PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, C07K 14/47, A01K 67/027 | **A1**

(11) International Publication Number: **WO 99/29852**

(43) International Publication Date: 17 June 1999 (17.06.99)

(21) International Application Number: PCT/US98/25841

(22) International Filing Date: 4 December 1998 (04.12.98)

(30) Priority Data:

60/067,610	5 December 1997 (05.12.97)	US
60/080,380	1 April 1998 (01.04.98)	US
60/080,384	1 April 1998 (01.04.98)	US

(71) Applicant: MEDICAL COLLEGE OF GEORGIA RESEARCH INSTITUTE, INC. [US/US]; 1120 15th Street, Augusta, GA 30912–4810 (US).

(72) Inventors: MUNN, David; 967 Meigs Street, Augusta, GA 30904 (US). MELLOR, Andrew; 480 Falcon Drive, Augusta, GA 30907 (US).

(74) Agent: PABST, Patrea, L.; Arnall Golden & Gregory, LLP, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309–3450 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HIGH–AFFINITY TRYPTOPHAN TRANSPORTER

(57) Abstract

A high–affinity and extremely selective tryptophan transport system present in human monocyte–derived macrophages is disclosed. Human monocyte–derived macrophages include two distinct transporters, a high–affinity (km=290 ± 160 nM) transporter that is highly specific for tryptophan and a low affinity (km=27 ± 4 μM) transporter that is less specific for tryptophan, consistent with the known system L. The tryptophan transport system is predominantly (86 %) sodium–independent. The high–affinity system is very specific for tryptophan and shows no transport of any other essential amino acids in the tryptophan transport concentration range. This high–affinity system is expressed at very low levels in fresh monocytes, but undergoes a 10–30 fold induction during macrophage differentiation. This high–affinity, tryptophan–selective transport system allows macrophages to take up tryptophan efficiently under conditions of very low substrate concentration, such as can occur at sites of inflammation.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

HIGH-AFFINITY TRYPTOPHAN TRANSPORTER

Background of the Invention

This application claims priority to U.S. Serial No. 60/067,610 entitled "Regulation of T Cell Activation" filed December 5, 1997; U.S. Serial No. 60/080,384 entitled "Regulation of Pregnancy" filed April 1, 1998; and U.S. Serial No. 60/080,380 entitled "IDO Inhibitors for Use as Antiviral Agents" filed April 1, 1998, by David Munn and Andrew Mellor.

This invention is generally in the field of tryptophan transporters, and more specifically is drawn to a novel tryptophan transport system.

The United States government has rights in this invention by virtue of grant K08 HL3395 from the National Institutes of Health.

Tryptophan occupies a unique position at the interface between the immune system and cellular metabolism. It is the only amino acid whose level is specifically and selectively modified in response to signals of infection and inflammation. This is accomplished by the enzyme indoleamine 2,3-dioxygenase (IDO), which rapidly and selectively degrades tryptophan following induction by interferon-γ (IFNγ) and other proinflammatory signals (Taylor and Feng, 1991 *FASEB J.* 5, 2516-2522). The IDO system, whose origins extend back to invertebrates (Suzuki, et al., 1996 *Biochem. Biophys. Acta* 1308, 41-48; Suzuki, 1994 J. Prot. Chem. 13, 9-13) is hypothesized to inhibit the replication of intracellular pathogens (Gupta, et al., 1994 *Infect. Immun.* 62, 2277-2284; Thomas, et al., 1993 *J. Immunol.* 150, 5529-5534) and to

participate in the antiproliferative effect of interferons on host cells (Aune and Pogue, 1989 *J. Clin. Invest.* 84, 863-875; Feng and Taylor 1989, *J. Clin. Invest.* 84, 863-875; Ozaki, et al., 1988, *Proc. Natl. Acad. Sci. USA* 85, 1242-1246). Tryptophan is also unique in that it is the only amino acid whose entry into the protein synthetic pathway is regulated by the immune system. Like IDO, the gene for tryptophanyl-tRNA synthetase is highly induced by IFNγ (Fleckner, et al., 1991 *Proc. Natl. Acad. Sci. USA* 88, 11520-11524; Fleckner, et al., 1991 *Proc. Natl. Acad. Sci. USA* 88, 11520-11524; Fleckner, et al., 1995 Cytokine 7, 70-77; Rubin, et al., 1991 *J. Biol. Chem.* 266, 24245-24248), which has been proposed to assist cells in competing for scarce supplies of tryptophan (Kisselev, 1993 *Biochimie* 75, 1027-103). These observations indicate that tryptophan metabolism plays an important role in host defense.

Activated macrophages display a marked induction of the tryptophan catabolic pathway (Werner, et al., 1987 *Life Sci.* 41, 273-280; Carlin, et al., 1989, *J. Leuk. Biol.* 45, 29-34). These cells are capable of reducing the tryptophan concentration in their local microenvironment to such a low level that cellular proliferation becomes impossible. This mechanism underlies the ability of certain types of macrophages (Munn, et al., 1996 *J. Immunol.* 156, 523-532) to inhibit T cell activation. In order to achieve this effect, however, macrophages must reduce the tryptophan concentration to the low nanomolar range (less than 50 nM), three orders of magnitude below its normal

level. Macrophages can continue to take up tryptophan efficiently at very low substrate concentrations, even when tryptophan is competing with multiple other amino acids, present at much higher concentrations, for transport into the cell.

Based on this analysis, it is postulated that there must be a specific, high affinity tryptophan transporter.

It is therefore an object of the present invention to provide a high-affinity amino acid transport system that is highly specific for tryptophan.

It is another object of the present description to provide methods and reagents for use in isolating and characterizing the cDNA and amino acid sequence of high affinity tryptophan transporters.

It is yet a still further object of the present invention to provide methods and reagents for designing and isolating molecules and drugs that can stimulate or inhibit the binding or transport of tryptophan with high affinity tryptophan transporters.

Summary of the Disclosure

A high affinity and extremely selective tryptophan transport system present in human monocyte-derived macrophages is disclosed. Human monocyte-derived macrophages include two distinct transporters, a high affinity (Km=290 ± 160 nM) transporter that is highly specific for tryptophan and a low affinity (Km=27 ± 4 μM) transporter that is less specific for tryptophan, consistent with

the known system L. The tryptophan transport system is predominantly (86%) sodium-independent. The high-affinity system is very specific for tryptophan and shows no transport of any other essential amino acids in the tryptophan transport concentration range. This high-affinity system is expressed at very low levels in fresh monocytes, but undergoes a 10-30 fold induction during macrophage differentiation. This high affinity, tryptophan-selective transport system allows macrophages to take up tryptophan efficiently under conditions of very low substrate concentration, such as can occur at sites of inflammation.

Methods for the isolation of the cDNA that codes for this high affinity tryptophan transport system are described along with methods for the purification of the high affinity transporter. Methods for obtaining and using molecules which interact with the high affinity transporter are also described. Molecules which are obtained by their competitive binding properties with tryptophan for the high affinity transporter tryptophan binding site are also described. Also described are compositions, and methods for using these compositions, which do not competitively interact with tryptophan for the high affinity transporter tryptophan binding site.

Brief Description of the Drawings

Figures 1a-c show saturation kinetics for uptake low substrate concentrations (less than 2.5 μM) of tryptophan transport for the high affinity transporter. Figure 1A is a graph of the [v] (pmol

Tryptophan/10^6/10 minutes) versus [S] (μM Tryptophan). Monocyte-derived macrophages were incubated with varying concentrations between 0 and 2.5 μM of radiolabeled tryptophan, and substrate uptake over 10 min (V, expressed in pmol tryptophan 10^6 $cells^{-1}$ · 10 min^{-1}) measured as a function of substrate concentration ([S], in μM). The inset is an Eadie-Hofstee plot of the uptake data, yielding a calculated Km of 230 nM in this experiment. The Km values are described in Table 1.

