Human microglia convert L-tryptophan into the neurotoxin quinolinic acid

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Immune activation leads to accumulations of the neurotoxin and kynurenine pathway metabolite quinolinic acid within the central nervous system of human patients. Whereas macrophages can convert L-tryptophan to quinolinic acid, it is not known whether human brain microglia can synthesize quinolinic acid. Human microglia, peripheral blood macrophages and cultures of human fetal brain cells (astrocytes and neurons) were incubated with [$^{13}C_6$]L-tryptophan in the absence or presence of interferon γ. [$^{13}C_6$]Quinolinic acid was identified and quantified by gas chromatography and electron-capture negative-chemical ionization mass spectrometry. Both L-kynurenine and [$^{13}C_6$]quinolinic acid were produced by unstimulated cultures of microglia and macrophages. Interferon γ, an inducer of indoleamine 2,3-dioxygenase, increased the accumulation of L-kynurenine by all three cell types (to more than 40 μM). Whereas large quantities of [$^{13}C_6$]quinolinic acid were produced by microglia and macrophages (to 438 and 1410 nM respectively), minute quantities of [$^{13}C_6$]quinolinic acid were produced in human fetal brain cultures (not more than 2 nM). Activated microglia and macrophage infiltrates into the brain might be an important source of accelerated conversion of L-tryptophan into quinolinic acid within the central nervous system in inflammatory diseases.

INTRODUCTION

Quinolinic acid is a neurotoxic metabolite that is normally present in low nanomolar concentrations in human brain and cerebrospinal fluid. Substantial increases in quinolinic acid concentrations, however, are found in the brain and cerebrospinal fluid of adult and paediatric patients with a broad spectrum of infectious and other inflammatory neurological diseases [1–4], and correlate with markers of immune activation such as neopterin [4–7]. Such increases in brain quinolinic acid levels to micromolar concentrations might be of neurological significance.

Quinolinic acid is derived from L-tryptophan via the kynurenine pathway. Indoleamine 2,3-dioxygenase is the first enzyme of the kynurenine pathway and converts L-tryptophan into L-kynurenine. Subsequent reactions catalysed by kynurenine 3-hydroxylase, kynureninase and 3-hydroxyanthranilate-3,4-dioxygenase convert L-kynurenine into quinolinic acid. Whereas the induction of indoleamine 2,3-dioxygenase by interferon γ increases the production of L-kynurenine in many different cell types, only certain cell types have been shown to produce quinolinic acid. Macrophages, which can infiltrate the brain in many conditions of brain inflammation, have a particularly high capacity for synthesizing quinolinic acid and might be important sources of quinolinic acid within the central nervous system. Microglia are the 'resident' macrophages within the brain and are also activated in many conditions of brain immune activation, including microbial infection and trauma. The purpose of the present study was to determine whether brain microglia can synthesize quinolinic acid from L-tryptophan.

MATERIALS AND METHODS

Chemicals

Quinolinic acid and 1,1,1,3,3,3-hexafluoropropan-2-ol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trifluoroacetylimidazole was acquired from Pierce Chemical Co. (Rockford, IL, U.S.A.). RPMI-1640, L-glutamine, fetal bovine serum, gentamicin, penicillin and streptomycin were obtained from Biofluids, Inc. (Rockville, MD, U.S.A.). L-[*ring*-$^{13}C_6$]Tryptophan was acquired from MSD Isotopes (Montreal, ON, Canada). Human recombinant interferon γ was obtained from Amgen Biologicals (Thousand Oaks, CA, U.S.A.) and the remaining reagents were obtained from Fisher (Fair Lawn, NJ, U.S.A.).

Preparation of human peripheral blood macrophage cells (PBMC) and brain microglia

PBMC from human volunteers were obtained from ABI Advanced Biotechnologies (Columbia, MD, U.S.A.). Macrophages were incubated in a 75 cm^3 plastic culture flask at a density of 2×10^5 cells per 1 ml of well tissue-culture medium consisting of RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 25 μg/ml gentamicin, 100 i.u./ml penicillin and 100 μg/ml streptomycin.

Human fetal microglia were isolated from a mixed brain-cell culture. Briefly, a single-cell suspension of dissociated human fetal cerebrum was cultured at a density of 2×10^6 cells/ml in two T-150 flasks. Cells were cultured in Dulbecco's modified Eagle's medium with Glutamax, 10% (v/v) pooled human serum and 10% (v/v) giant cell tumour extract, under 5% CO_2 in air. After 10 days *in vitro*, the flasks were placed on an orbital shaker and rotated at 200 rev./min for 3 h. Detached cells were harvested from the supernatant, counted and replated in 12-well plates at a density of 5×10^4 cells/ml per well. The purity of microglial cultures was confirmed by immunocytochemical staining with a variety of macrophage markers including HAM56, RCA-1 and CD68. Approx. 99% of the isolated cells were labelled intensely.

