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Induction of pulmonary indoleamine 2,3-dioxygenase by interferon

(lung slices/influenza virus/endotoxin)

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ABSTRACT **Pulmonary indoleamine 2,3-dioxygenase [indoleamine: oxygen 2,3-oxidoreductase(decyclizing)] has been found to be induced (30- to 100-fold) in the mouse after a single intraperitoneal administration of bacterial endotoxin [Yoshida, R. & Hayaishi, O. (1978) *Proc. Natl. Acad. Sci. USA* 75, 3998–4000] or during *in vivo* virus infection [Yoshida, R., Urade, Y., Tokuda, M. & Hayaishi, O. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4084–4086]. In the present study, an *in vitro* system with mouse lung slices was developed in which bacterial endotoxin (5 μg/ml) produced an induction (approximately 10-fold) of indoleamine 2,3-dioxygenase. The endotoxin was substituted by interferon from mouse L cells or mouse brain. The pulmonary enzyme activity increased almost linearly for 48 hr after addition of mouse interferon (10^4 units/ml) to lung slices. Interferon from mouse L cells or mouse brain produced a 10- to 15-fold increase in the enzyme activity, whereas that from human leukocytes was all but ineffective. The effect also was observed using highly purified L-cell interferon, prepared by poly(U) affinity column chromatography. When interferon was treated either by heat, α-chymotrypsin, or anti-interferon serum, such increase in the enzyme activity was diminished essentially to the same extent as seen in the antiviral activity. The increase in the enzyme activity was blocked when actinomycin D or cycloheximide was added to the slices before interferon treatment. These results suggest that the enzyme induction was produced by interferon and not by possible contaminants in the interferon preparations.**

Indoleamine 2,3-dioxygenase [indoleamine:oxygen 2,3-oxidoreductase (decyclizing)] (IDOase) is a hemoprotein (1) that catalyzes the incorporation of the superoxide anion as well as molecular oxygen (2, 3) into the pyrrole moiety of various indoleamine derivatives (4, 5). The pulmonary IDOase is dramatically induced (30- to 100-fold) in the mouse after a single intraperitoneal administration of bacterial endotoxin [lipopolysaccharide (LPS)] (6) or during virus infection (7). To determine the precise mechanisms of IDOase induction, we developed an *in vitro* system with lung slices from mice and examined the effects of various substances, including the superoxide anion, indoleamines, and interferons, on the enzyme activity. We report herein that the IDOase activity was increased approximately 10- to 15-fold within 48 hr after the addition of mouse interferon (10^4 units/ml) to mouse lung slices. A species-specificity of interferons and the effects of inhibitors of protein synthesis are presented also.

MATERIALS AND METHODS

Chemicals. L-[*ring*-2-^{14}C]Tryptophan (36 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels) was purchased from Commisariat á l'Energie Atomique, France, and was purified by column chromatography with Dowex 50W-X2 (H^+ form) (0.5 × 1.0 cm), as described (8). LPS from *Escherichia coli* 055:B5, prepared by the Westphal method, was from Difco. LPS (2.5–500 μg/ml) was suspended in a nonpyrogenic, isotonic NaCl solution and was stored at 4°C in a screw-capped vial. An aliquot (0.1 ml) of the suspension was added to Eagle's minimum essential medium (4 ml) containing 5% fetal calf serum. Poly(U)-Sepharose 4B was from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade.

Animals. Male slc:ICR mice weighing 31 ± 2 g were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan).The animals were raised under specific pathogen-free conditions at 25° ± 2°C and ≈50% humidity in an airconditioned room in the Institute of Laboratory Animals, Kyoto University.

Interferons. (*i*) L-cell interferon was induced on monolayers of L_{929} cells infected with Newcastle disease virus (Miyadera strain). The culture supernatant was acidified to pH 2.0 with 1 M HCl and was allowed to stand for 72 hr at 4°C to inactivate residual virus. The supernatant solution then was adjusted to pH 7.0, dialyzed against LPS-free distilled water, lyophilized, and stored at −80°C. The specific activity of mouse L-cell interferon was 1.4 × 10^6 units/mg of protein by the microassay method described below. (*ii*) The interferon preparation was further purified by poly(U) affinity column chromatography as described by Maeyer-Guignard *et al.* (9). The specific activity of this interferon was 2 × 10^8 units/mg of protein. (*iii*) An extract from the brain of ICR mice inoculated with Japanese encephalitis virus (JaG Ar01) contained interferon with a specific activity of 6 × 10^4 units/mg of protein. (*iv*) Human leukocyte interferon (provided by A. Matsuo of Green Cross Corp., Osaka, Japan) was derived from the nutrient medium of leukocyte suspensions inoculated with Sendai virus and was purified by the method of Cantell and Hirvonen (10). The specific activity of this purified preparation was 10^6 units/mg of protein.