Figure 1B is a graph of the [v] (pmol Tryptophan/10^6/10 minutes) versus v/[S]. The data was derived from an extended titration of tryptophan over a three log range (32 nM to 64 μM), demonstrating the presence of two transport systems with markedly different affinities (all data taken from a single experiment using the same macrophage preparation). The superimposed lines represent predicted uptake at concentrations of less than 1 μM and greater than 8 μM, based on the Km and Vmax values derived from the Lineweaver-Burk plots shown in Figure 1C.

Figure 1C is a Lineweaver-Burk plot for the high affinity data and the low affinity data shown in Figure 1B, demonstrating that each system followed Michaelis-Menten kinetics over the range of concentrations surrounding its Km value.

Figures 2A and 2B are graphs of 1/v (pmol amino acid 10^6 $cells^{-1}$ · 10 min^{-1}) versus 1/[S] (μM) for tryptophan transport of leucine and phenylalanine via the low affinity transport system. The affinity constants for uptake of

radiolabeled (Figure 2A) leucine (26 ± 6 μM) and (Figure 2B) phenylalanine (29 ± 2 μM) were determined by saturation kinetics as described in Figure 1.

Figure 2C is a saturation graph of tryptophan competition with phenylalanine for uptake. The dotted line shows the predicted inhibition curve calculated from Equation 1 based on the observed IC_{50} for the low affinity system L.

Figure 3 is a bar graph of the amount of uptake of different amino acids based on the % of tryptophan uptake. Cross-competition studies were performed using radiolabeled tryptophan (125 nM) versus a panel of unlabeled substrates (8 mM each). All data are expressed as tryptophan uptake relative to controls without added competitor ("CTL").

Figure 4A is a graph of [^{3}H]Tryptophan (pmol Tryptophan/10^6/10 minutes) versus [S] (μM Tryptophan) plotted on a semilog plot. Unlabeled 2-aminobicyclo(2,2,1)-hepatane-2-carboxylic acid (BCH) (diamonds) or tryptophan (squares) were allowed to compete for uptake with radiolabeled tryptophan (125 nM). The dotted lines show the predicted inhibition curves calculated from Equation 1 for single isolated systems, based on the observed IC_{50} values in experiments of 360 nM and 37 μM for tryptophan and BCH, respectively.

Figure 4B and 4C are graphs of V versus V/[S] at a Tryptophan concentration of greater than 8 μM (Figure 5B) and less than 1 μM (Figure 4C). Saturation kinetics for tryptophan uptake were performed as described with reference to Figure 1,

in the presence (circles) or absence (squares) of 500 μM unlabeled BCH. In the concentration range where system L predominated (greater than 8 μM tryptophan) BCH acted as a competitive inhibitor. However, in the range where the high-affinity system predominated (less than 1 μM), BCH acted only as a weak non-competitive inhibitor.

Figure 5 is a graph of ^{3}H]Tryptophan (%control) versus [unlabeled competitor] (μM). Unlabeled phenylalanine (open symbols) was allowed to compete for uptake with radiolabeled tryptophan (125 nM). Equation 1 was used to calculate the predicted inhibition curves (dotted lines) assuming that labeled and unlabeled substrate competed for a single system, with Ki in each case equal to the observed IC_{50}. This model correctly predicted the behavior of unlabeled tryptophan competing for its own uptake (competition for the high-affinity system, solid diamonds), and of phenylalanine at low concentrations (competition for system L, open symbols). However, at high concentrations of phenylalanine the observed data departed significantly from that predicted for a single system. Each of the 3 open symbols show data from separate experiments. A total of 11 experiments were performed using phenylalanine, leucine, tyrosine, or cysteine as competitors, all with similar departure from the predicted single-system kinetics.

Figure 6 is a bar graph of the amount of uptake of different amino acids based on the % of tryptophan uptake. Cross-competition studies were performed as described for Figure 3, but using a

concentration of unlabeled competitor (4 μM) predicted to affect only the high-affinity system.

Figure 7 is a graph of percent labeled tryptophan (250 nM radiolabelled tryptophan) taken up by the high affinity transporter as a function of concentration of either unlabelled tryptophan (diamonds) or unlabeled 1-methyl tryptophan (triangles) of different concentrations. The solid line through the diamonds shows the predicted inhibition curve if tryptophan competed with a Ki equal to the measured Km of the high affinity system (290 nM). The solid line through the triangles shows the predicted inhibition curve if 1-methyl tryptophan competed with a Ki of 30 μM, the measured Km of system L.

Figure 8 is a graph of tryptophan uptake inhibition at 25°C (squares) and 0°C (circles), one of three experiments.

Figure 9A and 9B are graphs of V versus V/[S] Peripheral blood monocytes were allowed to differentiate in vitro for up to 7 days under the influence of MCSF. Saturation kinetics for tryptophan uptake were performed as in Figure 1, over the range of 0.032 -1 μM substrate. (Figure 9A) day 0 (mean Vmax 0.16 ± 0.15 pmol 10^6 $cells^{-1}$· 10 min^{-1}, n = 4 vs. (Figure 9B) day 4-7 (mean Vmax 2.3 ± 0.9, n = 6).

Figure 9C is a graph of Vmax (high affinity transporter) versus Time (days). This graph illustrates the upregulation that takes place during macrophage differentiation.

Detailed Description of the Disclosure

Tryptophan transport Systems in Macrophages

The known sodium-independent systems are summarized in Kakuda J. Exp. Biol. 1994;196:93-108; McGivan Biochem. J. 1994;299:321-324. There is an IFNγ-inducible, high-affinity, tryptophan-selective uptake system in MCSF-derived macrophages.A common feature of both sodium-dependent and sodium-independent systems identified to date is that they all display a relatively modest affinity for substrate (Km of even the "high-affinity") systems in the range of 10-30 μM, and of the low-affinity systems 100-500 μM). This affinity is consistent with the fact that amino acids are normally found *in vivo* in this concentration range. Indeed, given the normal physiologic levels of tryptophan (50 μM), even the possibility of an amino acid transport system with nanomolar affinity seems paradoxical, since it would presumably always exist in a saturated state.

However, the local concentration of a substance in tissues may be markedly different from its concentration in the circulation, due to the limited rate of diffusion through the extracellular space (Casciari, et al., 1988 *Cancer Res.* 48, 3905-3909). Data from multicellular spheroid models show that the distance over which even small molecules can be supplied by diffusion in the face of ongoing consumption is limited to 100-200 micrometers (Li 1982 *Cancer* 50, 2066-2073). Thus, local concentrations of tryptophan in tissues could be much lower than those in plasma if the rate of consumption is high enough to exceed delivery.

This has been verified empirically *in vivo* by Burke, et al. 1995 *Int. J. Cancer* 60, 115-122. Consumption of tryptophan by activated macrophages can be very high. As a part of their role in host defense, macrophages possess the inducible enzyme, indoleamine 2,3-dioxygenase (IDO), which rapidly and selectively degrades tryptophan in response to infection and inflammation (Taylor and Fong 1991 *FASEB J.* 5, 2516-2522). Activated macrophages degrade tryptophan via IDO at a rate which is 100 times their already high rate of tryptophan consumption by cellular metabolism. Thus, it is readily conceivable that tryptophan degradation could outstrip diffusion-limited substrate delivery in tissues, resulting in very low local tryptophan concentrations within the immediate microenvironment of the macrophage.

