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Impact of tumour necrosis factor-α and interferon-γ on tetrahydrobiopterin synthesis in murine fibroblasts and macrophages

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Tumour necrosis factor-α causes an up to 30-fold induction of GTP cyclohydrolase I (EC 3.5.4.16) activity in murine dermal fibroblasts in a dose-dependent manner. Owing to the high constitutive activities of 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase (EC 1.1.1.153), this potentiates biosynthesis of tetrahydrobiopterin. Murine macrophages already contain high activities of GTP cyclohydrolase I when unstimulated, and this is further augmented up to 4-fold by tumour necrosis factor-α/interferon-γ. In Western blots an antiserum to murine liver GTP cyclohydrolase I does not stain cell extracts with high enzyme activities, suggesting that the cytokine induced peripheral form of GTP cyclohydrolase I might differ from the liver form.

INTRODUCTION

Tetrahydrobiopterin is synthesized from GTP by a sequence of three enzymes: GTP cyclohydrolase I (EC 3.5.4.16), 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase (EC 1.1.1.153) [1]. The metabolic roles of tetrahydrobiopterin in mammals include action as cofactor in phenylalanine-4-, tyrosine-3- and tryptophan-5-mono-oxygenases, thus being essential for the control of blood phenylalanine levels and neurotransmitter synthesis [2]. In addition, the cleavage of glyceryl ethers in rat liver microsomes [3] and the formation of nitrogen oxides from arginine in cytokine-treated murine cells [4–6] have been found to require tetrahydrobiopterin for full activity.

In humans, neopterin is excreted in increased amounts in patients with diseases challenging the cell-mediated immunity [7,8]. Neopterin is a degradation product of 7,8-dihydroneopterin triphosphate, the first biosynthetic intermediate of tetrahydrobiopterin synthesis [1]. *In vitro*, human macrophages produce high amounts of neopterin when stimulated with interferon-γ derived from activated T-lymphocytes [9]. Interferon-γ activates GTP cyclohydrolase I in human macrophages [10], fibroblasts and tumour-cell lines up to 40-fold [11], in T-cell lines 2.5-fold [12]. This triggers the synthesis of tetrahydrobiopterin inside the cells. Depending on the constitutive activity of the subsequent enzyme, 6-pyruvoyltetrahydropterin synthase, this tetrahydrobiopterin synthesis is accompanied by accumulation of neopterin derivatives [11]. After cleavage of the phosphate moieties by phosphatases the neopterin derivatives leak from the cells. This gives rise to the up-to-100-fold increases of neopterin concentrations in body fluids of patients suffering from diseases with endogenous cytokine formation, e.g. in endotoxic shock (see [8] for a review).

In contrast with the situation in humans, body fluids of mice do not contain neopterin [13], nor is its excretion enhanced in a way similar to humans by immunological challenge. Although minor increases in GTP cyclohydrolase I activity has been observed by treating a murine T-cell line with interleukin 2 [14], the question remained open as to whether murine cells could also be triggered to potentiate their GTP cyclohydrolase I activity in a manner similar to interferon-γ-treated human cells and which cytokine might be capable of doing so. In the present paper we show that tumour necrosis factor-α is a potent GTP cyclohydrolase I-inducing cytokine in murine fibroblasts and, to a lesser extent, in murine macrophages. We compared its action with interferon-γ, the most powerful GTP cyclohydrolase I stimulator in humans. In addition, we investigated the impact of both cytokines on the subsequent enzyme activities of the tetrahydrobiopterin-biosynthetic pathway and tested immunological reactivity of the cell homogenates with an antiserum to murine liver GTP cyclohydrolase I in Western-blot experiments.

EXPERIMENTAL

Materials

Recombinant murine interferon-γ with a specific activity of 10^7 i.u./mg of protein (produced in Chinese-hamster ovary cells) was obtained from Holland Biotechnology (Leiden, The Netherlands). Recombinant murine tumour necrosis factor-α (4×10^7 i.u./mg of protein; expressed in *Escherichia coli*) with an endotoxin content of < 10 pg/mg of protein, as determined by the *Limulus* amoebocyte lysate test, was generously given by Dr. G. R. Adolph (Bender and Co., Vienna, Austria). A rabbit antiserum neutralizing murine tumour necrosis factor-α was purchased from Innogenetics (Antwerp, Belgium). Human recombinant interferon-γ with a specific activity of 2×10^7 i.u./mg of protein was kindly provided by Bioferon (Laupheim, Germany), and human tumour necrosis factor-α (2×10^7 i.u./mg of protein) was obtained from Sigma (Munich, Germany). Roswell Park Memorial Institute tissue-culture medium type 1640 (RPMI 1640) (endotoxin concentration < 1 pg/ml), Dulbecco's modified Eagle's medium, minimum essential medium (Eagle) with Earle's salts, and fetal-calf serum were from Biochrome (Berlin, Germany). All other cell-culture supplements were from Sigma. GTP cyclohydrolase I was purified from *Escherichia coli* (strain B) as described in [15]. Sepiapterin reductase was purified from *Drosophila* (strain Oregon R) as previously described [11]. Pteridines