Inhibitors. Actinomycin D was dissolved in 95% ethanol (1 mg/ml) and then diluted in nonpyrogenic saline to a desired concentration. Actinomycin D solution (8 μg/16 μl) was added to the medium (4 ml). Cycloheximide was dissolved in nonpyrogenic saline (1 mg/ml) and was applied in a dose of 50 μl per dish.

Incubation of Tissues. Mice were sacrificed by dislocation of the neck. The lungs were rapidly and aseptically removed, and slices weighing 4–6 mg each (about 1.5 × 1.5 × 2 mm) were prepared with a razor. Approximately 50 pieces of these slices (0.2–0.3 g, wet weight) were placed in a petri dish (58 × 10 mm) (Nunc, Denmark) containing 4 ml of Eagle's medium supplemented with kanamycin (60 μg/ml) and 5%-(vol/vol) fetal calf serum (Flow, Stanmore, Australia). The fetal calf serum was heat-inactivated at 56°C for 30 min before use. Incubation was carried out at 37°C in a humidified atmosphere of 5% CO_2/95% air.

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Abbreviations: IDOase, indoleamine 2,3-dioxygenase [indoleamine: oxygen 2,3-oxidoreductase (decyclizing)]; LPS, lipopolysaccharide.

Enzyme Assay. After incubation, slices were separated from the medium frozen in a dry ice/ethyl alcohol bath, and stored at −80°C for subsequent assays of IDOase activity. The tissues were weighed and homogenized with 2 vol of ice-cold 0.14 M KCl/0.02 M potassium phosphate buffer, pH 7.0, by using a Kinematica Polytron homogenizer (Lucerne, Switzerland). Homogenates were centrifuged at 30,000 × *g* for 30 min at 4°C in a Sorvall model RC-2B centrifuge. The resulting supernatant was used as the enzyme source and was assayed as described (11).

Interferon Assay. Interferon activity was assayed by the microassay method (12) on monolayer cultures of mouse L_{929} cells. Mouse L_{929} cell suspension (50 μl;10^6 cells per ml) was dispersed into a well of the microplate. Twenty-five microliters of interferon solution diluted on the transfer plate was added to the confluent monolayers of L_{929} cells, and the culture was incubated at 37°C in a humidified 5% CO_2/95% air atmosphere for 24 hr. The medium containing interferon was discarded, and 50 $TCID_{50}$ (50% tissue culture infectious dose) of vesicular stomatitis virus (New Jersey strain) was then challenged. Cytopathic effect was observed 48 hr later and interferon titer was calculated by using the definition that 1 unit of interferon inhibits 50% of the prescribed cytopathic effect.

α-Chymotrypsin Treatment. α-Chymotrypsin treatment was performed as described by Braude *et al.* (13) with a slight modification. Two milliliters of mouse L-cell interferon containing 1.6 × 10^5 units and 1.92 mg of protein (bovine serum albumin) were dialyzed against 10 mM potassium phosphate buffer pH 7.6). After the sample was equilibrated at 4°C, 0.192 mg of α-chymotrypsin (Sigma) in 0.1 ml of 1 mM HCl (pH 3.0) was added. The pH was maintained at 7.6 by titration with 0.1 M NaOH, and the temperature was kept at 4°C. Aliquots (0.5 ml) were removed 6 hr later and added to the culture medium supplemented with 5% fetal calf serum.

Immunological Procedures. Antibody against L-cell interferon was prepared by injection of 30 mg of L-cell interferon with a specific activity of 2 × 10^6 units/mg of protein in complete Freund's adjuvant (Difco) in the back of rabbits, as described by Leskowitz and Waksman (14). The rabbit was bled at 2-wk intervals. The serum (30 ml) was mixed with a sonic extract of L cells (4 × 10^8). After incubation at 4°C for 5 days, precipitates formed by centrifugation at 30,000 × *g* for 30 min were discarded. This serum preparation neutralized 8 units of L-cell interferon at a dilution of 1:200,000. Control antisera were obtained from the untreated rabbits. One milliliter of the interferon preparation (4 × 10^3 units) was mixed with 30 μl of the anti-interferon or control serum, which had been diluted in 1 ml of Eagle's medium. After incubation at 25°C for 30 min and at 4°C for 1 hr, tubes were centrifuged at 10,000 × *g* for 15 min. Supernatant solution was removed, diluted in 2 ml of Eagle's medium, and then used for the culture medium.