Human fetal brain tissue was derived from a 17-week gestational age fetus [8], and consisted of a heterogeneous population of cells identified as astrocytes (approx. 65%) and neurons (approx. 25%) by using a monoclonal antibody to either the intermediate filaments of glial fibrillary acidic protein (Lab Systems, Amsterdam, The Netherlands) or neurofilament (Boehringer, Mannheim, Germany). Although no myelin or

Abbreviation used: PBMC, peripheral blood macrophage cells.

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myelin basic protein is detected in these cultures, a few cells (less than 1%) are gal/C positive and have been referred to as oligodendrocyte progenitor cells. Cells were expanded in culture into 25 cm^3 flasks and used at the third passage. Each flask contained approx. 2.3×10^6 cells. At this generation period there is no evidence for microglial cells [9] when tested for Fc receptors and reactivity to macrophage markers, including Mac 1. Cultures were incubated in Eagle's minimal essential medium supplemented with 10% (v/v) fetal bovine serum (containing approx. 20 μM unlabelled L-tryptophan) and 25 μg/ml gentamicin.

All cultures were incubated with 50 μM L-[$^{13}C_6$]tryptophan and either with or without interferon γ (100 i.u./ml) at 37 °C in humidified air containing 5% CO_2. Samples of media were collected every 24 h until L-tryptophan was depleted (L-kynurenine + [$^{13}C_6$]quinolinic acid concentrations more than 90% of available L-[$^{13}C_6$]tryptophan). Peak values of [$^{13}C_6$]quinolinic acid and L-kynurenine are presented.

Quantification of L-kynurenine and [$^{13}C_6$]quinolinic acid

L-Kynurenine was measured by HPLC with UV absorption spectrometry as described previously [10]. The amounts of [$^{13}C_6$]quinolinic acid released into the incubation medium were determined by gas chromatography and electron-capture negative-ion chemical ionization mass spectrometry with [$^2H_3$]quinolinic acid as internal standard [11]. Quinolinic acid, [$^2H_3$]quinolinic acid and [$^{13}C_6$]quinolinic acid were esterified to their dihexafluoropropan-2-ol esters without any back-exchange and extracted into 50–500 μl of heptane. Aliquots (1 or 2 μl) of each sample were injected into a 1 m × 0.53 mm internal diam. fused silica pre-column at 80 °C, which was sealed to a 15 m × 0.25 mm internal diam. DB-5 analytical column (J & W Scientific, Folsom, CA, U.S.A.). Column eluates were passed directly into the ion source of a Hewlett-Packard 5988 quadrupole mass spectrometer. Ions corresponding to the intact molecular anions of quinolinic acid (m/z 467), [$^2H_3$]quinolinic acid (m/z 470) and [$^{13}C_6$]quinolinic acid (m/z 473) were quantified as peak areas with high sensitivity (not more than 50 fg per injection).

RESULTS

[$^{13}C_6$]Quinolinic acid was identified in incubation media from both unstimulated and stimulated cultures of both microglia at 96 h and macrophages at 48 h. There was sufficient [$^{13}C_6$]quinolinic acid to obtain a mass spectrum that had ions corresponding to m/z 473, m/z 322 and m/z 167 in proportions typical of quinolinic acid (see [11–13]). Minute ion currents at m/z 473 and m/z 322 were detected in cultures of stimulated human fetal brains at 168 h.

The concentrations of L-kynurenine and [$^{13}C_6$]quinolinic acid, and the ratios of [$^{13}C_6$]quinolinic acid to L-kynurenine levels in the incubation media at equilibrium, are presented in Table 1. High concentrations of L-kynurenine were detected in the medium of control microglia, whereas baseline production in PBMC and fetal brain cultures was minimal. Interferon γ increased L-kynurenine concentrations in the media of all three cell cultures to approximately the same degree at steady-state values (48 h for PBMC, 96 h for microglia and 168 h for human fetal brain cultures), whereas L-tryptophan concentrations were depleted to less than 2%. High concentrations of [$^{13}C_6$]quinolinic acid were found in PBMC and microglia cultures. In contrast, the concentrations of quinolinic acid achieved in fetal brain cultures were substantially smaller.

DISCUSSION

In all three human cell types studied, interferon γ, a potent inducer of indoleamine 2,3-dioxygenase activity, increased the accumulation of L-kynurenine in the incubation medium. The quantities of L-kynurenine produced were similar in the three cultures and accounted for the largest proportion of the L-tryptophan in the original medium. In contrast, the amounts of quinolinic acid accumulated by these three culture types were strikingly different. PBMC accumulated the largest amount of quinolinic acid and had the highest ratio of quinolinic acid to L-kynurenine (0.044). Microglia produced a smaller proportion of quinolinic acid than macrophages (ratio 0.011), whereas the ratio of L-kynurenine to quinolinic acid in fetal brain cultures was 0.000036, a value 1/305 that in microglia. L-Kynurenine is converted into quinolinic acid by reactions catalysed by kynurenine 3-hydroxylase, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase. The ability of interferon γ-stimulated macrophages to convert L-tryptophan into quinolinic acid is in accordance with their high activities of indoleamine 2,3-dioxygenase, kynurenine 3-hydroxylase, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase (M. P. Heyes and K. Saito, unpublished work). Interferon γ-stimulated human fetal brain cultures contained a very high activity of indoleamine 2,3-dioxygenase, which explains their ability to convert L-tryptophan into L-kynurenine. However, human fetal brain cultures had undetectable activities of kynurenine 3-hydroxylase and kynureninase, although they did contain measurable 3-hydroxy-

