Induction of pterin synthesis is not required for cytokine-stimulated tryptophan metabolism

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Activation of the immune system which occurs in inflammatory diseases leads to parallel increases in pterin synthesis and increased production of neuroactive L-tryptophan metabolites. Several model systems were studied to determine whether pterins, which are cofactors for hydroxylation reactions, could be required in the oxidative kynurenine pathway of L-tryptophan degradation. Treatment of mice with interferon- γ increased L-tryptophan metabolism without any corresponding change in tissue bipterin concentrations. Cytokine-treated human fibroblasts, macrophages and glioblastoma cells all showed increases in kynurenine production, which were completely independent of

pterin synthesis. When pterin synthesis *de novo* was blocked, either by an inhibitor of GTP cyclohydrolase or because of a genetic deficiency of one of the enzymes of the pathway of pterin biosynthesis, cytokine-stimulated increases in tryptophan metabolism were unaffected. Furthermore, increasing intracellular tetrahydrobiopterin concentrations by treating cells with sepiapterin also had no effect on markers of tryptophan metabolism. Therefore, both normal and cytokine-stimulated L-tryptophan metabolism appears to be completely independent of pterin biosynthesis.

INTRODUCTION

Interferon- γ (IFN- γ), produced during immune activation in inflammatory diseases, stimulates the kynurenine pathway of L-tryptophan metabolism (Pfefferkorn et al., 1986). L-Tryptophan depletion, mediated via IFN- γ -dependent activation of indoleamine 2,3-dioxygenase (IDO), may play a role in the anti-proliferative effect of IFN- γ on cells and on invading organisms (Taylor and Feng, 1991). As shown in Figure 1, pterin biosynthesis is another metabolic pathway that is stimulated by the pleiotropic action of IFN- γ which also increases GTP cyclohydrolase activity (Werner et al., 1989; Werner-Felmayer et al., 1989). This stimulation results in an increase in the rate of conversion of GTP into neopterin, a pteridine with no known biological activity, which has nevertheless proven to be a useful 'surrogate' marker for immune activation (for a review, see Muller et al., 1991).

Numerous studies have demonstrated strong correlations in AIDS (acquired immunodeficiency syndrome) and other infectious diseases between IFN- γ levels, disease progression, production of the potential neuroactive L-tryptophan metabolites kynurenine acid and quinolinic acid, and neopterin levels (Heyes et al., 1992b). D-retrovirus-infected macaques have recently received attention as a model system to explore the pathophysiological effects of HIV (human immunodeficiency virus) infection in humans. Cerebrospinal-fluid levels of quinolinic acid and neopterin in infected macaques were significantly elevated and highly correlated with each other and with the stage of the disease (Heyes et al., 1991). Although no biochemical connections between neopterin and L-tryptophan metabolism have yet been established, changes in neopterin levels in extracellular fluids of humans and primates are thought to reflect alterations in rates of intracellular synthesis of tetrahydrobiopterin (BH₄), the cofactor

required for aromatic amino acid hydroxylations. IDO catalyses the superoxide-dependent oxidation of L-tryptophan to N-formylkynurenine (Yamamoto and Hayaishi, 1967). Because BH₄ can function as a cofactor in IDO assays *in vitro*, albeit with lower activity than ascorbate (Nishikimi, 1975; Ozaki et al., 1986), we investigated the possibility that IFN- γ -induced BH₄ synthesis might play a direct cofactor role in the kynurenine pathway. The stimulation of these two pathways by cytokines was studied in an animal model as well as in several types of cells in culture in which BH₄ levels could be altered by genetic or chemical means. Our results strongly suggest that tryptophan metabolism is independent of pterin synthesis, confirming a proposal based on a preliminary study in a T-cell line (Werner et al., 1991).

EXPERIMENTAL

Materials

Recombinant mouse and human IFN- γ and human tumour necrosis factor- α (TNF- α) were from R&D Systems, Minneapolis, MN, U.S.A. 2,4-Diamino-6-hydroxypyrimidine (DAHP) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sepiapterin (6-lactoyl-7,8-dihydropterin) was from B. Schircks Laboratory, Jona, Switzerland. All other reagents were of highest purity available and obtained from various local sources.

