Influence of interferon-gamma and extracellular tryptophan on indoleamine 2,3-dioxygenase activity in T24 cells as determined by a non-radiometric assay

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The indoleamine 2,3-dioxygenase (EC 1.13.11.17) activity in human T24 cells has been investigated in cell extracts by using a non-radioactive assay. It is enhanced in a dose-dependent manner up to 25-fold by interferon- γ . The maximum reaction velocity is increased rather than the K_m , which remains at 4 $\mu\text{mol/l}$. Induction of activity starts 3 h after stimulation and reaches a plateau at 21–48 h. Decreased stimulation was observed in the presence of high L-tryptophan concentrations.

INTRODUCTION

A tryptophan-cleaving enzyme has been isolated from rabbit intestine and was found to differ from the liver tryptophan pyrrolase in several properties [1]. Owing to its wide substrate specificity [2] this tryptophan cleaving enzyme has been termed indoleamine 2,3-dioxygenase (IDO; EC 1.13.11.17). IDO purified from mouse epididymis has been studied in detail recently and flavin mononucleotide (FMNH₂) has been suggested as co-factor in its reaction [3].

IDO activity can be markedly increased by interferon as was first reported for mouse lung slices in 1981 [4]. Interferon- γ was also found to induce IDO in human fibroblasts [5] and in several, but not all, other tested human cells *in vitro* [6,7]. These findings explain the enhanced excretion of tryptophan metabolites observed in a group of diseases which are characterized by an activation of the cell-mediated immunity. Some features of interferon- γ -induced IDO activity have been investigated in human fibroblasts [8].

In contrast with the above-mentioned studies, which employ assays based on the use of radiolabelled substrates, we present here a non-radioactive assay for IDO activity in cell extracts. This method is applied to extracts of the human bladder carcinoma cell line T24. The influence of interferon- γ on K_m and maximal velocity V_{max} , as well as the dependence of the enzyme induction on the extracellular L-tryptophan concentration and the time course of the induction are described.

MATERIALS AND METHODS

Materials

L-Tryptophan was purchased from Calbiochem (Los Angeles, CA, U.S.A.). D-Tryptophan, DL-kynurenine, chicken liver dihydrofolate reductase (EC 1.5.1.3), sheep liver dihydropteridine reductase (EC 1.6.99.7), NADPH tetrasodium salt, trypsin/EDTA solution and 4',6-

diamidino-2-phenyl-indole (DAPI) were obtained from Sigma (Munich, F.R.G.). Bovine liver catalase (EC 1.11.1.6), lyophilized pure bovine serum albumin (BSA) and cell culture medium were from Serva (Heidelberg, F.R.G.). *Photobacterium fischeri* NAD(P)H:FMN oxidoreductase (EC 1.6.8.1) and FMN monosodium salt were from Boehringer (Mannheim, F.R.G.). Tetrahydrobiopterin dihydrochloride and tetrahydroneopterin dihydrochloride were from Schircks (Jona, Switzerland). Antibiotics were from Biological Industries (Beth HaEmek, Israel). Foetal calf serum (FCS) was a product of Biochrom (Berlin, F.R.G.). The protein assay dye reagent was obtained from Bio-Rad (Richmond, CA, U.S.A.). Recombinant human interferon- γ (rHuIFN- γ) with an activity of 2×10^7 i.u./mg of protein was a gift from Bioferon (Laupheim, F.R.G.). All other chemicals were products from Merck (Darmstadt, F.R.G.).

Cell culture

The human bladder carcinoma line T24 [9] was kindly provided by Dr. Feichtinger (Department of Pathology, University of Innsbruck, Innsbruck, Austria). The line was mycoplasma negative as tested by staining with DAPI [10]. Cells were grown in Eagle's minimum essential medium (MEM) with Earle's salts base containing 2 mM-L-glutamine, 100 i.u. of penicillin/ml, 0.1 ng of streptomycin/ml and 10% (v/v) heat-inactivated FCS. Cultures were kept at 37 °C in a humid atmosphere of 5% CO₂ in air and transferred weekly.

