Parallel induction of tetrahydrobiopterin biosynthesis and indoleamine 2,3-dioxygenase activity in human cells and cell lines by interferon- γ

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In all of eight tested human cells and cell lines with inducible indoleamine 2,3-dioxygenase (EC 1.13.11.17) tetrahydrobiopterin biosynthesis was activated by interferon- γ . This was demonstrated by GTP cyclohydrolase I (EC 3.5.4.16) activities and intracellular neopterin and biopterin concentrations. Pteridine synthesis was influenced by extracellular tryptophan. In T 24-cell extracts, submillimolar concentrations of tetrahydrobiopterin stimulated the indoleamine 2,3-dioxygenase reaction.

INTRODUCTION

L-erythro-5,6,7,8-Tetrahydrobiopterin (tetrahydrobiopterin) is an essential cofactor of phenylalanine-4-, tyrosine-3- and tryptophan-5-mono-oxygenase [1], but is also synthesized in tissues lacking either of these three mono-oxygenases, suggesting further, still unknown, metabolic functions of tetrahydrobiopterin [2]. The key step of tetrahydrobiopterin biosynthesis is the cleavage of GTP to 7,8-dihydroneopterin triphosphate by the enzyme GTP cyclohydrolase I (EC 3.5.4.16) [3].

D-erythro-6-(1',2',3'-Trihydroxypropyl)pterin (neopterin) accumulates in body fluids of patients suffering from an inborn deficiency in tetrahydrobiopterin biosynthesis downstream from the first intermediate, 7,8-dihydroneopterin triphosphate [4]. Besides this extremely rare inherited disorder, neopterin was shown to be elevated in the panel of diseases challenging cell-mediated immunity [5–7]. In contrast with deficiency of tetrahydrobiopterin synthesis, where biopterin levels are substantially lower than in healthy controls, the highly elevated neopterin levels in infectious diseases are paralleled by slightly elevated biopterin levels [8]. *In vitro*, large amounts of neopterin were found to be released from macrophages upon stimulation by interferon- γ (IFN- γ) derived from activated T-lymphocytes [9].

In macrophage cultures [10] as well as in body fluids of patients [11], IFN- γ -induced pteridine synthesis is closely correlated with degradation of the essential amino acid tryptophan. This degradation is initiated by indoleamine 2,3-dioxygenase (IDO) (EC 1.13.11.17), which cleaves tryptophan to *N*-formylkynurenine [12]. IDO is strongly induced by IFN- γ in various human cells and is thought to play a role in host defence mechanisms against parasites and tumours [13–15].

Tetrahydrobiopterin has been shown to act as cofactor in IDO preparations purified from rabbit intestine [16] and mouse epididymis [17], but the physiological

significance of this finding remained elusive. If tetrahydrobiopterin was functionally connected with IDO in human cells also, pteridine synthesis should be induced together with this enzyme not only in macrophages, but also in any other cell with inducible IDO activity.

We therefore investigated pteridine synthesis in six human tumour-cell lines of various tissue origin, in human dermal fibroblasts and in human peripheral-blood monocyte-derived macrophages, which all degrade tryptophan when treated with IFN- γ .

MATERIALS AND METHODS

Materials

Pteridine derivatives were from Schircks (Jona, Switzerland). Sephadex G-25, Ficoll-Paque and Percoll were from Pharmacia (Uppsala, Sweden). *L*-Tryptophan was from Calbiochem (Los Angeles, CA, U.S.A.). *D*-Tryptophan, *L*-kynurenine, 4',6-diamidino-2-phenylindole (DAPI), trypsin/EDTA solution, pyruvic acid and antibiotics were purchased from Sigma (Munich, Germany). Bovine liver catalase (EC 1.11.1.6), calf intestine alkaline phosphatase (EC 3.1.3.1), GTP disodium salt, Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (MEM) with Earle's salts base and pure bovine serum albumin were from Serva (Heidelberg, Germany). RPMI 1640 (low endotoxin) and foetal-calf serum (FCS) were products from Biochrom (Berlin, Germany). RPMI 1640 Select Amine Kit was from Gibco (Grand Island, NY, U.S.A.). Recombinant human IFN- γ with an activity of 2×10^7 i.u./mg of protein was a gift from Bioferon (Laupheim, Germany). Cell-culture plasticware was from Falcon (Becton-Dickinson, Lincoln Park, NJ, U.S.A.). The protein assay dye reagent was from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were products from Merck (Darmstadt, Germany).