Animals

Male C57BL/6 mice were housed and treated with IFN- γ as previously described (Saito et al., 1992). All studies were approved by the NIMH Animal Care and Use Committee, and the

Abbreviations used: IFN- γ , interferon- γ ; TNF- α , tumour necrosis factor- α ; DAHP, 2,4-diamino-6-hydroxypyrimidine; IDO, indoleamine 2,3-dioxygenase; DMEM, Dulbecco's minimum essential medium; BH₄, tetrahydrobiopterin; TDO, L-tryptophan 2,3-dioxygenase; 6PPH₄, 6-pyruvoyl-tetrahydropterin; DHPR, dihydropteridine reductase.

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investigators adhered to the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

Cells and cell culture

Normal human macrophages, purified by elutriation and cultured for 7–10 days in Dulbecco's minimal essential medium (DMEM) containing 20% fetal-bovine serum and 10% human AB type serum were purchased from ABI, Columbia, MD, U.S.A. Normal and BH₄-deficient human fibroblasts were obtained from Dr. George Hoganson, University of Chicago Medical Center, and were cultured in DMEM containing 10% fetal-bovine serum. Other cell lines were from the American Type Culture Collection and were grown as suggested by ATCC. Treatments with cytokines, pterins and inhibitors were as described in Table legends. Macrophages and THP-1 cells were harvested by scraping. Other cells were usually harvested with trypsin and sonicated in 50 mM Tris/HCl (pH 7.4)/1 mM dithiothreitol/0.1 mM EDTA. Proteins were measured with Coomassie Blue dye reagent (Bradford, 1976) from Pierce Chemical Co., Rockford, IL, U.S.A.

Measurement of L-kynurenine levels and activities of IDO and L-tryptophan 2,3-dioxygenase (TDO)

Kynurenine was measured in protein-free samples by h.p.l.c. as described previously (Heyes et al., 1990). Tissue IDO and TDO activities were determined exactly as described by Saito et al. (1991a,b).

Pterin determinations

Pterins were measured by h.p.l.c. with fluorescence detection after oxidation of reduced forms as previously described (Sakai et al., 1993). Usually, in fresh cerebrospinal fluid, serum and urine, most of the biopterin is in the fully reduced form, and about 60% of the total neopterin is in the dihydro form (Levine and Milstien, 1984). In many studies, only the levels of the fully oxidized form of neopterin have been measured, although this probably provides a reliable indication of the total neopterin pool (Fuchs et al., 1989). In the present paper, we will thus for convenience refer to neopterin in discussions, although we always determined total neopterin levels (dihydro plus oxidized).

RESULTS

Chronic treatment of mice with IFN- γ

Chronic treatment of C57BL6 mice with recombinant mouse IFN- γ has previously been shown to mimic the effects of chronic immune stimulation in humans and primates on accumulation of neuroactive tryptophan metabolites, which occurs in inflammatory diseases (Saito et al., 1992). In similar experiments to those reported previously (Saito et al., 1992), C57BL6 mice were treated daily for 6 days with 20000 i.u. of IFN- γ and then killed on day 7. In agreement with previous studies, there were significant increases in L-tryptophan metabolism, as determined by measurements of plasma kynurenine levels and tissue IDO activity (Table 1). The largest increases were found in the lung, where the IDO activity in IFN- γ -treated animals was 26-fold greater than in the controls. There were no significant increases in pterin synthesis, except in the lung, as determined by measure-

ments of plasma and tissue BH₄ levels (Table 1). However, BH₄ levels in lung only increased by 50%, a value considerably lower than the increase in IDO activity.

Effect of cytokines on synthesis of pterins and metabolism of tryptophan in human fibroblasts

It has previously been demonstrated that cultures of human fibroblasts treated with IFN- γ are stimulated to produce pterins (Werner-Felmayer et al., 1989). Large amounts of dihydro-neopterin are excreted into the medium, intracellular BH₄ increases dramatically from essentially undetectable levels, and there is a parallel increase in metabolism of L-tryptophan due to activation of IDO (Werner et al., 1989). In order to explore possible biochemical interdependencies between these two pathways in human cells, we examined the effects of interferences

Table 1 Effect of IFN- γ treatment on L-tryptophan metabolism and pterin synthesis in C57BL6 mice

Mice were injected intraperitoneally with 20000 units of IFN- γ daily for 6 days and killed on the next day. Total biopterin (BIO; pmol/ml in plasma or pmol/mg of protein in tissue extracts), kynurenine (KYN; μ M in plasma), IDO activity (nmol of KYN/h per g) and liver TDO activity (μ mol of KYN/h per g) were determined. The values in parentheses are the numbers of mice in each group. BIO was measured in different animals from those used for KYN, IDO and TDO. The results are expressed as means $\pm$ S.D.: * $P \leq 0.002$ compared with saline controls.