Treatment with rHuIFN- γ

Cells were seeded at a density of approx. $3 \times 10^4/\text{cm}^2$. Medium was changed on day 4 of culture. At this time monolayers had reached confluency. Monolayers were incubated for a further 3 days to increase cell density and were then stimulated for 48 h with fresh culture medium containing various rHuIFN- γ concentrations in the

Abbreviations used: IDO, indoleamine 2,3-dioxygenase (EC 1.13.11.17); rHuIFN- γ , recombinant human interferon- γ ; FCS, foetal calf serum; BSA, bovine serum albumin; PBS, Dulbecco's phosphate-buffered saline; MEM, Eagle's minimum essential medium; DAPI, 4',6-diamidino-2-phenyl-indole.

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range 10–1000 i.u./ml. In some experiments the medium L-tryptophan concentration was increased to 100, 200 or 500 μM . Control cells were treated the same way but without supplements.

Preparation of cell extracts

Interferon- γ -treated and control cells were detached with 0.5 g of trypsin/l plus 0.2 g of EDTA/l in phosphate-buffered saline (PBS), washed once with culture medium containing 10% (v/v) FCS and once with PBS. The pellet was resuspended in cold distilled water, transferred into Eppendorf vials and kept on ice for 20 min. Supernatant was collected for enzyme assay after centrifugation for 10 min at 10000 g. Cell extracts (1 ml) were obtained from two 75 cm² culture flasks (Falcon, Beckton Dickinson, New Jersey, U.S.A.), which gave a total number of $(2\text{--}3) \times 10^7$ cells, and were pooled and extracted.

Protein determination

Protein concentrations of cell extracts were determined according to the method of Bradford [11]. Lyophilized pure BSA was used as protein standard.

Enzyme assay

The IDO assay used here is based on the quantification of kynurenine by h.p.l.c. before and after the incubation of the cell extracts with L-tryptophan, Methylene Blue, ascorbic acid and catalase.

The standard reaction mixture for determining the IDO activity of cell extracts contained 0.1 M-potassium phosphate buffer, pH 7.6, 10 mM-ascorbic acid, 5 mM-EDTA, 20 μM -Methylene Blue, 50 μM -L-tryptophan, 200 μl of cell extract (approx. 200 μg of protein) and PBS to yield a final volume of 1 ml. Depending on the test, the amount of cell extract and the concentration of tryptophan were varied. Tryptophan was supplied just before the reaction was started by addition of 3×10^4 units of catalase suspended in 20 μl . Aliquots of 500 μl were taken immediately after mixing and the remaining assay mixture was incubated for 10 min at 37 °C in a waterbath. The reaction was terminated by adding 100 μl of 20% (v/v) perchloric acid to 500 μl of the reaction mixture. Under these conditions the *N*-formylkynurenine formed initially is converted to kynurenine. After removal of the protein precipitate by centrifugation (2 min, 10000 g) samples were either analysed at once or were stored at -20 °C. Kynurenine and tryptophan concentrations remained stable for at least 4 weeks. The IDO activity is calculated from the difference in the kynurenine concentrations before and after the incubation as measured by h.p.l.c. (see below).

For substitution of the electron donor, Methylene Blue and ascorbic acid were replaced by 200 μM -NADPH, 100 μM -FMN, 0.1 i.u. of NAD(P)H:FMN oxidoreductase/ml; 100 μM -NADPH, 100 μM -tetrahydrobiopterin, 1 i.u. of dihydropteridine reductase/ml; 100 μM -NADPH, 100 μM -tetrahydroneopterin, 1 i.u. of dihydropteridine reductase/ml; or 100 μM -NADPH, 100 μM -tetrahydroneopterin, 0.1 i.u. of dihydrofolate reductase/ml.