A second feature of most amino acid transporters is that they typically accept multiple amino acids. However, there also exist a small number of selective transport systems, which display relative specificity for a particular amino acid. These systems generally target amino acids with a special biologic role, e.g., glutamate transport in neurons, or tyrosine transport in melanosomes. In these specialized settings, the local concentration of the amino acid of interest may be low, and it must compete for uptake with other amino acids present in higher concentration. In this context, a selective system for a particular amino acid would be advantageous.

The data in Figures 1a,b,c indicates that tryptophan transport in macrophages is achieved

through two separate transporters. Uptake kinetics performed at the substrate concentrations of interest (sub-micromolar) revealed a saturable system with extremely high affinity for tryptophan. (Figure 1A) Studies over a more extended range of concentrations revealed that uptake did not follow simple Michaelis-Menton kinetics, but appeared to be due to the presence of two separate transport activities. (Figure 1B and 1C).

One system displayed a Km value in the range of 20-30 micromolar and a pattern of substrate specificity consistent with system L (Figure 1B).

Characterization of the High Affinity Transporter

The other tryptophan transporter is a high affinity transport activity with a Km value in the nanomolar range (Km=290 to 340 nM, n=18 experiments with 4 donors), which does not correspond to any known tryptophan transporter (Figure 1C and Table 1). The data shown in Figures 3 and 5 indicate that the transport of tryptophan into macrophages is not completely inhibited by the addition of very high concentrations of similar amino acids. Therefore, macrophages must contain a transporter specific for tryptophan. The data in Figures 4a,b,c confirm that there are two separate transporters for tryptophan present in macrophages. The low affinity transporter is a non-specific transporter of amino acids because BCH competitively inhibits tryptophan transport (Figure 4B). This type of transport is similar to system L. The high affinity system is specific for tryptophan because BCH is not able to compete for transport at

concentrations below which the low affinity transporter can function (Figure 4C).

The high affinity transporter is very specific for tryptophan transport. Other amino acids, including neutral amino acids, do not compete with tryptophan for transport into macrophages, by the high affinity system. The data shown in Figure 6 indicate that unlabeled competitor amino acids, used at 4 micromolar, which should significantly inhibit a system with nanomolar affinity, have little impact on the high affinity transporter in transporting tryptophan. System L with a Km approximately 30 micromolar for both the competitor amino acids and tryptophan is not affected at 4 μM competitor. The uptake of radiolabeled tryptophan (125 nM) was markedly inhibited by 4 micromolar cold tryptophan indicating that 4 μM ligand is sufficient to compete with the radiolabeled ligand as long as the transporter is capable of binding the ligand. In contrast, 4 μM of any of the tested amino acids (including BCH) was unable to out compete the tryptophan transport.

The 5-hydroxy derivative of tryptophan, which differs from native tryptophan only in a single substitution on the side chain, similarly failed to compete for transport via the high-affinity system (Figure 7), indicating the exquisite specificity of the high-affinity system for tryptophan. Some amino acids showed non-competitive inhibition of the high-affinity system at high concentrations. However, even at millimolar concentrations 20,000 times greater than the Km of the high-affinity system, no competing amino acid except tryptophan

itself could fully inhibit uptake of labeled tryptophan.

The data shown in Figure 8 indicates that the higher association of tryptophan, relative to other amino acids, with macrophages is caused by increased relative transport at low concentrations, not simple binding to the macrophage surface. If the observed results are due to simple binding, a reduction in temperature should slow the rate of binding, but the reaction should come to equilibrium. The data in Figure 8 indicate that the amount of tryptophan associated with the macrophages is decreased and it is not in equilibrium, indicating a reduced rate of influx. The Vmax of tryptophan transport by the high affinity transporter, normalized by the number of cells, increases when peripheral blood monocytes are induced to differentiate by growth in the presence of macrophage colony stimulating factor (MCSF) Figures 9a,b,c). This increase in velocity is likely caused by an upregulation of the expression of the high affinity tryptophan transporter in macrophages, relative to the progenitor peripheral blood cells.

The high affinity transporter represents an excellent target for the inhibition or activation of tryptophan transport. Tryptophan uptake in macrophages represents a special case in which both high affinity and high selectivity are necessary. At nanomolar substrate concentrations, tryptophan uptake via system L would become inefficient, and it would be rendered even less efficient by the fact that multiple other substrates would compete

for uptake. When Equation 1 is used to predict the uptake of low concentrations of tryptophan via system L alone (50 nM tryptophan, 1 mM total other competing amino acids, and Km and Vmax values as in Figure 1) uptake is calculated to be 1.5 fmol 10^6 $cells^{-1}$ $10\ min^{-1}$. The presence of a selective high-affinity system as described herein would increase tryptophan transport greater than 100-fold, to 70 fmol 10^6 $cells^{-1}$ $10\ min^{-1}$. It is postulated that macrophages represent a specialized cell type which due to their own high IDO activity must be prepared to function in settings where the local tryptophan concentration is extremely low. A selective, high-affinity transport system would allow macrophages to continue to take up tryptophan efficiently, even under conditions where other cell types could not. Such a transporter could play an important role in supplying substrate to the IDO enzyme system for degradation, and also in allowing macrophages to compete with other cells for available tryptophan.

One significant feature of the transport system is its selective upregulation during macrophage differentiation. Unlike system L, which is constitutively present in fresh monocytes and only modestly increased in mature macrophages (approximately proportional to the increase in cell size), the high-affinity system undergoes 10- to 30-fold upregulation over the same period. This differentiation step from monocyte to macrophages is also known to produce massive increases in both IFNγ-inducible IDO activity (Carlin, et al., 1989 *J. Leuk. Biol.* 45, 29-34) and IFNγ-inducible tryptophanyl-tRNA synthetase activity (Krause, et

al., 1996 *J. Leuk. Biol.* 60, 540-545). Thus, the high-affinity tryptophan transporter is coordinately regulated during macrophage differentiation with two other genes known to be involved in immunoregulated tryptophan metabolism, indicating that the transporter participates in the tryptophan-mediated Mϕ immune response.

The following examples examine expression of the high-affinity transporter in macrophages, where it was first identified. However, it is expected to be present in other cell types which may also have specialized needs for tryptophan transport, such as cells of the nervous system, placenta, or blood-brain barrier.

Isolation of the High Affinity Transporter

The transporter can be isolated using standard techniques, based on the presence relative to the cell walls of the macrophages. Examples of techniques that can be used include differential display and macrophage expression libraries which are screened based on the high affinity and selectivity for tryptophan binding, as described in the examples, then purified using traditional chromatography, membrane fractionation, or other standard techniques. Alternatively, the gene encoding the transporter can be cloned as described below, and the gene (or cDNA) expressed to produce a recombinant protein.

Cloning of the High Affinity Transporter

The unusual features of the transporter can be used to clone the transporter. The transporter is unique in its extremely high affinity for tryptophan, and its selectivity for tryptophan over

other amino acids. The previously identified amino acid transporters which accept tryptophan all have affinities in the micromolar-to-milimolar range, and all accept other amino acids which compete with tryptophan for uptake. Thus, under conditions in which the tryptophan concentration is very low (<100 nM) and the total concentration of competing amino acids is very high (greater than 1 mM), cells without the high-affinity transporter would be unable to take up tryptophan efficiently and could not proliferate. However, under these same conditions cells with a high-affinity, tryptophan-selective transporter would still be able to take up tryptophan. Thus, the high-affinity transporter would confer a proliferative advantage under the conditions described.