Tissue	Amount or activity	Saline controls	IFN- γ -treated
Plasma	BIO	225 $\pm$ 58.9 (6)	263 $\pm$ 56.6 (5)
	KYN	1.04 $\pm$ 0.14 (4)	3.71 $\pm$ 0.72* (6)
Brain	BIO	21.0 $\pm$ 3.1 (6)	21.9 $\pm$ 5.4 (6)
	IDO	2.62 $\pm$ 0.53 (8)	4.17 $\pm$ 0.73* (9)
Lung	BIO	24.9 $\pm$ 2.2 (6)	37.4 $\pm$ 7.0* (6)
	IDO	22.0 $\pm$ 5.7 (8)	581 $\pm$ 111* (8)
Liver	BIO	113 $\pm$ 22.5 (6)	118 $\pm$ 27.4 (6)
	TDO	3.33 $\pm$ 0.23 (7)	2.52 $\pm$ 0.38 (8)

Table 2 Effect of IFN- γ and TNF- α on pterin synthesis and L-tryptophan metabolism in human fibroblasts

Confluent cultures of human skin fibroblasts were cultured for 48 h in the absence (None) or presence of cytokines: IFN- γ (250 units/ml) plus TNF- α (100 units/ml). Cells were harvested and kynurenine (KYN; μ M) in media and intracellular neopterin (NEO) and biopterin (BIO) (pmol/mg) measured as described in the Experimental section. The data are expressed as means $\pm$ S.D. from triplicate determinations: ND, none detected (< 1.0 pmol/mg). All KYN values from cytokine-treated fibroblasts were significantly greater than control values ($P \leq 0.001$). Def., deficient.

Fibroblasts	Treatment	Pterin		Tryptophan metabolism KYN
		NEO	BIO	
Control	None	ND	ND	0.6 $\pm$ 0.2
	Cytokines	43.7 $\pm$ 3.3	1141 $\pm$ 141	67.2 $\pm$ 15.0
	Cytokines + DAHP	3.4 $\pm$ 0.4	6.7 $\pm$ 1.3	62.4 $\pm$ 2.1
6PPH ₄ synthase Def.	None	ND	ND	0.7 $\pm$ 0.2
	Cytokines	660 $\pm$ 41.2	ND	47.3 $\pm$ 1.3
	Cytokines + SP	55.6 $\pm$ 4.8	1921 $\pm$ 81.6	46.6 $\pm$ 2.3
DHPR Def.	None	ND	ND	0.5 $\pm$ 0.02
	Cytokines	194 $\pm$ 31.6	1068 $\pm$ 254	66.0 $\pm$ 3.2

Figure 1 Metabolism of tryptophan and biosynthesis of BH₄

IFN- γ stimulates both IDO and GTPCH, the rate-limiting enzymes of each pathway. GTP cyclohydrolase (GTPCH) is inhibited by DAHP. The synthesis of unconjugated pterins in rodents is subject to different rate-limiting factors from those in humans and primates, since rodents have high 6PPH₄ synthase activity relative to GTPCH activity and do not produce any dihydroneopterin as a by-product of BH₄ synthesis, even after immune stimulation (Wachter et al., 1992). In humans and primates, a significant fraction of the dihydroneopterin triphosphate is dephosphorylated to dihydroneopterin, which is then transported to the extracellular space, where some becomes oxidized, either enzymically or non-enzymically, to neopterin. Reactions 1, 2 and 3 are catalysed by the enzymes 6PPH₄ synthase, sepiapterin reductase and dihydrofolate reductase respectively.