Table 1 Accumulations of L-kynurenine and [$^{13}C_6$]quinolinic acid, and the ratio of the concentrations of L-kynurenine and [$^{13}C_6$]quinolinic acid in the media of human peripheral blood macrophages (at 48 h), human fetal brain microglia (at 96 h) and mixed cultures of human fetal brain cells (astrocytes and neurons, at 168 h)

The ratio data are from interferon γ-stimulated cells. Cells were incubated with 50 μM L-[$^{13}C_6$]tryptophan (and 5 μM unlabelled L-tryptophan contained within the added serum) either with or without (control) 100 i.u./ml interferon γ. Concentrations are expressed as means ± S.E.M. for three incubation wells for each time. The minimum sensitivities for L-kynurenine and [$^{13}C_6$]quinolinic acid were 0.05 μM and 0.2 nM respectively. Note that baseline L-kynurenine levels in the serum added to the incubation media (approx. 0.05 μM) have been subtracted. For statistical comparisons, undetectable amounts were set at the minimum detectable values. Abbreviation: n.d., not detected. * $P < 0.001$.

Culture	L-Kynurenine (μM)		Quinolinic acid (μM)		Ratio of L-kynurenine to quinolinic acid
	Control	Interferon γ	Control	Interferon γ	
Blood macrophages	2.0 ± 0.5	35.0 ± 3.0*	0.02 ± 0.02	1.41 ± 0.21	0.040
Brain microglia	10.3 ± 0.4	37.5 ± 3.7*	0.23 ± 0.01	0.44 ± 0.02	0.012
Astrocytes and neurons	0.5 ± 0.1	55.1 ± 3.0*	n.d.	0.003 ± 0.001	0.000036

anthranilate 3,4-dioxygenase activity; this explains their relatively low capacity for converting L-kynurenine into quinolinic acid.

Brain microglia are derived from the bone marrow during embryonic development and are viewed as the 'resident' macrophages of the brain. Generally accepted estimates of the prevalence of microglia in the normal brain are approx. 3–4% [14]. Although usually quiescent cells with little volume in the normal brain, once activated this network of cells can represent a prominent volume of brain tissue. Autoimmune disease, physical trauma and infection of the brain with micro-organisms all activate brain microglia. In such neuropathological conditions, quinolinic acid levels in cerebrospinal fluid and brain tissue are increased [1–6]. Quinolinic acid concentrations in brain tissue exceed those in cerebrospinal fluid, and the levels in both brain and cerebrospinal fluid can exceed those in blood by a substantial margin [1,6]. These observations support the notion that brain quinolinic acid derives from intracerebral synthesis rather than simply being derived from blood or the meninges. Clinical studies of humans and non-human primates with inflammatory neurological conditions have shown that the concentrations of quinolinic acid increase to a greater extent than the accumulations in L-kynurenine [5,13,15]. The lower ratio of quinolinic acid to L-kynurenine in the incubation medium (Table 1) might be secondary to leakage of L-kynurenine and other intermediates out of the cells and into the relatively enormous volume of incubation media. It is also possible that the brain transport systems are most efficient in the transfer and metabolism of L-kynurenine rather than quinolinic acid.

When immune activation is restricted largely to the central nervous system compartment, such as in poliovirus infection [13], spinal cord injury [16] and ischaemic brain injury [17], large increases in brain indoleamine 2,3-dioxygenase activity accompany substantial accumulations of quinolinic acid in brain tissue. Macrophage infiltrates and activated microglia are likely to be important sources of quinolinic acid in conditions of brain immune activation. In mice and gerbils, large increases in indoleamine 2,3-dioxygenase activity and quinolinic acid concentrations are also found in brain after systemic immune activation in response to intraperitoneal injections of endotoxin, pokeweed mitogen or interferon γ [18–20]. Macrophage infiltrates are not a feature of these animals. Therefore microglia, as well as entry of quinolinic acid from the blood into the brain, might be an important source of quinolinic acid synthesized within the brain in such circumstances. It remains to be determined whether there are circumstances under which quinolinic acid production in astrocytes can be increased to amounts comparable with those in macrophages and microglia. Although the present study has established that human brain microglia and blood macrophages have a high capacity for producing quinolinic acid, this property might or might not be a feature of microglia and macrophages from other species.

Received 22 May 1996/9 July 1996; accepted 30 July 1996

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