H.p.l.c. methods

The h.p.l.c. system (Varian, Palo Alto, CA, U.S.A.) consisted of a Vista 5500 liquid chromatograph equipped with an automatic sampler 8000, a UV-200 detector, an LS 4 fluorescence detector (Perkin Elmer, Beaconsfield,

U.K.) controlled by a Vista 402 data system. Ready-to-use cartridges (125 mm $\times$ 4 mm, LiChroCART, Merck, Darmstadt, F.R.G.) of octadecyl-modified silica (LiChroSORB RP-18, 7 μm , Merck) were protected by disposable guard columns (4 mm $\times$ 4 mm) of the same material. The column was eluted isocratically at a flow rate of 0.8 ml/min with 0.015 M-potassium phosphate buffer, pH 6.4, containing 1.8% (v/v) acetonitrile. This system allows the separation and detection of several tryptophan metabolites by u.v. or fluorescence (FI) detection as indicated: tryptophan (u.v. 280 nm, FI 285, 365 nm), kynurenine (u.v. 260 nm), *N*-formylkynurenine (u.v. 280 nm), anthranilic acid (FI 300, 405 nm), 3-hydroxy-anthranilic acid (FI 320, 415 nm). The deproteinized acidic incubation mixtures were injected into the h.p.l.c. system without further treatment.

RESULTS

Characteristics of the non-radioactive assay

Fig. 1 shows typical chromatograms of the assay before (left-hand side) and after (right-hand side) 10 min of incubation at 37 °C under standard assay conditions. In this case an extract of T24 cells incubated with 25 i.u. of interferon- γ /ml was used. The concentrations of kynurenine and tryptophan before the incubation period (left) were 0.52 μM and 48.9 μM , and after the incubation period were (right) 5.88 μM and 43.1 μM , respectively. With a 500 μl assay mixture containing 111 μg of cell protein, an activity of 2.41 nmol/min per mg of protein was obtained. An incubation time of 10 min was used, since the reaction rate is constant up to 20 min and then begins to decrease (results not shown). The enzyme activity increases linearly with increasing amounts of protein in the assay mixture in both interferon- γ -treated (six observations, 10–341 μg of protein, linear correlation coefficient = 0.995) and untreated cells (six observations, 63–1260 μg of protein, linear correlation coefficient = 0.997). The sensitivity of the assay is determined by the detection limit of kynurenine in the h.p.l.c. system, which is 0.2 μM at a signal:noise ratio of 5:1. Variation coefficients range from 18% at low activity (0.183 nmol/min per mg of protein) to 11% at medium activity (0.620 nmol/min per mg of protein) and 6% at high activity (4.060 nmol/min per mg of protein), five observations each. Cell extracts could be frozen for at least 2 weeks at -20 °C or released from low-molecular-mass compounds by Sephadex G-25 gel filtration without loss of activity (results not shown).

Influence of interferon- γ on the IDO reaction in T24 cell extracts

The time course of the IDO reaction in extracts of T24 cells stimulated with 250 i.u. of interferon- γ /ml is shown in Fig. 2 (each point is the mean of five measurements $\pm$ s.d.). The enzyme activity begins to increase 3 h after stimulation and reaches a plateau at 21–48 h. The influence of interferon- γ on the affinity of IDO for L-tryptophan was investigated in extracts of untreated and of stimulated (250 i.u. of rHuIFN- γ /ml) T24 cells. Extracts of interferon- γ -stimulated cells (80–160 μg of protein) and extracts of unstimulated cells (600–1200 μg of protein) were incubated with 2, 5, 10, 25 and 50 μM -L-tryptophan under standard assay conditions. Two such series with untreated cells yielded K_m values of 4.6 and 3.9 μM (mean 4.2 μM) L-tryptophan. Four series with

Fig. 1. Chromatograms of the IDO assay in T24 cell extracts before (right-hand) and after (left-hand) the incubation

T24 cell monolayers were cultivated with 25 i.u. rHuIFN- γ /ml for 48 h. Cells were harvested, extracted with distilled water and 200 μ l containing 222 μ g of protein was used for the standard assay, which requires incubation for 10 min at 37 $^{\circ}$ C. K denotes the kynurenine peak and T the tryptophan peak. H.p.l.c. conditions are described in the Materials and methods section. The detector wavelength was set at 260 nm from 0 to 8 ml elution volume for the detection of kynurenine and then switched to 280 nm from 8 to 13 ml elution volume for the detection of tryptophan. One scale unit on the A axis corresponds to 0.001 absorbance unit (AU) for kynurenine and to 0.002 AU for tryptophan, which is monitored with twice the recorder attenuation.

interferon- γ -stimulated cells yielded a mean K_m of 4.1 ± 0.5 μ M-L-tryptophan.