Abbreviations used: MHC, major histocompatibility complex; RPMI 1640, Roswell Park Memorial Institute tissue-culture medium type 1640.

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were obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). The other compounds were of *Pro Analysi* quality from Merck (Darmstadt, Germany). Tissue-culture plasticware was from Falcon (Beckton–Dickinson, Lincoln Park, NJ, U.S.A.). The protein dye reagent was from Bio-Rad (Richmond, CA, U.S.A.).

H.p.l.c. apparatus

An h.p.l.c. system, consisting of a liquid chromatograph (LC 5500; Varian, Palo Alto, CA, U.S.A.), an LS 4 fluorescence detector (Perkin–Elmer, Beaconsfield, U.K.) and an AASP module (Varian) for direct insertion of solid-phase cartridges, was used for detection of pteridines in enzyme incubation mixtures and cell extracts. Fluid connections of the AASP instrument were modified as described in [16]. Reversed-phase columns were Lichrosorb products (RP18; 7 μm particle size) from Merck; strong-cation-exchange cartridges for solid-phase extraction were from Varian.

Tissue culture

The murine macrophage cell lines P388D$_1$ and J774A.1 were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were maintained in RPMI 1640 medium containing 2 mM-L-glutamine, penicillin (100 i.u./ml), streptomycin (0.1 mg/ml) and 10% (v/v) heat-inactivated fetal-calf serum. Peritoneal exudate cells for purification of resident peritoneal macrophages were obtained from 8–10-week-old Balb/c mice by peritoneal lavage with phosphate-buffered saline (130 mM-NaCl/2 mM-KCl/6 mM-Na_2HPO_4/1 mM-KH_2PO_4), pH 7.4. The thus-obtained peritoneal exudate cells from 30–40 mice for each experiment were kindly provided by Dr. E. Kämpgen, Department of Dermatology, University of Innsbruck. Cells were sedimented at 200 *g*, washed once with RPMI 1640 supplemented as described above and seeded at a density of 10^6/ml. To allow for macrophage adherence, cells were incubated for 2 h at 37 °C in CO_2/air (1:19). Plates were then washed with warm phosphate-buffered saline (see above) to remove non-adherent cells. The cell population obtained by this procedure was about 90% macrophages, as determined by morphology and α-naphthyl acetate esterase staining (Sigma). Murine fibroblasts, kindly provided by Dr. C. Heufler, Department of Dermatology, University of Innsbruck, were isolated from ear dermis explants of 8–10-week-old Balb/c mice. These were allowed to grow for 3–4 weeks with a change of medium every week, using RPMI 1640, supplemented as described above. Using this procedure, only fibroblasts survive in the culture. For experiments, cells of passages 10–20 were used. Human dermal fibroblasts, T 24 bladder carcinoma cells and THP-1 myelomonocytoma cells were grown and stimulated as previously described [11]. For stimulation, cells were cultivated in cytokine-containing medium for 48–72 h. All cells were mycoplasma-negative, as judged by staining with 4′,6-diamidino-2-phenylindole [17].

Preparation of cell extracts

Adherent cells were collected after trypsin treatment or scraped off the plates (P388D$_1$ and J774.A1). Cell extracts were obtained by repeated freezing and thawing of pelleted phosphate-buffered-saline-washed cells in 500 μl of distilled water. Peritoneal macrophages were directly extracted off the plates with distilled water. After centrifugation at 10000 *g* for 10 min, the supernatant was used for determination of intracellular pteridines and enzyme assays.

Determination of intracellular pteridines and of enzyme activities

Intracellular concentrations of reduced and oxidized pteridines were determined by oxidation with iodine in acidic or basic pH as described in [18], and then measured by h.p.l.c. with fluorescence detection [16]. For determination of enzyme activities, protein fractions of cells freed from low-molecular-mass compounds by Sephadex G-25 chromatography, and enzyme assays adapted from previous work were used as described [11]. Briefly, GTP cyclohydrolase I activities were measured by incubation with 2 mM-GTP in the presence of 5 mM-EDTA, the resulting dihydroneopterin triphosphate oxidized with acidic iodine solution, the phosphates cleaved with alkaline phosphatase and the resulting neopterin determined by h.p.l.c. Owing to the presence of EDTA, 6-pyruvoyltetrahydropterin synthase is inhibited and the 7,8-dihydroneopterin triphosphate formed by GTP cyclohydrolase I is not further metabolized.