with pterin synthesis *de novo* by genetic or chemical means on the stimulated L-tryptophan-oxidative pathway in cultured human skin fibroblasts. Treatment of control fibroblasts with 5 mM DAHP, an inhibitor of GTP cyclohydrolase, prevented the cytokine-dependent increase in both dihydroneopterin and BH₄ without significantly affecting the stimulated rate of production of kynurenine (Table 2). Fibroblasts from a patient with a genetic deficiency of BH₄, resulting from an absence of 6-pyruvoyl-tetrahydropterin (6PPH₄) synthase (Niederwieser et al., 1985), were still stimulated by cytokines to produce large amounts of kynurenine. As expected (see Figure 1), stimulation of pterin synthesis in 6PPH₄ synthase-deficient cells, results in a large increase in the production and release of dihydroneopterin in the absence of any significant production of BH₄. Furthermore, supplementation of 6PPH₄ synthase-deficient fibroblasts with sepiapterin, which results in large increases in intracellular BH₄ levels produced via the salvage pathway (Figure 1) (Nichol et al., 1983), also had no effect on the rate of formation of kynurenine in these mutant cells. It should be noted that sepiapterin, dihydrobiopterin and tetrahydrobiopterin have all been shown to be inhibitors of GTP cyclohydrolase (Shen et al., 1988), and, in these studies, also prevented the cytokine-induced increase in neopterin. Additional evidence for a lack of any connection between the synthesis of BH₄ and the formation of kynurenine was demonstrated by the absence of an effect of dihydropteridine reductase (DHPR) deficiency on kynurenine production in cultures of fibroblasts from a DHPR-deficient patient. Since DHPR plays a major role in maintaining BH₄ in its fully reduced tetrahydro form (Kaufman et al., 1975), in these DHPR-deficient fibroblasts 50–75% of the cytokine-stimulated biopterin was present as the oxidized, hydroxylation-cofactor-inactive, dihydro form.

Effect of cytokines on human macrophages

Macrophages are considered to be the major source of the IFN- γ -dependent increase in circulating neopterin levels following immune activation (Huber et al., 1984). The oxidation of L-tryptophan to kynurenine in macrophages has also been found

Table 3 Effect of cytokines on levels of pterins and L-tryptophan metabolism in human monocyte/macrophages

Human monocyte/macrophages (2×10^7 cells) cultured in 25 cm² flasks were treated for 48 h (a) with the indicated concentrations of IFN- γ , or for 48 h (b) in 12-well clusters (2.5×10^6 cells/well) with IFN- γ (100 units/ml) in the absence or presence of 5 mM DAHP or 50 μ M sepiapterin (SP). Kynurenine (KYN; μ M) in the media and intracellular neopterin (NEO) and biopterin (BIO) (pmol/mg in a and pmol/well in b) were measured as described in the Experimental section. The data are expressed as means $\pm$ S.D. from triplicate determinations. ND, none detected (< 1.0 pmol/mg or < 0.1 pmol/well). This experiment was representative of results found with monocyte/macrophage cultures from three other individuals. All KYN values from IFN- γ -treated cells were significantly greater than control values ($P \leq 0.01$).

Treatment	Pterin		Tryptophan metabolism KYN
	NEO	BIO	
(a) None	66.0 $\pm$ 6.3	ND	8.8 $\pm$ 2.6
IFN- γ (10 units/ml)	379 $\pm$ 62.8	ND	40.4 $\pm$ 3.4
IFN- γ (100 units/ml)	507 $\pm$ 26.9	ND	49.4 $\pm$ 5.2
IFN- γ (1000 units/ml)	501 $\pm$ 139	ND	43.7 $\pm$ 5.5
(b) None	0.64 $\pm$ 0.07	ND	3.23 $\pm$ 0.1
IFN- γ (100 units/ml)	32.9 $\pm$ 0.95	ND	23.0 $\pm$ 2.1
IFN- γ (100 units/ml) + DAHP	4.44 $\pm$ 0.52	ND	26.7 $\pm$ 1.5
IFN- γ (100 units/ml) + SP	3.76 $\pm$ 1.12	446 $\pm$ 131	23.5 $\pm$ 0.8

to be stimulated by IFN- γ (Werner-Felmayer et al., 1989). As shown in Table 3, treatment of human macrophages with IFN- γ resulted in large increases in neopterin synthesis as well as kynurenine production. Macrophages have been shown previously to produce neopterin and release it into the medium (Huber et al., 1984). We also found large corresponding increases in release of neopterin into the medium after stimulation with IFN- γ (results not shown). Since BH₄ is usually retained within cells, in most experiments we have reported intracellular pterin levels as an index of pterin synthesis rates.