Applications for the high affinity transporter with the cloned or purified transporter

The transporter, or gene, can be used to isolate other transporter proteins, to screen for inhibitors of the transporter, which are useful in altering tryptophan transport or T cell activation, and in screening of patient samples for defects in the transporter, which may be indicative of genetic defects, viral or parasitic infections, or malignancy. The transporter protein is also be useful for making antibodies which can be used in diagnostic assays to measure efficacy of tryptophan transport and the effect of compounds on the transporter, as well as isolation of the transporter.

Screening for Compounds which Modulate Tryptophan Transport Via the High Affinity Transporter

There are three basic mechanisms which can be used to modulate tryptophan catabolism in cells: modulation of IDO concentration or activity; modulation of the high affinity tryptophan transporter; and modulation of tryptophan concentration in the relevant cellular environment. A number of techniques are known for obtaining compounds which can be used to modulate IDO expression or activity, tryptophan levels, or the activity or expression of the high affinity tryptophan transporter, which in turn regulates the tryptophan levels intracellularly.

Assays for testing compounds for useful activity can be based solely on interaction with IDO, the high affinity tryptophan transporter, or enzymes involved in tryptophan metabolism ("the enzymes"), or alternatively, the assays can be based on interaction with the gene sequence encoding the enzymes. For example, antisense which binds to the regulatory sequences, and/or to the protein encoding sequences can be synthesized using standard oligonucleotide synthetic chemistry. The antisense can be stabilized for pharmaceutical use using standard methodology (encapsulation in a liposome or microsphere; introduction of modified nucleotides that are resistant to degradation or groups which increase resistance to endonucleases, such as phosphorothioates and methylation), then screened initially for alteration of enzyme activity in transfected or naturally occurring

cells which express the enzyme, then *in vivo* in laboratory animals. Typically, the antisense would inhibit expression. However, sequences which block those sequences which "turn off" synthesis can also be targeted.

Random generation of enzyme or enzyme encoding sequence binding molecules.

Oligonucleotide molecules with a given function, catalytic or ligand-binding, can be selected for from a complex mixture of random oligonucleotides in what has been referred to as "in vitro genetics" (Szostak, TIBS 19:89, 1992). One synthesizes a large pool of molecules bearing random and defined sequences and subjects that complex mixture, for example, approximately 10^{15} individual sequences in 100 μg of a 100 nucleotide RNA, to some selection and enrichment process. For example, by repeated cycles of affinity chromatography and PCR amplification of the molecules bound to the ligand on the column, Ellington and Szostak (1990) estimated that 1 in 10^{10} RNA molecules folded in such a way as to bind a given ligand. DNA molecules with such ligand-binding behavior have been isolated (Ellington and Szostak, 1992; Bock et al, 1992). Techniques aimed at similar goals exist for small organic molecules, proteins, antibodies and other macromolecules known to those of skill in the art. Screening sets of molecules for a desired activity whether based on small organic libraries, oligonucleotides, or antibodies is broadly referred to as combinatorial chemistry. Combinatorial techniques are particularly suited for defining binding interactions between molecules.

Using methodology well known to those of skill in the art, in combination with various combinatorial libraries, one can isolate and characterize those compounds which bind to or interact with the desired target. The relative binding affinity of these compounds can be compared and optimum compounds identified using competitive binding studies which are well known to those of skill in the art.

Screening molecules similar to tryptophan for inhibition of tryptophan binding or transport is a method of isolating desired compounds. The high affinity tryptophan transporter is highly specific for tryptophan transport.

Molecules isolated which inhibit tryptophan transport can either be competitive inhibitors of the high affinity tryptophan transporter or non-competitive inhibitors. In one embodiment the molecules are competitive inhibitors of tryptophan transport by the high affinity transporter. It is preferred that competitive inhibitors of tryptophan transport via the high affinity transporter do not get transported via the low affinity transporter. It is most preferred that competitive inhibitors of tryptophan transport via the high affinity transport system do not get transported via either the high affinity or low affinity transporters. This prevents unwanted intracellular buildup of tryptophan analogs.

In another embodiment the inhibitors of tryptophan transport by the high affinity transporter are non-competitive inhibitors of tryptophan transport. One type of non-competitive

inhibitor will cause allosteric rearrangements which prevent the transporter from binding or transporting tryptophan. Another type of non-competitive inhibitor of tryptophan transport by the high affinity transporter binds the high affinity transporter in a place other than the tryptophan binding site. However, it prevents tryptophan from accessing the tryptophan binding site because of stearic hinderance.

Non-competitive inhibitors specific for the high affinity tryptophan transporter are preferred.

Computer assisted drug design

Computer modeling technology allows visualization of the three-dimensional atomic structure of a selected molecule and the rational design of new compounds that will interact with the molecule. The three-dimensional construct typically depends on data from x-ray crystallographic analyses or NMR imaging of the selected molecule. The molecular dynamics require force field data. The computer graphics systems enable prediction of how a new compound will link to the target molecule and allow experimental manipulation of the structures of the compound and target molecule to perfect binding specificity. Prediction of what the molecule-compound interaction will be when small changes are made in one or both requires molecular mechanics software and computationally intensive computers, usually coupled with user-friendly, menu-driven interfaces between the molecular design program and the user.

Examples of molecular modelling systems are the CHARMm and QUANTA programs, Polygen

Corporation, Waltham, MA. CHARMm performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modelling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen, et al., 1988 *Acta Pharmaceutica Fennica* 97, 159-166; Ripka, *New Scientist* 54-57 (June 16, 1988); McKinaly and Rossmann, 1989 *Annu. Rev. Pharmacol._Toxiciol.* 29, 111-122; Perry and Davies, OSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 1989 *Proc. R. Soc. Lond.* 236, 125-140 and 141-162; and, with respect to a model enzyme for nucleic acid components, Askew, et al., 1989 *J. Am. Chem. Soc.* 111, 1082-1090. Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc., Pasadena, CA., Allelix, Inc, Mississauga, Ontario, Canada, and Hypercube, Inc., Cambridge, Ontario. Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or

synthetic chemicals, and biologically active materials, including proteins, for compounds which alter substrate binding or enzymatic activity.

Generation of nucleic acid regulators

Nucleic acid molecules containing the 5' regulatory sequences of the enzyme or transporter genes can be used to regulate or inhibit gene expression *in vivo*. Vectors, including both plasmid and eukaryotic viral vectors, may be used to express a particular recombinant 5' flanking region-gene construct in cells depending on the preference and judgment of the skilled practitioner (see, e.g., Sambrook et al., Chapter 16). Furthermore, a number of viral and nonviral vectors are being developed that enable the introduction of nucleic acid sequences *in vivo* (see, e.g., Mulligan, 1993 *Science*, 260, 926-932; United States Patent No. 4,980,286; United States Patent No. 4,868,116; incorporated herein by reference). Nucleic acid can be encapsulated in cationic liposomes which can be injected intravenously into a mammal. This system has been used to introduce DNA into the cells of multiple tissues of adult mice, including endothelium and bone marrow (see, e.g., Zhu et al., 1993 *Science* 261, 209-211).

The 5' flanking sequences of the enzyme gene can also be used to inhibit the expression of the enzyme. For example, an antisense RNA of all or a portion of the 5' flanking region of the enzyme gene can be used to inhibit expression of the enzyme *in vivo*. Expression vectors (e.g., retroviral expression vectors) are already available in the art which can be used to generate

an antisense RNA of a selected DNA sequence which is expressed in a cell (see, e.g., U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286). Accordingly, DNA containing all or a portion of the sequence of the 5' flanking region of the enzyme gene can be inserted into an appropriate expression vector so that upon passage into the cell, the transcription of the inserted DNA yields an antisense RNA that is complementary to the mRNA transcript of the enzyme gene normally found in the cell. This antisense RNA transcript of the inserted DNA can then base-pair with the normal mRNA transcript found in the cell and thereby prevent the mRNA from being translated. It is of course necessary to select sequences of the 5' flanking region that are downstream from the transcriptional start sites for the enzyme gene to ensure that the antisense RNA contains complementary sequences present on the mRNA.