6-Pyruvoyltetrahydropterin synthase was measured by incubation with dihydroneopterin triphosphate (freshly prepared by the use of GTP cyclohydrolase I from *E. coli*) in the presence of excess sepiapterin reductase, NADPH and Mg^{2+}. The 6-pyruvoyltetrahydropterin initially formed is thus converted into tetrahydrobiopterin, which is determined by h.p.l.c. after oxidation in acidic iodine solution.

Sepiapterin reductase was measured by incubation with sepiapterin and NADPH, followed by oxidation of the resulting tetrahydrobiopterin to biopterin and determination by h.p.l.c. [16].

Protein determination

Protein concentrations of cell extracts and eluates were determined as described by Bradford [19]. Pure BSA (Serva, Heidelberg, Germany) was used as standard.

Detection of major-histocompatibility-complex (MHC) antigens

MHC antigens were detected by enzyme-linked immunofluorescence assay as described in [20], using purified anti-IA^d antibody (Beckton–Dickinson, Mountain View, CA, U.S.A.) and undiluted supernatant from 34-1-2S hybridoma (anti-H2-K^d/D^d; American Type Culture Collection). A horseradish-peroxidase-conjugated antibody to mouse IgG whole molecule (Sigma) served as second antibody.

Western blotting

GTP cyclohydrolase I was purified from mouse liver homogenates (strain C_3H) by $(NH_4)_2SO_4$ fractionation, Ultrogel Ac-A34, DEAE-Trisacryl and GTP–agarose chromatography. Purified protein (150 μg) was used for immunizing New Zealand White rabbits [21]. Fresh livers from three Balb/c mice were washed in 0.1 M-Tris/HCl (pH 7.8)/5 mM-EDTA/0.3 M-KCl/10% (v/v) glycerol, frozen for 1 h at −80 °C and then homogenized under liquid N_2 by means of a pestle. The material was then mixed with 10 ml of the above-described Tris/HCl buffer and cells were disrupted by sonification. After centrifugation at 10000 *g* for 30 min, the homogenate was purified by passing it through Sephadex G-25 columns. Human liver material was generously provided by Dr. E. Ambach, Institute of Forensic Medicine, University of Innsbruck. It was obtained (with informed consent of the next-of-kin) from a 30-year old male 18 h after he had died in an accident. The material was stored frozen at −20 °C for another 20 h. About 10 g of frozen material were then treated as described for mouse liver. Material from cultured cells (murine and human) was derived as described above, using 0.1 M-Tris/HCl, pH 7.8, supplemented as described for mouse liver, instead of distilled water for preparation of cell extracts.

GTP cyclohydrolase I activity was determined by using an aliquot of freshly prepared Sephadex G-25 eluates.

Samples were applied to 10% polyacrylamide gels containing 0.4% SDS as described in [22]. A discontinuous pH-gradient from pH 6.8 to pH 8.8 was used. Samples were denatured by heating to 100 °C for 2 min in the presence of 3% (v/v) SDS and 5% (v/v) 2-mercaptoethanol. Up to 100 μg of total protein were applied per lane. Mouse GTP cyclohydrolase I prepared for immunization was used as positive control and for spiking unknown samples. Prestained molecular-mass markers (Amersham International, Amersham, Bucks., U.K.) were used for calibration. Proteins were blotted to nitrocellulose filters (Schleicher and Schüll; BA 83) as described by Towbin *et al.* [23]. Filters were blocked for 1 h with phosphate-buffered saline (see above), containing 0.02% (v/v) Tween 20, 10% (v/v) fetal-calf serum, 1% (v/v) BSA and 0.5% (v/v) ovalbumin. Filters were then allowed to react with the antiserum to mouse GTP cyclohydrolase I (1:5000) for 90 min and for a further 90 min with an affinity-purified human-serum-adsorbed goat anti-[rabbit-IgG,-A,M(H+L)] second antibody (5 mg/ml, diluted 1:5000), which was conjugated to alkaline phosphatase (Tago, Burlingame, CA, U.S.A.). Antibodies were diluted in the above-described blocking buffer, except that only 1% (v/v) fetal-calf serum was used. After each incubation step, filters were washed for 3 × 15 min with phosphate-buffered saline, containing 0.02% (v/v) Tween 20. Alkaline phosphatase activity was revealed with Nitroblue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate toluidine salt (Sigma) as described in [24].