Abbreviations used: IDO, indoleamine 2,3-dioxygenase (EC 1.13.11.17); IFN- γ , recombinant human interferon- γ ; FCS, foetal-calf serum; PBS, Dulbecco's phosphate-buffered saline; MEM, Eagle's minimum essential medium; DMEM, Dulbecco's modified Eagle medium; RPMI 1640, Roswell Park Memorial Institute tissue-culture medium type 1640; DAPI, 4',6-diamidino-2-phenylindole.

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Cell culture

Human cell lines A 431 (epidermoid carcinoma) [18], A 498 (kidney carcinoma) [18], A 549 (lung carcinoma) [19], SK-HEP-1 (liver carcinoma) [20], U 138 MG (glioblastoma) [21], T 24 (bladder transitional-cell carcinoma) [22] and normal dermal fibroblasts were either obtained directly from the American Type Culture Collection (Rockville, MD, U.S.A.) or kindly supplied by various institutions of the University of Innsbruck. Buffy coats from blood of healthy donors and human serum were generously provided by the Blood Transfusion Center of the University Hospital of Innsbruck.

T 24 was grown in MEM with Earle's-salts base supplemented with 10% (v/v) heat-inactivated FCS, 2 mM-L-glutamine, 100 i.u. of penicillin/ml and 0.1 ng of streptomycin/ml. The same medium with a further addition of 1 mM-pyruvic acid was used for A 498 and U 138 MG. A 431, A 549 and fibroblasts of passages 16–23 were cultivated in DMEM with supplements as described for T 24. For SK-HEP-1, DMEM with the same supplements but a further addition of 1 mM-pyruvic acid, was used. The pyruvic acid added is required for optimal growth of the respective cell lines. All cell lines were mycoplasma-negative as tested by staining with DAPI [23] and have been reported to be free of HeLa-cell markers [24]. Depending on the cell line used, the seeding density was $(1-5) \times 10^4$ cells/cm².

Peripheral-blood-derived monocytes were enriched from buffy coats of healthy blood donors by a two-step density-gradient centrifugation over Ficoll-Paque [25] and a discontinuous Percoll gradient [26]. Cells were seeded in 10 ml of RPMI 1640 (low endotoxin) with 10% heat-inactivated human pooled serum, 2 mM-L-glutamine and antibiotics (see above) at a density of 10^6 /ml in plastic Petri dishes and allowed to adhere. Washing of plates with warm phosphate-buffered saline (PBS) after 2 and a further 24 h was performed to remove non-adherent cells. The resulting cell population consisted of more than 99% esterase-positive cells on day 3 of culture. Cells were then referred to as 'macrophages'.

All cultures were kept at 37 °C in humidified air containing 5% CO₂.

Confluent monolayers of cell lines on day 7 of culture and macrophages on day 3 were stimulated with 250 i.u. of IFN- γ /ml in fresh medium for 48–72 h. In some experiments, increasing concentrations of L-tryptophan were added to the culture concurrently with IFN- γ . With macrophages the effect of increasing D- and L-tryptophan concentrations was tested using tryptophan-free RPMI 1640 culture medium (Select Amine Kit; Gibco), supplemented as complete RPMI 1640 (see above).

Preparation of cell extracts

An extract (1 ml) was prepared from $(1-2) \times 10^7$ cells. Cells except macrophages were harvested by trypsin treatment, washed once with culture medium containing 10% FCS and once with PBS. Cells were resuspended in 0.1 M-Tris/HCl (pH 7.8)/2.5 mM-EDTA/0.15 M-KCl/5% (v/v) glycerol and disrupted by freezing, thawing and vigorous pipetting. As macrophages cannot be recovered without fatal damage from plastic surfaces after several days of culture, these cells were washed twice with warm PBS when still adherent to the culture plate and then removed in 600 μ l of Tris buffer (see above) by vigorous scraping with a rubber

policeman. Suspensions of interferon-treated and control plates were pooled separately. Cell viability after applying either of these procedures was less than 5% as determined by staining with Trypan Blue.