Antisense RNA can be generated *in vitro* also, and then inserted into cells. Oligonucleotides can be synthesized on an automated synthesizer (e.g., Model 8700 automated synthesizer of Milligen-Biosearch, Burlington, MA or ABI Model 380B). In addition, antisense deoxyoligonucleotides have been shown to be effective in inhibiting gene transcription and viral replication (see e.g., Zamecnik et al., 1978 *Proc. Natl. Acad. Sci. USA* 75, 280-284; Zamecnik et al., 1986 *Proc. Natl. Acad. Sci.*, 83, 4143-4146; Wickstrom et al., 1988 *Proc. Natl. Acad. Sci. USA* 85, 1028-1032; Crooke, 1993 *FASEB J.* 7, 533-539. Inhibition of expression of a gene by antisense oligonucleotides is possible

if the antisense oligonucleotides contain modified nucleotides (see, e.g., Offensperger et. al., 1993 *EMBO J.* 12, 1257-1262 (*in vivo* inhibition of duck hepatitis B viral replication and gene expression by antisense phosphorothioate oligodeoxynucleotides); Rosenberg et al., PCT WO 93/01286 (synthesis of sulfurthioate oligonucleotides); Agrawal et al., 1988 *Proc. Natl. Acad. Sci. USA* 85, 7079-7083 (synthesis of antisense oligonucleoside phosphoramidates and phosphorothioates to inhibit replication of human immunodeficiency virus-1); Sarin et al., 1989 *Proc. Natl. Acad. Sci. USA* 85, 7448-7794 (synthesis of antisense methylphosphonate oligonucleotides); Shaw et al., 1991 *Nucleic Acids Res* 19, 747-750 (synthesis of 3' exonuclease-resistant oligonucleotides containing 3' terminal phosphoroamidate modifications); incorporated herein by reference).

The sequences of the 5' flanking region of enzyme gene can also be used in triple helix (triplex) gene therapy. Oligonucleotides complementary to gene promoter sequences on one of the strands of the DNA have been shown to bind promoter and regulatory sequences to form local triple nucleic acid helices which block transcription of the gene (see, e.g., 1989 Maher et al., *Science* 245, 725-730; Orson et al., 1991 *Nucl. Acids Res.* 19, 3435-3441; Postal et al., 1991 *Proc. Natl. Acad. Sci. USA* 88, 8227-8231; Cooney et al., 1988 *Science* 241, 456-459; Young et al., 1991 *Proc. Natl. Acad. Sci. USA* 88, 10023-10026; Duval-Valentin et al., 1992 *Proc. Natl. Acad. Sci. USA*

89, 504-508; 1992 Blume et al., *Nucl. Acids Res.* 20, 1777-1784; 1992 Grigoriev et al., *J. Biol. Chem.* 267, 3389-3395.

Both theoretical calculations and empirical findings have been reported which provide guidance for the design of oligonucleotides for use in oligonucleotide-directed triple helix formation to inhibit gene expression. For example, oligonucleotides should generally be greater than 14 nucleotides in length to ensure target sequence specificity (see, e.g., Maher et al., (1989); Grigoriev et al., (1992)). Also, many cells avidly take up oligonucleotides that are less than 50 nucleotides in length (see e.g., Orson et al., (1991); Holt et al., 1988 *Mol. Cell. Biol.* 8, 963-973; Wickstrom et al., 1988 *Proc. Natl. Acad. Sci. USA* 85, 1028-1032). To reduce susceptibility to intracellular degradation, for example by 3' exonucleases, a free amine can be introduced to a 3' terminal hydroxyl group of oligonucleotides without loss of sequence binding specificity (Orson et al., 1991). Furthermore, more stable triplexes are formed if any cytosines that may be present in the oligonucleotide are methylated, and also if an intercalating agent, such as an acridine derivative, is covalently attached to a 5' terminal phosphate (e.g., via a pentamethylene bridge); again without loss of sequence specificity (Maher et al., (1989); Grigoriev et al., (1992)).

Methods to produce or synthesize oligonucleotides are well known in the art. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see

e.g., Sambrook et al., Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (see also, Ikuta et al., in *Ann. Rev. Biochem.* 1984 53, 323-356 (phosphotriester and phosphite-triester methods); Narang et al., in Methods Enzymol., 65, 610-620 (1980) (phosphotriester method). Accordingly, DNA sequences of the 5' flanking region of the enzyme gene described herein can be used to design and construct oligonucleotides including a DNA sequence consisting essentially of at least 15 consecutive nucleotides, with or without base modifications or intercalating agent derivatives, for use in forming triple helices specifically within the 5' flanking region of a enzyme gene in order to inhibit expression of the gene.

In some cases it may be advantageous to insert enhancers or multiple copies of the regulatory sequences into an expression system to facilitate screening of methods and reagents for manipulation of expression.

Preparation of Protein Fragments or Amino Acid Analogs

Compounds which are effective for blocking binding of the transporter or IDO can also consist of protein fragments, expressed recombinantly and cleaved by enzymatic digest or expressed from a sequence encoding a peptide of less than the full length protein, or tryptophan analogs which compete with tryptophan for binding and/or uptake by the high affinity tryptophan transporter. It is a routine matter to make appropriate protein

fragments, and test for inhibition of activity of the protein in the presence of the fragments. The preferred fragments are of human origin, in order to minimize potential immunological response. The peptides can be as short as five to eight amino acids in length and are easily prepared by standard techniques. They can also be modified to increase *in vivo* half-life, by chemical modification of the amino acids or by attachment to a carrier molecule or inert substrate. The peptides can also be conjugated to a carrier protein such as keyhole limpet hemocyanin by its N-terminal cysteine by standard procedures such as the commercial Imject kit from Pierce Chemicals or expressed as a fusion protein, which may have increased efficacy. As noted above, the peptides can be prepared by proteolytic cleavage of the proteins, or, preferably, by synthetic means. These methods are known to those skilled in the art. An example is the solid phase synthesis described by J. Merrifield, 1964 *J. Am. Chem. Soc.* 85, 2149, used in U.S. Patent No. 4,792,525, and described in U.S. Patent No. 4,244,946, wherein a protected alpha-amino acid is coupled to a suitable resin, to initiate synthesis of a peptide starting from the C-terminus of the peptide. Other methods of synthesis are described in U.S. Patent No. 4,305,872 and 4,316,891. These methods can be used to synthesize peptides having identical sequence to the proteins described herein, or substitutions or additions of amino acids, which can be screened for activity as described above.

The peptide can also be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

Peptides containing cyclopropyl amino acids, or amino acids derivatized in a similar fashion, can also be used. These peptides retain their original activity but have increased half-lives *in vivo*. Methods known for modifying amino acids, and their use, are known to those skilled in the art, for example, as described in U.S. Patent No. 4,629,784 to Stammer.

Pharmaceutical Compositions

Compounds which alter enzyme activity are preferably administered in a pharmaceutically acceptable vehicle. Suitable pharmaceutical vehicles are known to those skilled in the art. For parenteral administration, the compound will usually be dissolved or suspended in sterile water or saline.