RESULTS

Table 1 shows GTP-cyclohydrolase I, 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase activities as well as intracellular biopterin concentrations in extracts of murine fibroblasts, peritoneal macrophages and macrophage lines ($P388D_1$ and J774A.1). Untreated cells were compared with cells treated with murine tumour necrosis factor-α (500 i.u./ml), interferon-γ (50 i.u./ml), and a combination of both stimuli. These doses had been previously found to be optimal for pteridine-dependent nitric oxide formation [6]. 6-Pyruvoyltetrahydropterin synthase and sepiapterin reductase were present in a comparable range in all four investigated cells and remained unchanged upon treatment with murine interferon-γ or murine tumour necrosis factor-α. By contrast, GTP cyclohydrolase I was low in untreated fibroblasts and strongly stimulated by tumour necrosis factor-α, leading to a substantial increase in intracellular biopterin levels. GTP cyclohydrolase I activity was only marginally stimulated by 50 i.u./ml of murine interferon-γ; higher doses of murine interferon-γ (500 i.u./ml and 1000 i.u./ml) did not show a stronger stimulation than 50 i.u./ml in any of the tested cell types. Peritoneal macrophages as well as the macrophage lines $P388D_1$ and J774A.1 showed considerable GTP-cyclohydrolase I activity even when untreated. This was slightly enhanced by murine tumour necrosis factor-α or murine interferon-γ and the combination of both stimuli in freshly isolated peritoneal macrophages, but not in the permanent macrophage lines $P388D_1$ and J774A.1. Intracellular levels of biopterin, which occurred to more than 95% as tetrahydro derivative, accordingly showed little change in the murine macrophages and macrophage lines. None of the investigated murine cells contained detectable quantities of neopterin.

Since in human cells interferon-γ is the strongest stimulus of GTP cyclohydrolase I, we wanted to confirm the activity of the murine interferon-γ preparation by measuring induction of MHC-antigen expression [25]. As little as 5 i.u./ml of interferon-γ was able to induce significantly increased expression of MHC class II antigens in $P388D_1$ and J774A.1 (6-fold as compared with controls) as well as MHC class I antigens in fibroblasts (4-fold as compared with controls). Thus the interferon-γ preparation used was highly active on the cells, while having little impact on GTP cyclohydrolase I activities.

Time- and dose-dependence of GTP cyclohydrolase I stimu-

Table 1. Enzyme activities of tetrahydrobiopterin biosynthesis and intracellular biopterin concentrations in murine fibroblasts, murine macrophages and macrophage cell lines with and without cytokine treatment

Murine fibroblasts and macrophages were isolated and grown as detailed in the Experimental section. About 10^7 cells were incubated for 48 h with either medium alone or in the presence of 50 i.u. of interferon-γ (IFN-γ)/ml, 500 i.u. of tumour necrosis factor-α (TNF-α)/ml, or a combination of both. Cells were then harvested, disrupted, and Sephadex G-25 eluates were prepared. Enzyme activities were determined as detailed in the Experimental section. Mean values ± S.D. for three determinations are shown, except for single observations in the case of intracellular biopterin concentrations of peritoneal macrophages. Significance (Student's t test): $^{**}P < 0.01$; $^{*}P < 0.0001$.

Cell type	Treatment	Enzyme activity (pmol·min^{-1}·mg^{-1}) GTP cyclohydrolase I	6-Pyruvoyltetrahydropterin synthase	Sepiapterin reductase	Intracellular biopterin (pmol·mg^{-1})
Fibroblasts	None	0.05 ± 0.01	39.2 ± 3.1	451 ± 31	10 ± 3 *
	TNF-α	1.52 ± 0.05 **	35.8 ± 1.7	466 ± 18	134 ± 18 *
	IFN-γ	0.12 ± 0.01	40.6 ± 2.9	431 ± 28	15 ± 1
	IFN-γ + TNF-α	2.06 ± 0.03	31.6 ± 1.3	475 ± 9	161 ± 18
Peritoneal macrophages	None	1.22 ± 0.41 **	43.3 ± 2.1	189 ± 15	63
	TNF-α	2.55 ± 0.07	42.5 ± 0.9	226 ± 11	102
	IFN-γ	1.64 ± 0.09 *	41.3 ± 1.2	196 ± 7	73
	IFN-γ + TNF-α	4.46 ± 0.07	39.9 ± 1.4	214 ± 8	132
$P388D_1$	None	6.72 ± 0.46	60.4 ± 2.5	299 ± 11	476 ± 38
	TNF-α	6.60 ± 0.10	54.8 ± 4.5	311 ± 22	477 ± 27
	IFN-γ	5.87 ± 0.08	57.2 ± 1.7	293 ± 14	385 ± 43
	IFN-γ + TNF-α	5.76 ± 0.34	57.8 ± 3.1	310 ± 19	456 ± 53
J774A.1	None	1.18 ± 0.13	24.5 ± 2.0	302 ± 37	264 ± 39
	TNF-α	1.14 ± 0.02	32.6 ± 1.8	339 ± 33	276 ± 43
	IFN-γ	1.02 ± 0.06	26.9 ± 1.4	282 ± 52	275 ± 27
	IFN-γ + TNF-α	0.98 ± 0.05	27.1 ± 5.6	300 ± 12	261 ± 31