Protein Determination. Protein concentration was determined by the method of Lowry *et al.* (15), with bovine serum albumin as a standard.

RESULTS

In order to clarify the mechanism of the *in vivo* induction of IDOase by LPS (6) or during virus infection (7), an *in vitro* system with mouse lung slices was developed. When lung slices were incubated in the presence of LPS under the conditions described under *Materials and Methods*, IDOase activity in the supernatant fraction from high-speed centrifugation increased for at least 48 hr and gradually decreased. As shown in Fig. 1, a dose-dependent increase in the specific activity of IDOase was observed below 1 μg of LPS per ml, and a maximum induction (approximately 10-fold) was obtained at approximately 5 μg of LPS per ml (20 μg/0.2–0.3 g of lung slices), a dose almost identical to that which had been used *in vivo* (20 μg per mouse, 0.2–0.3 g of lung) (6). A similar increase in the enzyme activity was obtained by addition of poly(I)·poly(C) (525 μg/4 ml), a synthetic double-stranded RNA, to the lung slices (data not included).

In these *in vitro* systems, various other substances, such as the superoxide anion or indoleamines [which are utilized by IDOase (2–5)] were examined for effects on the enzyme activity. Methyl viologen (40 μg/ml to 1.25 mg/ml), a potent inducer of the superoxide anion in the lung (16); 5-OH-L-tryptophan (80 μg/ml to 2.25 mg/ml), a precursor of serotonin; or serotonin (0.352–352 μg/ml) caused no significant effect on the enzyme activity (data not included). However, when interferon (10^4 units/ml) from mouse L cells was added to the medium containing mouse lung slices, a 10- to 15-fold increase in the IDOase activity was observed within 48 hr (Fig. 2). The specific activity of IDOase in the high-speed supernatant fraction of lung slices increased almost linearly for 48 hr, then began to decrease, and reached a normal value after about 7 days. In the absence of interferon, the enzyme activity increased slowly and reached a

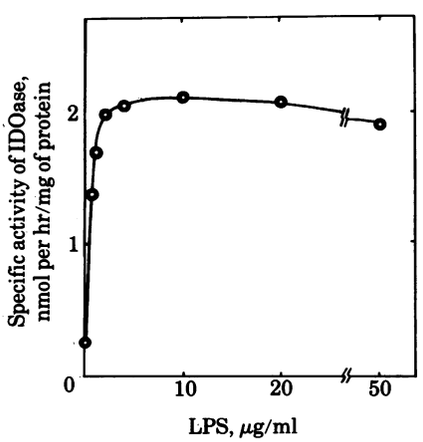

FIG. 1. Effect of LPS on IDOase activity in mouse lung slices. LPS (0–50 μg/ml) was added to the culture medium at zero time, and incubation was carried out for 36 hr. The enzyme activity is expressed as nmol of product formed per hr/mg of protein. Each point represents the mean value in duplicate.

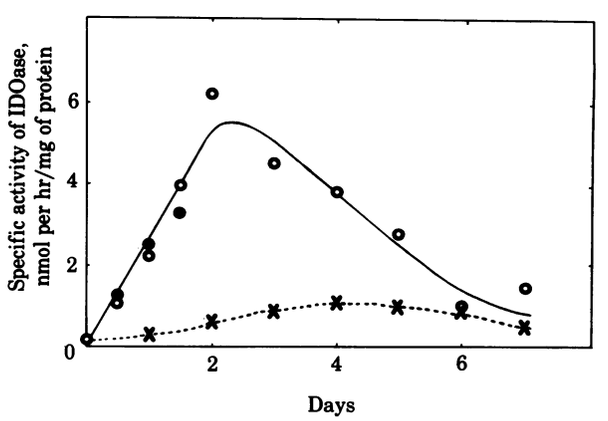

FIG. 2. Effects of mouse interferon on the IDOase activity of mouse lung slices. Mouse interferons were added to the culture medium of mouse lung slices, at zero time. The enzyme activity is expressed as nmol of product formed per hr/mg of protein. Each point represents the mean value in duplicate. ○, L-cell interferon (10^4 units/ml); ●, purified L-cell interferon (10^4 units/ml); ×, no addition.