Inhibition of macrophage GTP cyclohydrolase activity with DAHP (5 mM), which inhibited neopterin synthesis by more than 85%, had no effect on kynurenine production. Since the highly enriched human macrophages used in these studies did not produce or release detectable amounts of BH₄, we also examined the effect of increasing intracellular BH₄ levels with exogenous sepiapterin. Addition of sepiapterin to the cells increased the intracellular level of BH₄, presumably via the sequential action of sepiapterin reductase and dihydrofolate reductase (Nichol et al., 1983), and inhibited neopterin production by its effect on GTP cyclohydrolase as described above, yet also had no effect on the rate of kynurenine production.

Effect of IFN- γ on pterins and L-tryptophan metabolism in other human cell lines

Since macrophages apparently synthesize little, if any, BH₄, we examined the effects of IFN- γ on several other human cell lines known to contain the pathway for pterin biosynthesis *de novo*. In U373 glioblastoma cells, both the synthesis of BH₄ and the production of kynurenine are strongly stimulated by IFN- γ (Table 4). However, as with both human fibroblasts and macrophages, inhibition of BH₄ synthesis with DAHP, or increasing BH₄ levels with sepiapterin, had no effects on the rate of kynurenine production. The monocytic leukaemia cell line THP-1 does not produce pterins in the absence of stimulation. After treatment with IFN- γ , large amounts of neopterin and smaller amounts of biopterin are produced concurrently with increased kynurenine. THP-1 cells, however, showed a similar lack of

Table 4 Effect of IFN- γ on pterin synthesis and L-tryptophan metabolism in human glioblastoma U373 and monocytic THP-1 cells

U373 and THP-1 cells, $(1-2) \times 10^6$ /well in 6-well clusters, were treated for 48 h with IFN- γ (100 units/ml) in the absence or presence of 5 mM DAHP or 100 μ M sepiapterin (SP). The media were removed and cells were washed and harvested. Intracellular biopterin (BIO) and neopterin (NEO) (pmol/mg) and kynurenine (KYN; μ M) in the medium were measured as described in the Experimental section. The data are expressed as means $\pm$ S.D. from triplicate determinations and are from a typical experiment repeated three times: ND, none detected (< 1.0 pmol/mg). All KYN values from IFN- γ -treated cells were significantly greater than control values ($P \leq 0.001$).

Cells	Treatment	Pterin		Tryptophan metabolism KYN
		NEO	BIO	
U373	None	ND	ND	2.05 $\pm$ 0.08
	IFN- γ	ND	12.9 $\pm$ 0.60	38.4 $\pm$ 0.65
	IFN- γ + DAHP	ND	ND	37.3 $\pm$ 0.68
	IFN- γ + SP	ND	11 184 $\pm$ 261	34.5 $\pm$ 0.81
THP-1	None	ND	ND	0.88 $\pm$ 0.07
	IFN- γ	222 $\pm$ 17.1	3.37 $\pm$ 0.57	12.3 $\pm$ 0.50
	IFN- γ + DAHP	9.68 $\pm$ 2.58	ND	14.0 $\pm$ 0.68
	IFN- γ + SP	11.9 $\pm$ 1.43	14 551 $\pm$ 679	13.8 $\pm$ 1.80

response to alterations of intracellular pterin levels, where the rate of the IFN- γ -stimulated formation of kynurenine was also completely unaffected by the presence or absence of intracellular BH₄ or by inhibition of IFN- γ -induced neopterin production.