Generation of Transgenic Animals for Screening

With the knowledge of the cDNA encoding the high affinity tryptophan transporter and regulatory

sequences regulating expression thereof, it is possible to generate transgenic animals, especially rodents, for testing the compounds which can alter enzyme expression, translation or function in a desired manner.

There are basically two types of animals which are useful: those not expressing the high affinity transporter, and those which overexpress the high affinity transporter, either in those tissues which already express the protein or in those tissues where only low levels are naturally expressed. The animals in the first group are preferably made using techniques that result in "knocking out" of the gene for enzyme, although in the preferred case this will be incomplete, either only in certain tissues, or only to a reduced amount. These animals are preferably made using a construct that includes complementary nucleotide sequence to the enzyme gene, but does not encode functional enzyme, and is most preferably used with embryonic stem cells to create chimeras. Animals which are heterozygous for the defective gene can also be obtained by breeding a homozygote normal with an animal which is defective in production of enzyme.

The animals in the second group are preferably made using a construct that includes an unregulated promoter or one which is modified to increase expression as compared with the native promoter. The regulatory sequences for the enzyme gene can be obtained using standard techniques based on screening of an appropriate library with the cDNA encoding enzyme. These animals are most preferably made using standard microinjection techniques.

These manipulations are performed by insertion of cDNA or genomic DNA into the embryo using microinjection or other techniques known to those skilled in the art such as electroporation, as described below. The DNA is selected on the basis of the purpose for which it is intended: to inactivate the gene or to overexpress the gene. The enzyme encoding gene can be modified by homologous recombination with a DNA for a defective enzyme, such as one containing within the coding sequence an antibiotic marker, which can then be used for selection purposes.

Animals suitable for transgenic experiments can be obtained from standard commercial sources. These include animals such as mice and rats for testing of genetic manipulation procedures, as well as larger animals such as pigs, cows, sheep, goats, and other animals that have been genetically engineered using techniques known to those skilled in the art. The procedures for manipulation of the embryo and for microinjection of DNA are described in detail in Hogan et al. Manipulating the mouse embryo, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1986), the teachings of which are incorporated herein. These techniques are readily applicable to embryos of other animal species, and, although the success rate is lower, it is considered to be a routine practice to those skilled in this art. Methods for the culturing of ES cells and the subsequent production of transgenic animals, the introduction of DNA into ES cells by a variety of methods such as electroporation, calcium phosphate/DNA

precipitation, and direct injection are described in detail in Teratocarcinomas and embryonic stem cells, a practical approach, ed. E.J. Robertson, (IRL Press 1987), the teachings of which are incorporated herein. Transfection is carried out by one of several methods described in detail in Lovell-Badge, in Teratocarcinomas and embryonic stem cells, a practical approach, ed. E.J. Robertson, (IRL Press 1987) or in Potter et al Proc. Natl. Acad. Sci. USA 81, 7161 (1984). Calcium phosphate/DNA precipitation, direct injection, and electroporation are the preferred methods. DNA molecules introduced into ES cells can also be integrated into the chromosome through the process of homologous recombination, described by Capecchi, (1989). Direct injection results in a high efficiency of integration. Desired clones are identified through PCR of DNA prepared from pools of injected ES cells. Positive cells within the pools are identified by PCR subsequent to cell cloning (Zimmer and Gruss, Nature 338, 150-153 (1989)).

Once the transgenic animals are identified, lines are established by conventional breeding and used as the donors for tissue removal and implantation using standard techniques for implantation into humans.

Gene Therapy

Altered levels of transporter can also be achieved using gene therapy, either to administer nucleotide molecules which inhibit transporter activity, or to increase transporter activity either through direct alteration of activity, for

example by increasing substrate binding affinity, or through overexpression.

Examples

The following methods and materials were used in the examples.

Materials. Recombinant human macrophage colony-stimulating factor (MCSF) was the gift of Genetics Institute, Cambridge MA. Recombinant human interferon-γ (IFNγ) was the gift of Genentech, South San Francisco, CA. Radiolabeled [^{3}H]tryptophan (21 Ci/mmol) was obtained from Amersham USA. All preparations were adjusted with their respective unlabeled amino acids to a specific activity of 20 Ci/mmol before use. Unlabeled amino acids and all other reagents were obtained from Sigma unless otherwise specified. Tyrosine and other sparingly-soluble amino acids were dissolved in 0.1 M NaOH prior to dilution.

Monocyte isolation and culture. Human peripheral blood monocytes were isolated from healthy volunteer donors by leukocytapheresis and counterflow centrifugal elutriation, following appropriate informed consent under a protocol approved by our Institutional Review Board. Monocytes of greater than 95% purity by cell surface markers (Munn and Armstrong 1993 *Cancer Res*. 53, 2603-2613) were cultured as described by Munn and Cheung 1989 *J. Exp. Med*. 170, 511-526, for 7 days in the presence of MCSF (200 U/ml). The monocytic leukemia lines THP-1 and U-937 were obtained from the American Type Culture Collection, Rockville, MD.

Uptake measurements. Single cell suspensions were obtained by treating macrophage monolayers with 2 mM EDTA in Hanks balanced salt solution for 15 minutes at room temperature. Cells were washed twice in Tris-choline buffer (150 mM choline chloride, 10 mM tris, pH 7.4), suspended in Tris-choline at between one and 2 x 10^6 cells/ml, and 100 µl/well added to 96 well microtiter plates. Titrations of radiolabeled amino acids were then transferred to the assay plate and uptake allowed to proceed for 10 minutes (the linear range). At the end of the assay period, wells were harvested simultaneously onto glass-fiber filters and washed vigorously for 30 seconds with phosphate buffered saline solution using a Tomtec 96 well parallel harvester. Nonspecific binding of radioactivity to the filters (based on wells without cells) was typically less than 10% of the total signal, and was subtracted from each data point. All assays were performed in triplicate; where error bars are not shown the SD between replicates was less than 10%. In all assays, the uptake rate (V) was normalized per 1 x 10^6 cells (expressed as pmol 10^6 $cells^{-1} \cdot 10\ min^{-1}$). Normalization to milligrams protein was considered less informative because protein content can change markedly during macrophage differentiation, independently of other functional attributes.

Analysis of saturation kinetics. Uptake kinetics were analyzed using an Eadie-Hofstee transformation (V/[S] vs. V) to identify linear concentration ranges for each transporter. Data from these two ranges were then analyzed separately

using double-reciprocal (Lineweaver-Burk, 1/[S] vs. 1/V) plots to determine Km and Vmax. In all cases, comparable results were obtained if these parameters were estimated from the Eadie-Hofstee plots. Regression analysis was performed using the Microsoft Excel spreadsheet program. Kinetic parameters were calculated on a minimum of 4-6 points, and were considered reliable if the correlation coefficient of the regression line was greater than 0.95.

For competitive inhibition studies, the Ki value for each competitor was estimated by fitting the measured inhibition curve to the formula in Equation 1 (Cheng and Prusoff 1973, *Biochem. Pharmacol.* 22, 3099-3108).

EQUATION 1:

$$Vi = \frac{Vmax \bullet [S]}{Km \left(1 + \frac{[I]}{Ki}\right) + [S]}$$

where [S] is the concentration of labeled substrate, [I] is the concentration of unlabeled inhibitor, Vi is the observed rate of uptake in the presence of inhibitor, Ki is the putative affinity constant of the inhibitor for the transporter, and Km and Vmax have their customary meanings. This equation assumes a single transport system and so does not fully model the situation in macrophages, where two transporters are present. However, the affinity constants of the two systems differed so widely (100-fold) that the data closely fit a single-system model at substrate concentrations surrounding the respective Km values.

Example 1: Tryptophan transport is sodium-independent and occurs via two transport systems.