Extracts were applied to Sephadex G-25 columns (3 cm $\times$ 0.5 cm) after centrifugation for 10 min at 10000 g. The protein fraction was eluted with 0.1 M-Tris/HCl (pH 7.8)/5 mM-EDTA/0.3 M-KCl/10% (v/v) glycerol.

Only freshly harvested cell extracts were used, as GTP cyclohydrolase I activity and the tetrahydrobiopterin content decreased in some cases to less than 30% already after overnight storage at –20 °C.

Protein determination

Protein contents of cell extracts and eluates were determined as described by Bradford [27] using freeze-dried pure bovine serum albumin as standard.

Enzyme assays

GTP cyclohydrolase I assay was carried out as described by Viveros *et al.* [28] using 100–900 μ g of cell protein. Briefly, the eluate was incubated with 2 mM-GTP for 90 min at 37 °C. The 7,8-dihydroneopterin triphosphate thus formed was then first oxidized to neopterin triphosphate by addition of acidic iodine solution and then dephosphorylated to neopterin by alkaline phosphatase. Neopterin in incubation mixtures was quantified by solid-phase-extraction on-line-elution h.p.l.c. [29]. Under these conditions, reaction rates increased linearly up to 4.5 mg of protein [30].

Assay for IDO activity was performed in eluates of cell extracts using a method based on the quantification of kynurenine by h.p.l.c. as previously described [31]. The reaction mixture contained 0.1 M-potassium phosphate buffer, pH 7.6, 10 mM-ascorbic acid, 5 mM-EDTA, 20 μ M-Methylene Blue, 50 μ M-L-tryptophan, 3×10^4 units of catalase/ml and 200–800 μ g of cell protein. The incubation time was 10 min at 37 °C.

Pteridine determinations

Total neopterin and biopterin in cell extracts and supernatants were quantified after acid-iodine oxidation by solid-phase-extraction on-line-elution h.p.l.c. [29]. Briefly, 100 μ l of cell extracts (500 μ l in the case of macrophages) or supernatants were mixed with 5 μ l of 10 M-H₃PO₄ and 5 μ l of 0.1 M-KI/I₂ and incubated for 1 h in the dark. For determination of the amount of biopterin and 7,8-dihydrobiopterin, H₃PO₄ was replaced by 5 μ l of 1 M-NaOH. For the measurement of neopterin without inclusion of reduced derivatives, 100 μ l of the sample was incubated with 10 μ l of 0.1 M-Fe³⁺-EDTA. After incubation, the reaction mixture was acidified by addition of 5 μ l of 10 M-H₃PO₄ and 5 μ l of 1 M-ascorbic acid, which also served to destroy remaining iodine. The incubation mixtures were then extracted with AASP-SCX cartridges (Analytichem, Harbor City, CA, U.S.A.) and the pteridines directly eluted on to a reversed-phase h.p.l.c. column and quantified by fluorescence detection as described in [29].

Determination of kynurenine in culture supernatants

Kynurenine in supernatants was quantified by reversed-phase h.p.l.c. with u.v. detection as described in [10].

Table 1. Activities of IDO, GTP cyclohydrolase I and pteridine concentrations in extracts of human cells after IFN- γ treatment as compared with untreated controls

Cells were cultivated for 48 h with 250 i.u. of IFN- γ /ml or without IFN- γ , and cell extracts were obtained as detailed in the Materials and methods section. Activity of IDO was assessed by h.p.l.c. measurement of the kynurenine formed from tryptophan in 10 min at 37 °C. Activity of GTP cyclohydrolase I (GTP-CH) was determined by quantification of the amount of neopterin derivatives formed from GTP in 90 min at 37 °C. Neopterin derivatives were measured as neopterin by h.p.l.c. after oxidation with acidic iodine and dephosphorylation with alkaline phosphatase. Intracellular neopterin and biopterin concentrations were determined by h.p.l.c. after oxidation by acidic iodine. Data expressed in the Table are means $\pm$ S.D. for four experiments. For macrophages, five observations made on cells of three different donors are reported. Significance of difference between stimulated and control cells was calculated by Student's *t* test: *, $P < 0.0001$; ●, $P < 0.001$; ○, $P < 0.01$; ◇, $P < 0.02$; ◆, $P < 0.05$.