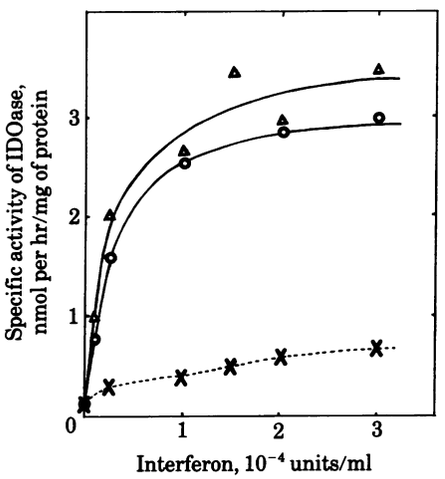

FIG. 3. Dose dependency of interferons on the IDOase activity. After addition of interferons (0–3 × 10^4 units/ml), lung slices were incubated for 36 hr. The enzyme activity is expressed as nmol of product formed per hr/mg of protein. Each value represents the mean value in duplicate. ○, Mouse L-cell interferon; △, mouse brain interferon; × human leukocyte interferon.

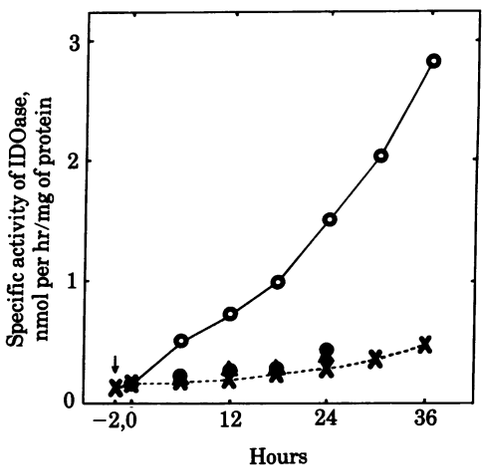

FIG. 5. Effects of actinomycin D and cycloheximide on the increase in the pulmonary IDOase activity induced by interferon treatment. Arrow indicates the time when these agents were added to the culture medium: 2 hr before interferon treatment (10^4 units/ml). Each point represents the mean value in duplicate. ○, Mouse L-cell interferon only (10^4 units/ml); ●, interferon plus actinomycin D (2 μg/ml); ▲, interferon plus cycloheximide (12.5 μg/ml); ×, no addition.

plateau after about 4 days, when the increment was about 4-fold. Although this observation was also reproducible, the exact nature is at present unknown, because there was no increase in interferon titer. Fig. 2 also shows that a similar effect was observed with highly-purified L-cell interferon (10^4 units/ml), prepared by poly(U) affinity column chromatography.

When lung slices were incubated with increasing amounts of interferon (0–30,000 units/ml), a dose-dependent increase in the specific activity of IDOase was observed by either mouse L-cell or mouse brain interferon, reaching a plateau in the range of about 10^4 units/ml (Fig. 3). Human leukocyte interferon showed little effect on the enzyme activity, indicating that these effects of interferons are probably species-specific.

In an attempt to determine whether the increase in the enzyme activity was due to interferon itself or to some possible contaminants, the effects of heat, α-chymotrypsin, and anti-interferon serum treatment were individually examined (Fig. 4). After heat treatment for 30 min at 60°C or 80°C, the extents of inactivation of the ability to increase the IDOase activity and the antiviral activity were essentially the same. Fig. 4 also shows that incubation with α-chymotrypsin for 6 hr reduced the activity of mouse L-cell interferon (10^4 units/ml) by more than 99% ($<10^2$ units/ml) and that the ability to increase the IDOase activity was all but lost by this treatment. However, the effect of LPS or poly(I)·poly(C) on the IDOase activity was retained intact by the treatment (data not included). Furthermore, when interferon was preincubated with anti-interferon serum, the antiviral activity of interferon (10^3 units/ml) was almost completely abolished (<5 units/ml) and the IDOase activity did not increase significantly. These results suggest that the increase in the IDOase activity is caused by the biological activities of interferon and not by possible contaminants, such as LPS or double-stranded RNA.