Monocyte derived macrophages were incubated with radiolabeled tryptophan (125 nM) for 2, 4, 6 or 10 minutes, either in buffered saline solution (150 mM NaCl) or buffered choline chloride. The majority of tryptophan uptake is sodium-independent. Uptake in the absence of sodium was 86 ±6% of uptake in saline, n=4 Therefore all subsequent assays were performed in the absence of sodium.

Saturation kinetics at low substrate concentrations (less than 2.5 μM) indicated that the tryptophan transport system is a saturable system with very high affinity (Figures 1A). Studies over an extended range of concentrations revealed that tryptophan uptake did not follow simple Michaelis-Menten kinetics. Analyzing the data with Eadie-Hofstee (V/[s] vs. V) plots suggested the presence of two transport systems, as indicated by the non-linear nature of the transformed data (Figure 1B). The transport kinetics were analyzed separately at high (8-64 μM) and low (less than 1 μM) substrate concentrations, using Lineweaver-Burk plots (Figure 1C). Individually the data indicated two independent, saturable transport systems, the first with a low affinity (Km of 20-30 μM, which is in the usual range for previously described "high-affinity" amino acid transporters (Sanches del Pino, et al., 1995, *J. Biol. Chem.* 270, 14913-14918; Mokrzan, et al., 1995, *J. Pharmacol. Exp. Ther.* 272, 1277-1284;

Low, et al., 1993, *J. Cell. Physiol.* 156, 626-634; Prasad, et al., 1994, *Endocrinol.* 134, 574-581; and Low, et al., 1994, *J. Biol. Chem.* 269, 32098-32103)), and the second system with a very high affinity (Km of approximately 300 nM). When the Km and Vmax constants obtained from these separate analyses were employed with the Michaelis-Menten equation to generate Theoretical predicted uptake curves for each system were generated, using the Km and Vmax values derived from the independent analysis of the low affinity data set and the high affinity data set. The observed data over the range of concentrations surrounding each Km fit well to the generated curves (the predicted regression lines are superimposed on Figure 1B). Therefore, tryptophan uptake in macrophages was modeled best as two independent transport systems, each following classical Michaelis-Menten kinetics but with markedly different affinities for substrate.

Table I summarizes the high-affinity system in human macrophages and two human macrophage-like cell lines, THP-1 and U-937. Human macrophages were allowed to differentiate for seven days under the influence of macrophage colony stimulating factor (MCSF). Macrophages and cell lines were assayed for tryptophan uptake, and the Km value was determined for the range of substrate concentrations 32 nM - 1 μM.

Table 1: Presence of the high-affinity tryptophan transporter in macrophages and macrophage-like cell lines.

Cell type	Km (μM)	Experiments (n)
macrophages	0.29 ± 0.16	18
THP-1	0.25 ± 0.12	11
U-937	0.34 ± 0.33	3

Example 2: Uptake via the lower-affinity system is consistent with system L.

Of the known sodium-independent transport systems only system L accepts tryptophan with low-micromolar affinity (reported affinity as high as Km=8-30 μM). This was consistent with the values observed for the lower-affinity system (Km = 27 ± 4 μM, n=4, Figure 1) herein described. Leucine and phenylalanine, as shown in Figures 2a,b,c, which are both known substrates for system L, were transported via the low affinity system with comparable affinity. Tryptophan competed with phenylalanine for uptake via this system, with a Ki value similar to the Km value for its own uptake via the lower-affinity system (Figures 1B and 1C). Cross-competition experiments showed that tryptophan transport could be at least partially inhibited by a number of other neutral amino acids, which is also consistent with a contribution of system L in tryptophan transport.

BCH is a substrate which is used as the model substrate for system L transport. Figure 3

indicates that tryptophan transport is inhibited by BCH, but not by charged amino acids. Thus, the lower-affinity transport system present in macrophages displayed multiple features consistent with system L, and is very likely system L or a new transport system with characteristics much like those of system L.

Example 3: Uptake via the high-affinity system is distinct from system L and very specific for tryptophan.

Uptake via system L was distinguished from uptake via the previously unidentified high-affinity system. BCH was used to selectively compete for uptake via system L. As shown in Figure 4A, the IC_{50} for inhibition of tryptophan transport by BCH was approximately 100-fold greater than the IC_{50} for inhibition by tryptophan itself. Using Equation 1, the data for BCH fit well to a single system, competitive-inhibition model with a Ki equal to the observed IC_{50} of 37 μM. In contrast, inhibition by unlabeled tryptophan fit to a much higher affinity model (Ki = IC_{50} = 360 nM in the experiment shown), and BCH showed no evidence of competition for this system (as indicated by the lack of inhibition at competitor concentrations less than 1 μM).

The saturation kinetics of tryptophan transport were compared in the presence or absence of BCH. BCH was found to act as a competitive inhibitor over the range of substrate concentrations affected by system L, but it interacted only weakly (IC_{50} > 500 μM) with the high-affinity system (Figure 4C).

More importantly, the interaction between BCH and the high affinity system was strictly non-competitive. BCH did not compete with tryptophan for transport via the high-affinity system. Similar results were obtained for leucine and phenylalanine, indicating that leucine and phenylalanine do not compete with tryptophan for transport by the high affinity system. Thus, while tryptophan competed with multiple substrates for transport by system L, the high-affinity component of tryptophan transport is selective for tryptophan.

The data represented in Figure 2 indicated that even at 8000 μM competitor, tryptophan was not fully inhibited from being transported. Since tryptophan competed with these other substrates at approximately 30 μM (Figures 2 and 4), then a single system composed of system L under these conditions would have less than 1 % of the tryptophan being transported. Since there are two transport systems either the transport systems have the same specificity or different. If the high affinity transport system had specificity requirements similar to system L, then 8000 μM competitor should have reduced tryptophan transport to below 1 % of the control, as if there was only one system. Therefore, this data (Figures 2, 3, 4) also indicated that the transport of tryptophan is occurring via different pathways, one of which is highly specific for tryptophan, relative to other amino acids.

Extended titrations were performed using several unlabeled competitors (leucine,

phenylalanine, tyrosine, cysteine, and BCH) (Figure 3). Phenylalanine was identified as the most efficient competitor, and data for this representative substrate are shown in Figure 4. Like BCH, phenylalanine displayed no detectable competition for the high-affinity system (i.e., no inhibition at competitor concentrations less than 1 μM). Based on the IC_{50}, phenylalanine was predicted to compete with tryptophan with a Ki of approximately 10 μM. However, at high competitor concentrations the observed inhibition departed markedly from that predicted for a single system. Instead, tryptophan uptake continued at a rate significantly higher than expected, suggesting the presence of an additional transporter not subject to competition by phenylalanine. Titrations of leucine, tyrosine, cysteine, and BCH all showed similar patterns, with even less interaction with the tryptophan-selective component, which was consistent with the results shown in Figure 3.

The cross-competition studies shown in Figure 3 were repeated using unlabeled competitors at a concentration of 4 μM rather than 8000 μM. Based on Equation 1, this concentration was predicted to significantly inhibit the higher-affinity system (80% inhibition) but have little impact (less than 10% inhibition) on the lower-affinity system. As shown in Figure 6, tryptophan uptake was markedly inhibited by 4 μM unlabeled tryptophan, but BCH and all other substrates tested had minimal effect. Thus, when tested in isolation, the high-affinity system displayed a pattern of substrate specificity entirely different from that of system L or any of

the known sodium-independent transport systems, and appeared highly selective for tryptophan.

Example 4: Tryptophan does not compete with other substrates for the high-affinity system.

