydroxyapatite Chromatographies

The supernatant (4 ml) containing 0.2% (w/v) (+)-adenosine indicated absorption
at 260 nm by a Hitachi 300-20S spectrophotometer. After washing with 3 ml
of 0.1 M sodium acetate buffer (pH 6.0), the active fractions (about 4 ml) were
eluted with 15 ml of a linear gradient of 15 ml each of 0.1 M sodium acetate and
0.6 M sodium phosphate buffer (pH 6.0). The active fractions (about 4 ml) were
eluted with 15 ml of a linear gradient of 15 ml each of 0.1 M sodium acetate and
0.6 M sodium phosphate buffer (pH 6.0).

Step 4: 2. Phospho-cellulose Chromatography

The supernatant (20 ml) was applied to a column (2 × 25 cm) of DEAE-
fibrinogen (9.4 ml) equilibrated with 0.1 M sodium acetate buffer (pH 6.0). The
active fractions (about 20 ml) were pooled and concentrated to 1 ml and treated
with 1 ml of 0.1 M sodium acetate buffer (pH 6.0). The active fractions (about 20 ml) were pooled and concentrated to 1 ml and treated with 1 ml of 0.1 M sodium acetate buffer (pH 6.0).

Step 5: Gel Filtration

The active fractions (125 ml) after the phospho-cellulose column were concentrated to about 3 ml with a small column (3 × 3 cm) of hydroxyapatite and
Celite (Bio-Rad Laboratories, Richmond, Calif.) 2,000 ml and centrifuged at 10,000 × g for 30 min.

Step 6: Ileotropic Focusing

The active fractions (20 ml) after the gel filtration were pooled and concentrated to about 5 ml with a Centricon-20 (Amicon, Woburn, Mass.) and applied to a column (2 × 90 cm) of Staphylococcus aureus 13630 protein A-Sepharose 4B (Cleveland, Ohio) 8.4 ML/2,000 ml and centrifuged at 10,000 × g for 30 min.

Step 7: CM Affi-Gel Blue Chromatography

The active fractions (95820, 15 ml) after Step 6 were pooled, concentrated, and the solution of the enzyme was changed to the 0.25 M potassium phosphate buffer, pH 6.0, with a Centricon-25. After freezing the sample was applied to a column (0.67 × 7.5 cm) of TSK CM-SW5 (a weak cation exchanger), previously equilibrated with the same buffer. After washing with 5 ml of the same buffer, the enzyme was eluted with a linear gradient of 5 ml each of 20 and 200 mM potassium phosphate buffer, pH 6.0, at 20°C. The flow rate was 3 ml/min. The enzyme was eluted at about 100 ml of buffer concentration (Fig. 2).

Step 8: TSK CM-SW5 Chromatography

The active fractions (98240, 10 ml) after Step 7 were pooled, concentrated, and the solution of the enzyme was changed to the 0.25 M potassium phosphate buffer, pH 6.0, with a Centricon-25. After freezing the sample was applied to a column (0.67 × 7.5 cm) of TSK CM-SW5 (a weak cation exchanger), previously equilibrated with the same buffer. After washing with 5 ml of the same buffer, the enzyme was eluted with a linear gradient of 5 ml each of 20 and 200 mM potassium phosphate buffer, pH 6.0, at 20°C. The flow rate was 3 ml/min. The enzyme was eluted at about 100 ml of buffer concentration (Fig. 2).

APPENDIX

Induction of Indoleamine 2,3-Dioxygenase by Interferon-γ

As summarized in Table I, human indoleamine 2,3-dioxygenase was purified by
about 10,000-fold from crude extracts of the placenta by a series of purification steps with the recovery of 0.9%.

The preincubation was homogenized by SDS-PAGE and its Mr was calculated to be about 400,000 (Fig. 4).

The migration rate in SDS-PAGE was not affected by the reducing agent, 2-
mercaptoethanol (1 mM) or dithiothreitol (100 μM) (not shown), indicating the absence of intermolecular disulfide bonds. Analytical gel filtration with Sephadex G-100 in 50 mM
potassium phosphate buffer (pH 6.5) gave an identical Mr of about 40,000 (not shown). These results
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Induction of Indoleamine 2,3-Dioxygenase by Interferon-γ

Fig. 1. Isoelectric focusing. The detail was described under "MATERIALS and METHODS."

Fig. 2. Elution profile of human indoleamine 2,3-dioxygenase from CM Affi-Gel Blue column. The detail was described under "MATERIALS and METHODS."

Fig. 3. Elution profile of human indoleamine 2,3-dioxygenase from TSK CM-35W column. The detail was described under "MATERIALS and METHODS."

Fig. 4. SDS-PAGE of human indoleamine 2,3-dioxygenase purified from the placenta. The final preparation (50 ng) was applied on 10% (w/v) of polyacrylamide gel in the presence of 0.1% (w/v) SDS (37). Protein was stained by the silver stain (53).

Fig. 5. Absorption Spectrum of human indoleamine 2,3-dioxygenase purified from the placenta. Spectrum of the purified enzyme (0.17 mg) in 0.7 ml of about 0.13 M potassium phosphate buffer, pH 6.0, was recorded at 20 °C with a Hitachi 200-20 spectrophotometer in a cuvette with a 10 mm light path.