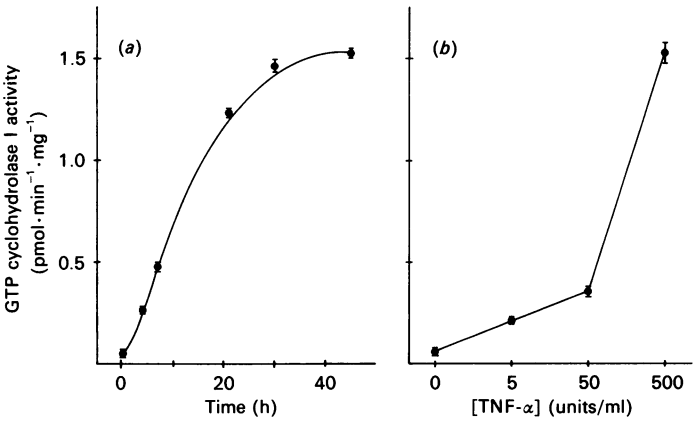

Fig. 1. Time-course (*a*) and dose-dependence (*b*) of GTP cyclohydrolase I activity induced by tumour necrosis factor-α in murine fibroblasts

Confluent monolayers of fibroblasts were treated with 500 i.u./ml of tumour necrosis factor-α at zero time. At the indicated time points (*a*), cells were harvested for determination of GTP cyclohydrolase I activity. For the dose-dependence curve (*b*), confluent monolayers were incubated with various doses of tumour necrosis factor-α for 48 h. GTP cyclohydrolase I activity was determined as detailed in the Experimental section. Values are means ± S.D. for three determinations.

lation in murine fibroblasts by murine tumour necrosis factor-α is presented in Fig. 1. Activity was raised as quickly as 4 h after the addition of murine tumour necrosis factor-α- and reached a plateau after 30 h. Tumour necrosis factor-α at 5 i.u./ml was sufficient to lead to a significant ($P < 0.0001$; Student's t test) stimulation of GTP cyclohydrolase I activity.

We also tested for specificity of the effect of tumour necrosis factor-α on murine fibroblasts by using a neutralizing antiserum to murine tumour necrosis factor-α. GTP cyclohydrolase I activity (pmol·mg^{-1}·min^{-1}) was 0.09 ± 0.002/0.11 ± 0.03 for untreated control cells and 1.53 ± 0.12/1.25 ± 0.02 for cells treated with 500 i.u./ml of murine tumour necrosis factor-α. Concurrent application of the neutralizing antiserum (1000 i.u./ml) completely reduced the enzyme activity to 0.11 ± 0.01/0.07 ± 0.01 (mean ± S.D. for three incubations; two experiments).

To test a previous suggestion of the mechanism of increased pteridine synthesis [14] we investigated the impact of cycloheximide on cytokine induction of pteridine synthesis. As shown in Table 2, cycloheximide inhibited GTP cyclohydrolase I activity in human cells (dermal fibroblasts and THP-1 monocytoma cells). However, in murine fibroblasts cycloheximide did not inhibit cytokine-stimulated GTP cyclohydrolase I activity, but clearly enhanced the enzyme activity.