Cell line	[IFN- γ] (i.u./ml)	IDO activity (nmol·min ⁻¹ · mg ⁻¹)	GTP-CH activity (pmol·min ⁻¹ · mg ⁻¹)	Neopterin (pmol/mg)	Biopterin (pmol/mg)
A 431 (epidermoid carcinoma)	250 0	1.479 $\pm$ 0.302 < 0.014	0.074 $\pm$ 0.007 0.016 $\pm$ 0.005]*	15.41 $\pm$ 5.40 1.46 $\pm$ 0.21]○	29.46 $\pm$ 4.19 1.69 $\pm$ 1.22]*
A 498 (kidney carcinoma)	250 0	0.662 $\pm$ 0.048 < 0.016	0.230 $\pm$ 0.054 < 0.002	8.54 $\pm$ 3.86 < 0.29	46.18 $\pm$ 11.83 < 0.74
A 549 (lung carcinoma)	250 0	1.558 $\pm$ 0.302 < 0.011	0.042 $\pm$ 0.001 0.009 $\pm$ 0.004]*	0.82 $\pm$ 0.12 < 0.22	5.55 $\pm$ 0.15 < 0.55
SK-HEP-1 (liver carcinoma)	250 0	0.119 $\pm$ 0.033 < 0.045	0.030 $\pm$ 0.011 < 0.004	1.90 $\pm$ 0.84 < 0.32	11.47 $\pm$ 7.11 < 0.82
U 138 MG (glioblastoma)	250 0	0.520 $\pm$ 0.071 < 0.057	0.181 $\pm$ 0.088 0.023 $\pm$ 0.010]◇	29.92 $\pm$ 3.04 < 0.42	37.40 $\pm$ 12.63 < 1.05
T 24 (bladder carcinoma)	250 0	3.140 $\pm$ 0.343]* 0.064 $\pm$ 0.042]*	4.232 $\pm$ 0.520 0.482 $\pm$ 0.262]*	5.64 $\pm$ 2.12 2.36 $\pm$ 1.48]◆	294.34 $\pm$ 21.81]* 33.67 $\pm$ 12.38]*
Fibroblasts (dermal, normal)	250 0	3.999 $\pm$ 1.130 < 0.079	0.447 $\pm$ 0.115 < 0.039	6.95 $\pm$ 0.41 < 0.72	28.26 $\pm$ 14.21 < 1.82
Macrophages (monocyte-derived)	250 0	0.466 $\pm$ 0.290 < 0.06	3.689 $\pm$ 1.087 < 0.03	159.63 $\pm$ 51.22 1.95 $\pm$ 1.27]●	2.19 $\pm$ 1.08 < 0.3

Derivatization of biopterin to pterin-6-carboxylic acid

Extract of T 24 cells treated with 250 i.u. of IFN- γ /ml (containing 2.5 mg of cell protein) (600 ml) was oxidized in acidic-iodine solution, extracted and eluted to the h.p.l.c. system as described above. The intense fluorescent peak at the retention time of biopterin was collected. Of this fraction, 100 μ l was then incubated with 10 μ l of 1 M-NaOH and 50 μ l of 0.02% KMnO₄ for 15 min at 60 °C. After addition of 2 μ l of 0.1 M-ascorbic acid and 10 μ l of 1 M-potassium phosphate, pH 6.0, pterin-6-carboxylic acid was quantified by direct injection into the h.p.l.c. system.