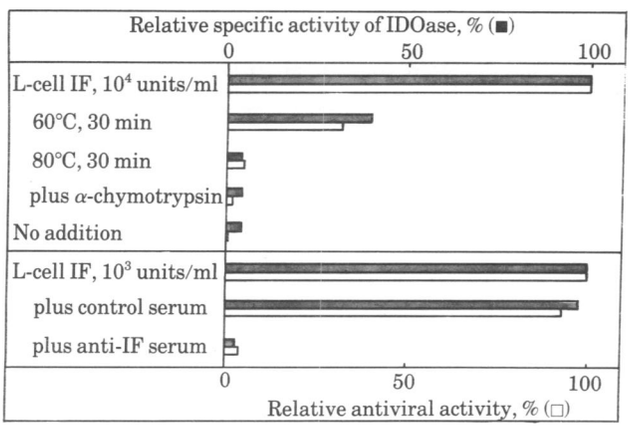

FIG. 4. Effect of heat, α-chymotrypsin, or anti-interferon serum treatment of interferon (IF) on the IDOase activity and the antiviral activity. After heat treatment (60°C or 80°C for 30 min), α-chymotrypsin treatment (for 6 hr), or anti-interferon serum treatment of interferon, the changes in the antiviral activity and in the ability to increase the IDOase activity were examined 36 hr later. The actual activities of IDOase induced by interferon at the concentrations of 10^4 and 10^3 units/ml were 2.4 and 0.76 (nmol per hr/mg of protein) for a mean value in duplicate, respectively. These values represent 100% activity. □, Antiviral activity; ■, specific activity of IDOase.

Actinomycin D (2 μg/ml) and cycloheximide (12.5 μg/ml) blocked the increase in the IDOase activity measured 6–24 hr after addition of interferon, if given 2 hr before the addition of interferon (Fig. 5), suggesting that the increase in this enzyme activity was probably because of net synthesis of the enzyme protein.

DISCUSSION

Pulmonary indoleamine 2,3-dioxygenase has been induced *in vivo* in the mouse by an intraperitoneal administration of bacterial LPS (6) or during virus infection (7). In this study, we developed an *in vitro* system with mouse lung slices in order to clarify the mechanism of IDOase induction. The maximum extent of the increase in the enzyme activity by LPS was essentially the same *in vitro* (10-fold) (Fig. 1) and *in vivo* (30-fold) (6). Dose dependency of LPS with lung slices (Fig. 1) was also almost identical to that observed *in vivo* (6). Thus, the *in vitro* system with lung slices described in this paper is expected to contribute to the detailed analysis of the mechanism of enzyme induction and to clarify the physiological significance of this enzyme.

In these *in vitro* systems, a 10- to 15-fold increase in the enzyme activity was observed by the addition of mouse interferon.

The time course of the interferon induction *in vivo* by LPS or during virus infection always preceded that of the induction of IDOase (refs. 17 and 18; unpublished data), and it was also shown in this *in vitro* system (unpublished data). The maximum level of the enzyme induction after interferon or LPS treatment *in vitro* was much the same (Figs. 1 and 3). Furthermore, preliminary experiments from our laboratory indicated that a remarkable enzyme induction was observed by the addition of interferon to the lung slices from C3H/HeJ mice, in which there was no increase in interferon titer by LPS, and LPS was all but ineffective on the enzyme activity *in vivo* and *in vitro* (unpublished data). These results suggest that the induction of pulmonary IDOase by LPS or during virus infection probably involves interferon.

Interferon has not only antiviral activity but other cellular and body functions, such as depression of the cell growth, cell division, and DNA synthesis (19); enhancement of phagocytosis by macrophages (20, 21); and immunoenhancing (22) or immunodepressing (23) effects. The molecular mechanisms are, however, poorly understood. Recently, Kimchi *et al.* reported that interferon (2×10^2 units/ml) produced a 6- to 7-fold increase in eukaryotic initiation factor 2 protein kinase and a 10- to 15-fold increase in oligoadenylate (an activator of endonuclease) synthetase in L cells (24). The antiviral activity of interferon has been assumed to be mediated by these enzymes because oligoadenylate and phosphorylated proteins were found to be synthesized in extracts of interferon-treated mouse L cells (25), chicken embryo cells (26), and in the reticulocyte lysates (27), if supplemented with double-stranded RNA and ATP. However, the interrelationships of these enzyme activities and relevance to the mechanism by which interferon blocks viral replication are unclear at present. On the other hand, no information on the molecular mechanism of interferon actions other than antiviral activity is available. In the present study, a high interferon titer (10^4 units/ml) was required for the IDOase induction as compared with the low titer (2×10^2 units/ml) for the antiviral activity (24). It may imply that IDOase either is related to other interferon actions, such as macrophage activation (enhancement of cytotoxic activity for tumor cells) (10^3–10^4 units/ml) (28), or is simply caused by the difference in the systems employed. The roles of the indoleamine 2,3-dioxygenase, the oligoadenylate synthetase, and protein kinase(s) and their relationships in various interferon actions remain to be established.

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