Finally, we tested cross-reactivity of murine and human cell and liver homogenates with an antiserum prepared against purified mouse liver GTP cyclohydrolase I in Western-blot experiments. As is seen from Fig. 2, human and murine liver homogenates show cross-reactivity with the antiserum (lanes b and g). In contrast, extracts of murine P388D$_1$ macrophages, tumour necrosis factor-α stimulated murine fibroblasts, human T 24 cells (control and interferon-γ treated) as well as human THP-1 cells did not cross-react (lanes c, e, h, i and l). These cell extracts had GTP cyclohydrolase I activities comparable with the liver specimens, and equivalent amounts (10 μg) of protein were applied to each lane. To exclude experimental artifacts, the extracts showing no cross-reactivity were spiked with purified murine GTP cyclohydrolase I, and the cross-reactivity was recovered (lanes d, f, k and m in Fig. 2). Using up to 100 μg of total cell protein did not result in any reaction with the antiserum to GTP cyclohydrolase I.

Table 2. Effect of cycloheximide on GTP cyclohydrolase I activity in murine (MuFB) and human (HFB) fibroblasts and in THP-1 cells

Confluent monolayers of murine or human fibroblasts and THP-1 monocytoma cells (10^7 in 20 ml) were treated with 1 μg of cycloheximide (CHX)/ml, alone or in the presence of cytokines: 50 i.u. of interferon-γ (IFN-γ)/ml plus 500 i.u. of tumour necrosis factor-α (TNF-α)/ml. GTP cyclohydrolase I activity was determined in Sephadex G-25 eluates as described in the Experimental section. Values are pmol of neopterin formed/min per mg of total cell protein. Results are means ± S.D. for three different incubations from one of three similar experiments.

Treatment	GTP cyclohydrolase I pmol·min^{-1}·mg^{-1}		
	MuFB	HFB	THP-1
Control	0.08 ± 0.02	0.02 ± 0.01	0.03 ± 0.01
CHX	0.29 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
IFN-γ/TNF-α	1.98 ± 0.10	2.31 ± 0.07	1.07 ± 0.01
CHX/IFN-γ/TNF-α	3.12 ± 0.06	1.22 ± 0.04	0.34 ± 0.01

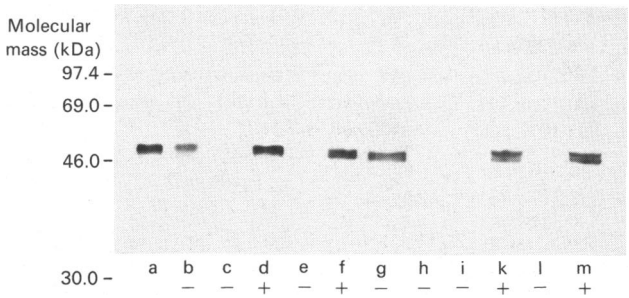

Fig. 2. Western-blot analysis of GTP cyclohydrolase I in murine and human liver homogenates as compared with extracts of cytokine-stimulated cells

Samples were resolved in 10% SDS/polyacrylamide gels and transferred to nitrocellulose. Bands were stained with a rabbit antiserum for GTP cyclohydrolase I of mouse liver and an alkaline phosphatase-conjugated second antibody (see the Experimental section). Purified murine liver GTP cyclohydrolase I (0.15 μg), used for immunization, was run as positive control (lane a). From liver homogenates and cell extracts (see the Experimental section) 10 μg of total protein was applied per lane; b, mouse liver homogenate; c and d, P388D$_1$; e and f, murine fibroblasts treated with 500 i.u. of tumour necrosis factor-α/ml for 48 h; g, human liver homogenate; h, T 24 control cells; i and k, T 24 cells treated with 250 i.u. of interferon-γ/ml for 48 h; and m: interferon-γ-stimulated (250 i.u./ml; 48 h) THP-1 cells. Lanes labelled with '+' had been spiked with 0.15 μg of purified enzyme corresponding to an activity of 0.24 pmol·min^{-1}. GTP cyclohydrolase I activities per lane were as follows: a, 0.24; b, 0.22; c, 0.42; d, 0.42 + 0.24; e, 0.15; f, 0.15 + 0.24; g, 0.05; h, 0.01; i, 0.13; k, 0.13 + 0.24; l, 0.12; and m, 0.12 + 0.24 pmol·min^{-1}. One of a series of five blots with individually prepared and freshly used cell preparations is shown here.