RESULTS

Concerted induction of tetrahydrobiopterin biosynthesis and IDO by IFN- γ

Table 1 shows IDO activity and pteridine synthesis of six human tumour-cell lines from various tissue origins, of human dermal fibroblasts and of human macrophages derived from peripheral-blood monocytes. Activity of GTP cyclohydrolase, the key enzyme of pteridine biosynthesis and intracellular concentrations of neopterin and biopterin were distinctly increased by IFN- γ in all cells investigated. T 24 cells, the only cells with constitutive IDO activity, showed considerable pteridine synthesis also when unstimulated. Chemical identity of biopterin was confirmed with a fraction purified by h.p.l.c. from cell extract of IFN- γ -stimulated T 24 cells, which showed fluorescence spectra identical with that of

Table 2. Pteridines released into the supernatant by IFN- γ stimulated cells

Neopterin and biopterin accumulated in the supernatant during 48 h by IFN- γ (250 i.u./ml)-treated human cells were determined after oxidation with acidic iodine by h.p.l.c. [29]. For tissue origin of the tumour-cell lines and cell-culture conditions, see the Materials and methods section. Levels in unstimulated cells did not differ from medium blanks, except for 47.8 $\pm$ 22.1 pmol of neopterin/mg formed by macrophages. Data are expressed as means $\pm$ S.D. for four experiments and for 15 experiments in the case of macrophages.

Cell line	Pteridine released (pmol/mg)	
	Neopterin	Biopterin
A 431	45.2 $\pm$ 7.8	< 37.5
A 498	116.2 $\pm$ 28.5	139.7 $\pm$ 30.9
A 549	< 17.7	< 44.2
Sk-HEP-1	< 22.6	< 56.5
U 138 MG	111.3 $\pm$ 20.1	102.9 $\pm$ 8.6
T 24	< 39.6	281.7 $\pm$ 70.9
Fibroblasts	39.6 $\pm$ 24.1	205.4 $\pm$ 12.6
Macrophages	867.3 $\pm$ 497.9	< 50

synthetic biopterin and was specifically derivatized by alkaline KMnO₄ to pterin-6-carboxylic acid (results not shown). As determined by the lability to alkaline oxidation [32], biopterin in homogenates occurs to more

than 95% as the tetrahydro derivative, which is the active cofactor form. In contrast, 34–46% of neopterin derivatives are already present in the fully aromatic, fluorescent form (results not shown). The pteridine pattern of macrophages is peculiar in that these cells produce a large excess of neopterin compared with biopterin, whereas, in all other investigated cells, the

Fig. 1. Capacity of tetrahydrobiopterin (BH₄, a) in comparison with ascorbic acid (Asc; b) to act as a cofactor of IDO

Extracts were prepared from IFN- γ (250 i.u./ml, 24 h)-stimulated T 24 cells, and the protein fraction was purified from low-molecular-mass compounds by Sephadex G-25 column chromatography. A 300–400 μ g portion of protein was used for the determination of IDO activity. Various concentrations of either tetrahydrobiopterin or ascorbic acid served as cofactor in the standard assay mixture, which contained 0.1 M-potassium phosphate buffer, pH 7.6, 50 μ M-L-tryptophan, 3×10^4 units of catalase and 20 μ M-Methylene Blue. Data are expressed as means $\pm$ S.D. for three experiments.

Fig. 2. Dependence of pteridine synthesis (a) and IDO activity (b) by human macrophages upon IFN- γ on extracellular L- and D-tryptophan concentration

Human macrophages were purified from peripheral blood as described in the Materials and methods section. On day 3, cells were treated with 250 i.u. of IFN- γ /ml for 72 h in medium containing various concentrations of L-tryptophan ($\blacktriangle$) and D-tryptophan ($\triangle$). D-Tryptophan-containing media were not totally devoid of the L-isomer due to the addition of human serum (final concn. 5 μ mol of L-tryptophan/l). Pteridine synthesis and IDO activity were determined by the formation of neopterin and kynurenine respectively, which were quantified in supernatants by h.p.l.c. (see the Materials and methods section for details). Data are expressed as means $\pm$ S.D. for three experiments.