To determine the influence of interferon- γ on the maximum velocity V_{max} , extracts of cells treated with 25, 100, 250 or 1000 i.u. of rHuIFN- γ /ml were incubated with 10, 25, 100 and 250 μ M-L-tryptophan. With all interferon- γ doses used, the velocity reached a maximum at 50 μ M-L-tryptophan (standard assay conditions), and declined at higher L-tryptophan concentrations. V_{max} increased in an interferon- γ dose-dependent manner (Table 1).

Behaviour of T24 cell extracts towards the D- and L-isomer of tryptophan

The activity of IDO towards 50, 100 and 500 μ M of the D- and L-isomer was studied in extracts of interferon- γ (250 i.u./ml)-stimulated cells. At 500 μ M the L-isomer, but not the D-isomer, shows a significant substrate inhibition ($P < 0.05$, Student's t test; Table 2). At 50 μ M the activity towards the D-isomer is about 33 times less than that towards the L-isomer.

Requirements for the IDO reaction in T24 cell extracts

In the standard assay mixture ascorbic acid, catalase and Methylene Blue were required for full activity. If any of these was omitted the activity dropped to less than 20%. If Methylene Blue and ascorbic acid were replaced

by FMN and NAD(P)H:FMN oxidoreductase, less than 7% of standard assay activity was observed (five experiments). Tetrahydrobiopterin and tetrahydro-neopterin coupled with dihydropteridine reductase or dihydrofolate reductase were also unable to substitute for the Methylene Blue/ascorbic acid system (activities less than 15%).

Effect of extracellular L-tryptophan on the IDO activity induced by interferon- γ

T24 cell monolayers were incubated for 48 h with 25 i.u. of rHuIFN- γ /ml in culture medium containing 50 or 500 μ M-L-tryptophan (four experiments each). When stimulation was carried out in the presence of 500 μ M-L-tryptophan the IDO activity determined in cell extracts (1.71 ± 0.21 nmol/min per mg of protein) was significantly lower than that in the presence of 50 μ M-L-tryptophan (2.59 ± 0.30 nmol/min per mg of protein, $P < 0.002$, Student's t test). This effect could also be observed by monitoring the amount of kynurenine released into the culture medium by cell monolayers in 48 h. Stimulation with 10 i.u. of rHuIFN- γ /ml was performed in the presence of 50, 100 and 200 μ M-L-tryptophan. The amounts of kynurenine in the culture medium dropped from 908 ± 88 to 513 ± 118 and 346 ± 94 nmol/48 h per mg of protein, respectively (mean $\pm$ S.D., three observations each).

Fig. 2. Time course of the induction of IDO by interferon- γ in T24 cell extracts

T24 cell monolayers were cultivated with 250 i.u. of rHuIFN- γ /ml added at 0 h. At the indicated time points, cells were harvested, extracted with distilled water and the standard assay procedure performed. IDO activity was calculated from the amount of kynurenine formed in 10 min at 37 °C measured by h.p.l.c. Mean $\pm$ S.D. values of five measurements are shown.

Table 1. Dependence of the maximum velocity $V_{\max}$ on the dose of interferon- γ in T24 cell extracts

T24 cell monolayers were cultivated with the indicated doses of rHuIFN- γ for 48 h. Cells were then harvested, extracted with distilled water and the standard assay procedure performed. The amount of kynurenine formed was quantified by h.p.l.c., and is given in nmol/min per mg of total cell protein. The data shown are means of three experiments $\pm$ S.D.