DISCUSSION

Pteridine metabolism in mice and other non-primate mammals differs from humans in that neopterin is not detected in body fluids of mice, and is found to be very low in some non-primate mammals [13]. In humans, neopterin concentrations show pronounced increases in clinical states leading to activation of the

cell-mediated immunity such as viral infections, allograft injections or cytokine treatments (reviewed in [8]). In non-primate mammals several findings suggested that, despite the lack of neopterin formation comparable with that in humans, the pteridine excretion might be stimulated by immunological challenge. This includes increased excretion of 7,8-dihydro-6-hydroxylumazine [26], biopterin [27] and several pteridines [28] in tumour bearing-mice, rats and dogs respectively. Small amounts of neopterin have been detected in sheep after immunization [29]. However, all the changes reported in these studies were of minor extent compared with the large changes seen in humans. On the basis of investigations with cultured cells from man and mouse, it had been argued that the high increase in neopterin in humans was due to the rise in intracellular GTP pools and the lack of feedback inhibition, rather than due to an increase in pteridine-biosynthetic activity [14].

The present study is the first detailed investigation of the impact of a cytokine on pteridine-biosynthetic activities in cultured non-primate, non-neopterin-forming cells. Our results clearly indicate that tumour necrosis factor-α induces in murine fibroblasts an up to 30-fold stimulation of GTP cyclohydrolase I activity, thus potentiating the biosynthesis of tetrahydrobiopterin. Since the enzyme assay incubations were done using protein fractions freed from low-molecular-mass compounds at saturating conditions with respect to GTP, this resembles increase in biosynthetic activity rather than feedback or substrate concentration effects, as suggested previously [14]. 6-Pyruvoyl-tetrahydropterin synthase activities in the cultured murine cells (Table 1) are found to be two orders of magnitude higher than in human cells [11]. Thus the 7,8-dihydroneopterin triphosphate formed by GTP cyclohydrolase I is efficiently converted into tetrahydrobiopterin by the murine cells irrespective of the activation state of GTP cyclohydrolase I. This explains the lack of neopterin accumulation in cytokine-treated murine cells, confirming previous observations made with homogenates of murine and human liver [30].

In contrast with human fibroblasts, GTP cyclohydrolase I activity is much less stimulated by murine interferon-γ in murine fibroblasts. Another difference to the human system is that murine macrophages already contain high GTP-cyclohydrolase I activities when unstimulated. This gives an enzymic basis for the high intracellular tetrahydrobiopterin concentrations in murine macrophage lines [4,5,14]. However, the general mechanism seems to be equivalent in both species: cytokines lead to an increase in GTP cyclohydrolase I activity and thus trigger increased synthesis of tetrahydrobiopterin. To date, the metabolic role of this synthesis has been demonstrated in murine cells only. The tetrahydrobiopterin thus formed provides a cofactor essential to the formation of nitric oxide from arginine [4–6]. This reaction is regulated by the same cytokines as pteridine synthesis. The unstable reaction product nitric oxide is involved in the cytotoxicity of cytokines and in the regulation of blood pressure (summarized in [31]).

The time course of the induction of GTP cyclohydrolase I activity (Fig. 1) would suggest synthesis *de novo* of GTP cyclohydrolase I protein after the cytokine treatment. In human fibroblasts and monocytes (THP-1), cycloheximide drastically reduced cytokine-induced activation of GTP cyclohydrolase I, whereas in murine fibroblasts the enzyme activity was further enhanced by cycloheximide. It was shown previously that transcription of genes induced by interferon-γ requires protein synthesis [32]. On the other hand, it was reported that inhibition of protein synthesis by cycloheximide can activate transcription factors such as NF-κB [33]. This factor is also induced by tumour necrosis factor-α, thus leading to cytokine-induced gene expression without involvement of protein synthesis [34]. Our results obtained with cycloheximide may therefore reflect different pathways of processing of the cytokine signal in the cell types used.

An antiserum prepared to murine liver GTP cyclohydrolase I did not stain a protein extracted from cultured cells in Western-blot experiments, even though a high activity was applied to each lane (Fig. 2). One means of explaining this finding is the assumption that GTP cyclohydrolase I might occur as two distinct molecules: a constitutive liver form, and a cytokine-inducible peripheral form. This would resemble very much the situation with dioxygenases cleaving tryptophan to *N*-formylkynurenine, which occur in a constitutive liver form (tryptophan pyrrolase) and an interferon-inducible peripheral form (indoleamine 2,3-dioxygenase) [35].