amounts of neopterin are less than, or equal to, that of biopterin (Table 1). Further, only in IFN- γ -treated macrophages was monapterin [L-threo-6-(1',2',3'-trihydroxypropyl)pterin] detected (11.12 ± 3.26 pmol/mg of protein⁻¹; mean $\pm$ S.D. of five observations). The magnitude of GTP-cyclohydrolase I, of intracellular neopterin and of intracellular biopterin concentrations was not significantly ($P > 0.10$) correlated with the magnitude of the IDO activity. The values for GTP cyclohydrolase correlated significantly only with the sum of neopterin plus biopterin ($P = 0.00019$), but not with neopterin ($P = 0.11$) or biopterin ($P = 0.06$) concentrations alone (linear correlation analysis; calculated from Table 1).

Release of pteridines into the supernatant by IFN- γ -treated human cells

To estimate the potential contribution of the various cells to the pteridine patterns observed in body fluids of patients, we examined the amounts of neopterin and biopterin released by IFN- γ -stimulated cells into the supernatant (Table 2). Macrophages are the most potent producers of neopterin. Although T 24 cells have an even higher GTP cyclohydrolase I activity and intracellular pteridine concentration than macrophages (Table 1), only a much smaller amount of biopterin leaks to the supernatant (Table 2). Comparing intracellular and extracellular concentrations of pteridines in A 431, A 498 and U 138 MG cells (Table 1) also indicates that biopterin derivatives synthesized are retained within the cells more efficiently than are neopterin derivatives.

Cofactor activity of tetrahydrobiopterin in the IDO reaction

Cofactor activity of tetrahydrobiopterin towards IDO was tested in human cell extracts, activity which had thus far only been described for rabbit [16] or mouse [17] tissue. For this purpose, eluates of IFN- γ -treated T 24 cells were incubated with Methylene Blue, catalase and various concentrations of either tetrahydrobiopterin or ascorbic acid (Fig. 1). Whereas tetrahydrobiopterin at 70 μ mol/l stimulated the IDO reaction to half-maximal activity, ascorbic acid at 1300 μ mol/l was required for half-maximal stimulation. At saturating conditions, tetrahydrobiopterin gave only one quarter of the turnover achieved by ascorbic acid (Fig. 1). When Methylene Blue was omitted, with both tetrahydrobiopterin and ascorbate the turnover fell sharply to 0.076 ± 0.010 and 0.091 ± 0.017 nmol/min per mg of protein respectively (mean $\pm$ S.D. for three observations each).

Influence of extracellular L-tryptophan on pteridine synthesis

Depending on the cell type tested, an increase of the extracellular tryptophan concentration has different effects on IDO activity. We investigated how pteridine synthesis was influenced by an alteration of IDO activity through raised L-tryptophan concentrations in the culture medium at a given dose of IFN- γ . The results of such experiments with macrophages are shown in Fig. 2. Increase in the L-tryptophan content of the culture medium was paralleled by both increased IDO activity and pteridine synthesis ($P < 0.0001$ and $P < 0.0002$; linear regression analysis). As a control, corresponding amounts of D-tryptophan did not show a comparable effect (Fig. 2). Addition of L-kynurenine, which is readily

metabolized by both stimulated and unstimulated macrophages [33] also failed to influence pteridine synthesis (results not shown).

In contrast with macrophages, increase of extracellular L-tryptophan leads to a decrease of IDO activity in T 24 cells [31]. In T 24 cells stimulated with IFN- γ (10 units/ml) for 24 h, GTP cyclohydrolase activities (in pmol/min per mg) were 1.11 and 1.13 at an extracellular L-tryptophan concentration of 50 μ M and decreased to 0.577 and 0.556 at 500 μ M extracellular L-tryptophan.