Dose of IFN- γ (i.u./ml)	$V_{\max}$ (nmol/min per mg)
0	0.183 $\pm$ 0.033
25	2.35 $\pm$ 0.72
100	5.49 $\pm$ 1.47
250	5.25 $\pm$ 0.63
1000	7.48 $\pm$ 1.40

DISCUSSION

The characteristics of IDO in T24 cells described here share several features with those described for this enzyme from other sources. The substrate inhibition with the L-, but not with the D-isomer, of tryptophan, as well as the requirement for ascorbic acid, Methylene Blue and catalase for full activity were reported in the first investigation of IDO from rabbit intestine [1]. Compared with the enzymes from rabbit intestine (K_m 45 μ M) and from mouse epididymis (K_m 10 μ M), the enzyme in human T24 cell extracts has a higher affinity for

Table 2. Formation of kynurenine (nmol/min per mg of protein) by T24 cell extracts incubated with different concentrations of D- and L-tryptophan

T24 cell monolayers were incubated with 250 i.u. of rHuIFN- γ /ml for 48 h. Cells were then harvested and extracted with distilled water. The standard assay procedure was performed with the indicated concentrations of L- and D-tryptophan, and the amount of kynurenine formed was quantified by h.p.l.c. The data shown are means $\pm$ S.D. of three experiments.

[Tryptophan] (μ M)	Kynurenine (nmol/min per mg)	
	L-Tryptophan	D-Tryptophan
50	3.26 $\pm$ 0.65*	0.099 $\pm$ 0.073
100	2.92 $\pm$ 0.81*	0.23 $\pm$ 0.13
500	2.14 $\pm$ 0.10*	0.84 $\pm$ 0.29

* $P < 0.05$ (Student's t -test).

L-tryptophan (K_m 4 μ M), which is comparable with the K_m of 3 μ M in human fibroblasts [8]. Serum tryptophan concentrations are about 60 μ M so that the enzyme will be saturated at physiological conditions. The time course of the induction of IDO in T24 cells shown here also resembles observations made with human fibroblasts [8].

T24 cell extracts, however, show an activity towards D-tryptophan, which was not seen with the fibroblasts. Furthermore, we could detect an IDO activity in untreated T24 cells, which was not observed by using a radioactive assay in whole T24 cells [12]. These discrepancies might originate from different properties of the assay used here and the radioassays used by the other groups, which might, because of baseline radiation, fail to detect low enzyme activities [12]. In T24 cell extracts FMNH₂ could not replace Methylene Blue as electron donor. This is in contrast with findings with IDO from mouse epididymis, where FMNH₂ led to a 4-fold greater increase in activity compared with Methylene Blue [3]. Tetrahydrobiopterin and tetrahydroneopterin also failed to show cofactor activity. These observations have to be regarded as preliminary, and the question of the natural electron donor of IDO in human tissues must be examined in experiments using homogenous enzyme preparations rather than crude extracts.

The biological significance of the induction of IDO by interferon- γ is a much discussed topic. It has been suggested to be responsible for the killing of intracellular parasites [5], for antitumour activity [7], and for immunological functions of interferon- γ -activated macrophages [13]. The lower activity induced by a specified dose of interferon- γ in the presence of high levels of extracellular L-tryptophan, as shown here for T24 cells, could be of physiological relevance. Provided that a similar mechanism is operating in mice, this finding could help to explain striking observations on the susceptibility of mice to L-tryptophan loads following immunological stimulation [14]. When mice received an L-tryptophan load concurrently with an LD₅₀ of endotoxin, which is a potent inducer of interferon- γ , the survival rate of the animals was not affected by the tryptophan load. In contrast, if the tryptophan was administered 10 h after

the endotoxin challenge, all animals died within 48 h [14]. This dependence of the lethal effect of L-tryptophan on the lag between endotoxin challenge and tryptophan load may be explained by the lower induction of IDO in the presence of high extracellular tryptophan, which we describe here. In the case of concurrent administration of endotoxin and tryptophan, the resulting IDO activity, and hence the amounts of toxic tryptophan metabolites formed, would be lower and this might protect the animals. It is striking that the critical lag time in this experiment *in vivo* is 4–10 h, which is about the time required for induction of IDO with interferon- γ (Fig. 2). However, whether the decreased induction of IDO in the presence of increased tryptophan is restricted to T24 cells, or whether it is a general feature of IDO induction by interferon- γ , remains to be demonstrated.

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