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REFERENCES

1. Nichol, C. A., Smith, G. K. & Duch, D. S. (1985) Annu. Rev. Biochem. **54**, 729–764
2. Kaufman, S. (1986) in Chemistry and Biology of Pteridines (Cooper, B. A. & Whitehead, V. M., eds.), pp. 185–200, Walter de Gruyter, Berlin and New York
3. Kaufman, S., Pollock, R. J., Summer, G. K., Das, A. K. & Hajra, A. K. (1990) Biochim. Biophys. Acta **1040**, 19–27
4. Tayeh, M. A. & Marletta, M. A. (1989) J. Biol. Chem. **264**, 19654–19658
5. Kwon, N. S., Nathan, C. F. & Stuehr, D. J. (1989) J. Biol. Chem. **264**, 20496–20501
6. Werner-Felmayer, G., Werner, E. R., Fuchs, D., Hausen, A., Reibnegger, G. & Wachter, H. (1990) J. Exp. Med. **172**, 1599–1607
7. Wachter, H., Hausen, A. & Grassmayr, K. (1979) Hoppe-Seyler's Z. Physiol. Chem. **360**, 1957–1960
8. Wachter, H., Fuchs, D., Hausen, A., Reibnegger, G. & Werner, E. R. (1989) Adv. Clin. Chem. **27**, 81–141
9. Huber, C., Batchelor, J. R., Fuchs, D., Hausen, A., Lang, A., Niederwieser, D., Reibnegger, G., Swetly, P., Troppmair, J. & Wachter, H. (1984) J. Exp. Med. **160**, 310–316
10. Schoedon, G., Troppmair, J., Adolf, G., Huber, C. & Niederwieser, A. (1986) J. Interferon Res. **6**, 697–703
11. Werner, E. R., Werner-Felmayer, G., Fuchs, D., Hausen A., Reibnegger, G., Yim, J. J., Pfleiderer, W. & Wachter, H. (1990) J. Biol. Chem. **265**, 3189–3192
12. Ziegler, I., Schott, K., Lübbert, M., Herrmann, F., Schwulera, U. & Bacher, A. (1990) J. Biol. Chem. **265**, 17026–17030
13. Duch, D. S., Bowers, S. W., Woolf, J. H. & Nichol, C. A. (1984) Life Sci. **35**, 1895–1901
14. Schoedon, G., Troppmair, J., Fontana, A., Huber, C., Curtius, H.-C. & Niederwieser, A. (1987) Eur. J. Biochem. **166**, 303–310
15. Yim, J. J. & Brown, G. M. (1976) J. Biol. Chem. **251**, 5087–5094
16. Werner, E. R., Fuchs, D., Hausen, A., Reibnegger, G. & Wachter, H. (1987) Clin. Chem. **33**, 2028–2033
17. Russel, W. C., Newman, C., Williamson, D. H. (1975) Nature (London) **253**, 461–462
18. Fukushima, T. & Nixon, J. C. (1980) Anal. Biochem. **102**, 176–188
19. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
20. Warren, M. K. & Vogel, S. N. (1985) J. Immunol. **134**, 982–989
21. Cha, K. W., Jacobson, K. B. & Yim, J. J. (1991) J. Biol. Chem. **266**, 12294–12300
22. Laemmli, U. K. (1979) Nature (London) **227**, 680–685
23. Towbin, H., Staehlin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
24. Leary, J. L., Brigati, D. J. & Ward, D. C. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 4045–4049
25. Wong, G. H. W., Clark-Lewis, I., Harris, A. W. & Schrader, J. W. (1984) Eur. J. Immunol. **14**, 52–56
26. Wachter, H., Grassmayr, K. & Hausen, A. (1979) Cancer Lett. **6**, 61–66
27. Mura, P., Morlier, J. P., Barriere, M., Bredon, P., Papet, Y. & Chameaud, J. (1990) Cancer Lett. **54**, 57–60
28. Goldberg, M., Gassner, F. & Merkenschlager, M. (1987) J. Vet. Med. A. **34**, 481–486

29. Rokos, H., Bienhaus, G. & Kunze, R. (1985) in Biochemical and Clinical Aspects of Pteridines (Wachter, H., Curtius, H. C. & Pfleiderer, W., eds.), pp. 313–318, Walter de Gruyter, Berlin and New York
30. Hasler, T. & Niederwieser, A. (1986) in Chemistry and Biology of Pteridines (Cooper, B. A. & Whitehead, V. M., eds.), pp. 319–322, Walter de Gruyter, Berlin and New York
31. Marletta, M. A. (1989) Trends Biochem. Sci. **14**, 488–492
32. Faltynek, C. R., McCandless, S., Chebath, J. & Baglioni, C. (1985) Virology **144**, 173–180
33. Osborn, L., Kunkel, S. & Nabel, G. J. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 2336–2340
34. Sen, R. & Baltimore, D. (1986) Cell (Cambridge, Mass.) **47**, 921–928
35. Watanabe, Y., Yoshida, R., Sono, M. & Hayaishi, O. (1981) J. Histochem. Cytochem. **29**, 623–632

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