DISCUSSION

The results presented here demonstrate that IFN- γ -induced pteridine synthesis is a more general phenomenon than production of neopterin derivatives by macrophages [9,34]. It is observed in a variety of human cells of various tissue origin and yields tetrahydrobiopterin, the active form of a hydroxylating cofactor. Compared with other inducers of GTP cyclohydrolase I, like reserpine or insulin, which in rat adrenal medulla were shown to lead to 2-fold increases of activity [28], IFN- γ is a far better inducer, causing 5–100-fold increases in GTP cyclohydrolase I activity. In contrast with previous observations [34], we were able to detect small amounts of biopterin derivatives in macrophages. This may be due to the more sensitive methods used here. The ratio of neopterin to biopterin in the cells varies from 0.02 in T 24 to 0.80 in U 138 and 72.9 in macrophages (calculated from Table 1). This may be caused by different relative activities of GTP cyclohydrolase I compared with the enzymes converting dihydroneopterin triphosphate to tetrahydrobiopterin. Depending on the cell type, this ratio might also be influenced by factors determining the chemical stability of dihydroneopterin triphosphate or tetrahydrobiopterin.

Dihydroneopterin and tetrahydrobiopterin were shown to be equally potent feedback inhibitors of GTP cyclohydrolase I [35]. This is consistent with our finding that GTP cyclohydrolase I activities correlated with the sum of neopterin plus biopterin rather than with neopterin or biopterin alone. In body fluids of patients treated with IFN- γ , pronounced increases in neopterin derivatives were accompanied only by much smaller rises in biopterin concentrations [11]. This is compatible with our observation that the neopterin derivatives formed upon IFN- γ treatment are more readily released than tetrahydrobiopterin. Increase in tryptophan in the culture medium led to decreased IDO and GTP cyclohydrolase activities in the tumour cell line T 24. Whether this is functionally related to the reported ability of tryptophan to inhibit the antiproliferative effect of IFN- γ [36] remains to be demonstrated. In contrast, macrophages appear to be peculiar in that both enzyme activities increase upon increase of extracellular tryptophan.

The physiological significance of IFN- γ -induced tetrahydrobiopterin biosynthesis remains to be established. It has never been reported, nor have we been able to detect increased activities of the known tetrahydrobiopterin-dependent aromatic amino acid hydroxylase reactions, i.e. phenylalanine-4-, tyrosine-3- and tryptophan-5-mono-oxygenase, in IFN-treated cells (E. R. Werner, G. Werner-Felmayer, D. Fuchs, A. Hausen, G. Reibnegger & H. Wachter, unpublished work). However, all these cells react to IFN- γ by induction of IDO. Further, we show here that alterations of IDO activity by the substrate

L-tryptophan are paralleled by changes in the activity of GTP cyclohydroxylase I, the key enzyme of tetrahydrobiopterin biosynthesis. We have therefore considered the possibility of an involvement of tetrahydrobiopterin in the IDO reaction. Tetrahydrobiopterin could contribute to this reaction by activation of one of the two oxygen atoms introduced into the tryptophan molecule by IDO, similar to the mechanism in pterin-dependent amino acid hydroxylases [1]. In addition, tetrahydrobiopterin might act by maintaining the catalytically active iron in the ferrous state.

The nature of the physiological cofactor of IDO has not yet been identified unambiguously. *In vitro*, IDO is known to require Methylene Blue and ascorbic acid for full activity. With enzyme purified from mouse epididymis, FMNH₂ has been found to lead to the highest turnover rates [37]. We could not confirm this stimulatory action of FMNH₂ in human T 24 cell extracts [31]. On the basis of investigations with IDO purified from rabbit intestine [16] or mouse epididymis [17], tetrahydrobiopterin has been suggested to be a physiological cofactor of the IDO reaction. We demonstrate here that, in extracts of human T 24 cells also, the IDO reaction is stimulated by submillimolar concentrations of tetrahydrobiopterin. However, the additional requirement of Methylene Blue indicates that the tetrahydrobiopterin-dependent contribution to the IDO reaction may be only a part of a complex mechanism.

Although our results provide several lines of evidence for a functional significance of tetrahydrobiopterin in the IDO reaction, it cannot be ruled out at present that tetrahydrobiopterin is involved in a reaction other than those discussed here, e.g. in the cleavage of ether lipids [38].

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