DISCUSSION

The mechanisms of the neurological dysfunctions, seizures and brain atrophy that occur in inflammatory neurological diseases are not understood. Overwhelming evidence has been presented recently linking increased L-tryptophan metabolism and subsequent formation and accumulation of neuroactive tryptophan metabolites, such as quinolinic acid and kynurenine, within the central nervous system and a broad spectrum of neurological diseases associated with stimulation of the immune system (Heyes et al., 1992b). The increased rate of flux of L-tryptophan through the kynurenine pathway appears to be dependent on IFN- γ -induced induction of IDO within the central nervous system (Heyes et al., 1992b). Decreased serum tryptophan levels accompanied by corresponding increased kynurenine levels have also been correlated with increased serum neopterin and endogenous IFN- γ levels in human HIV-1 infection (Fuchs et al., 1990). In addition, we have shown that there is a high degree of correlation between quinolinic acid and neopterin in cerebrospinal fluid and the clinical and viral status of D-retrovirus-infected rhesus macaques (Heyes et al., 1991). Numerous studies have now established that increased production of neopterin is a useful surrogate marker for activation of the immune system and increased production of IFN- γ (for review, see Wachter et al., 1989). Because of the parallel induction of both pterin synthesis and IDO activity by IFN- γ in human cells, and because BH₄ can serve as a cofactor in the IDO reaction (Werner et al., 1989), the latter authors suggested that increased neopterin could reflect increased intracellular synthesis of BH₄, which might be necessary to support the increased oxidation of L-tryptophan catalysed by IDO. This would be an important function for BH₄, since its biosynthesis *de novo* represents a pharmacologically accessible target.

IDO activity in mice has been shown previously to be increased by treatment with cytokines or immune stimulants, such as IFN- γ , pokeweed mitogen and lipopolysaccharide (Saito et al., 1992). In confirmation of those previous results, we found that tissue IDO activities were significantly increased in mice chronically treated with IFN- γ . However, there were no corresponding increases detectable in tissue biopterin levels. Even in the lung, where IDO activity was increased more than 26-fold, there were only small increases in biopterin concentrations. It should be noted that not only are immune responses somewhat different in rodents compared with humans and primates, but both the metabolism of L-tryptophan and the biosynthesis of pterins are also clearly different in these species. In order to explore further the possible link between increased pterin synthesis and increased L-tryptophan degradation in humans and primates, we examined several other model systems in which flux through the pathway for pterin biosynthesis *de novo* could be altered independently to test the effect on production of neuroactive L-tryptophan metabolites.

As originally shown by Werner et al. (1989), treatment of human fibroblasts with IFN- γ and TNF- α results in induction of pterin synthesis, with a corresponding increase in L-tryptophan metabolism (Table 2). Inhibition of GTP cyclohydrolase activity in the fibroblasts with DAHP almost completely blocks the cytokine-dependent production of neopterin and biopterin without decreasing the stimulated production of kynurenine. Furthermore, when skin fibroblasts from BH₄-deficient children were treated with cytokines, there also was no decrease in the cytokine-stimulated production of kynurenine. Even increasing intracellular levels of BH₄ by addition of sepiapterin had no effect. Thus, in fibroblasts, the induction of L-tryptophan metabolism by IFN- γ does not require the induction of pterin biosynthesis.

In humans and primates, macrophages are the primary source of extracellular neopterin (Huber et al., 1984) and also have the ability to convert tryptophan into quinolinic acid (Heyes et al., 1992a). However, either inhibiting synthesis of neopterin or increasing intracellular levels of biopterin through the salvage pathway in human monocytes/macrophages had no significant effect on the production of kynurenine. Finally, we studied the effects of IFN- γ on several other human cell lines which were also treated with either DAHP or sepiapterin. Again, we found that the cytokine-stimulated metabolism of tryptophan was independent of the level of pterins in either glioblastoma cells or monocytic leukaemia cells.

In conclusion, we have shown that cytokine-stimulated metabolism of tryptophan through the kynurenine pathway is apparently independent of pterin synthesis. Although it is potentially possible that the affinity of IDO for BH₄ *in vivo* is so high that its activity would not be decreased even when BH₄ levels are lower than we can measure, this seems unlikely, on the basis of kinetic measurements *in vitro* (Werner et al., 1989). Therefore the function, if any, of the increase in pterin synthesis found in human inflammatory diseases probably lies elsewhere. The demonstration that cytokine-induced NO production in macrophages is dependent on BH₄ synthesis suggests that this may be an important function for cytokine-induced increases in pterin synthesis (Sakai et al., 1993).

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