The data presented in Examples 1-3 is not consistent with a single system that has markedly higher affinity for tryptophan because tryptophan was not able to preferentially compete for uptake over other substrates. An additional study in which unlabeled tryptophan was allowed to compete for uptake of radiolabeled leucine, phenylalanine, or tryptophan, all at 125 nM shows that tryptophan competed with labeled leucine or phenylalanine only for the shared system L, and only with the expected affinity (Ki = 30 μM). There was no detectable high-affinity inhibition of phenylalanine or leucine uptake by tryptophan. In contrast, tryptophan competed for its own uptake with the predicted high affinity. Therefore, the data supported the existence of two separate transporters, with the high-affinity system being selective for tryptophan.

No other amino acid transporter accepting tryptophan has the same very high degree of specificity. As shown by Figure 7, comparing the amount of labeled tryptophan (250 nM radiolabelled tryptophan) taken up by the high affinity transporter as a function of concentration of either unlabelled tryptophan or unlabeled 1-methyl tryptophan at different concentrations. The solid line through the diamonds shows the predicted inhibition curve if tryptophan competed with a Ki

equal to the measured Km of the high affinity system (290 nM). The solid line through the triangles shows the predicted inhibition curve if 1-methyl tryptophan competed with a Ki of 30 μM, the measured Km of system L. There was no competition by 1-methyl tryptophan for the high affinity system.

The same type of experiment was done with D-tryptophan, serotonin, 5-hydroxy tryptophan and N-acetyl tryptophan. These additional substrates also did not compete with tryptophan for the high-affinity system.

Example 5: High-affinity tryptophan uptake reflects transmembrane transport.

To distinguish between transmembrane transport and binding of tryptophan to a high-affinity cell-surface receptor, the uptake of tryptophan at 25°C and 0°C was compared. As shown in Figure 8, the rate of tryptophan accumulation was reduced by 90% at 0°C, and this difference remained constant over an extended assay period (30 min). Thus, the accumulation of cell-associated tryptophan was consistent with transmembrane transport rather than surface-receptor binding.

Example 6: The high-affinity system is induced during macrophage differentiation.

Peripheral blood monocytes undergo many functional changes as they differentiate into macrophages. The high-affinity tryptophan transporter was regulated during macrophage differentiation. For these experiments, transporter expression was defined as the Vmax for the high affinity component derived from Eadie-

Hofstee plots (Figure 2). As shown in Figures 9a,b,c, the high affinity system underwent a 10-30 fold upregulation in Vmax during macrophage differentiation. By comparison, the system L component underwent only a 2- to 3-fold induction over the same period (from 14 ± 8 to 27 ± 4 pmol 10^6 $cells^{-1} \cdot 10\ min^{-1}$, n = 4). Thus, the high-affinity system was markedly and selectively induced during macrophage differentiation.

1. A high affinity transport system for tryptophan having a Km of between approximately 130 and 450 nM, which does not exhibit competitive inhibition by any other essential amino acids, and which is inducible during macrophage differentiation.

2. An isolated nucleotide molecule encoding a high affinity transport system for tryptophan having a Km of between approximately 130 and 450 nM, which does not exhibit competitive inhibition by any other essential amino acids, and which is inducible during macrophage differentiation.

3. An animal in which expression is altered of a high affinity transport system for tryptophan having a Km of between approximately 130 and 450 nM, which does not exhibit competitive inhibition by any other essential amino acids, and which is inducible during macrophage differentiation.

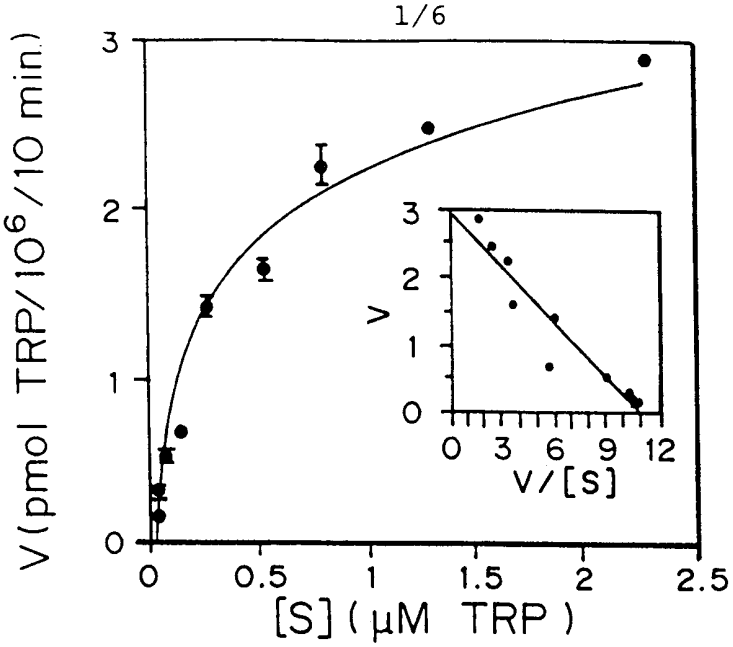


FIG. 1A

FIG. 1B

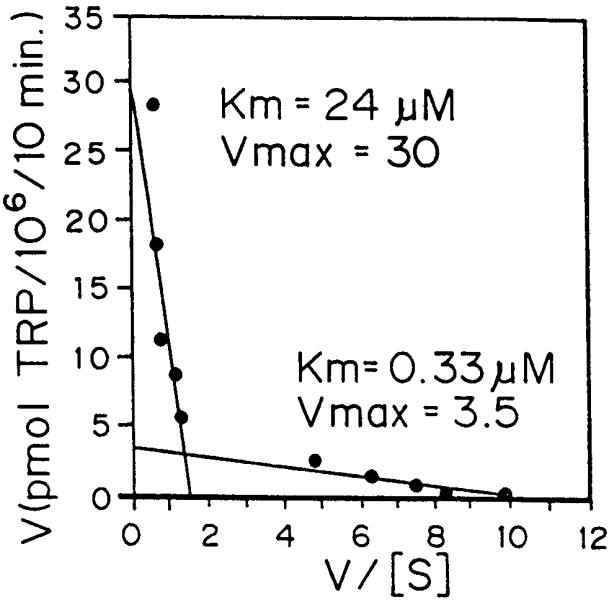


FIG. 1C

[TRP]<1 μM [TRP]>8 μM

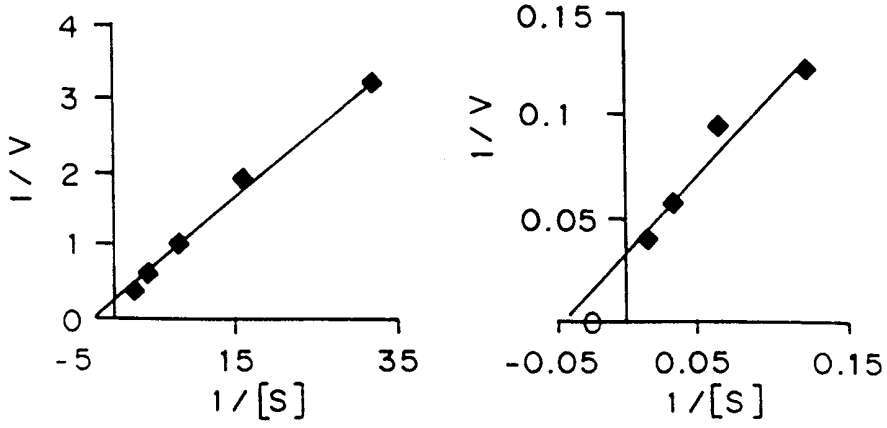


FIG. 2A

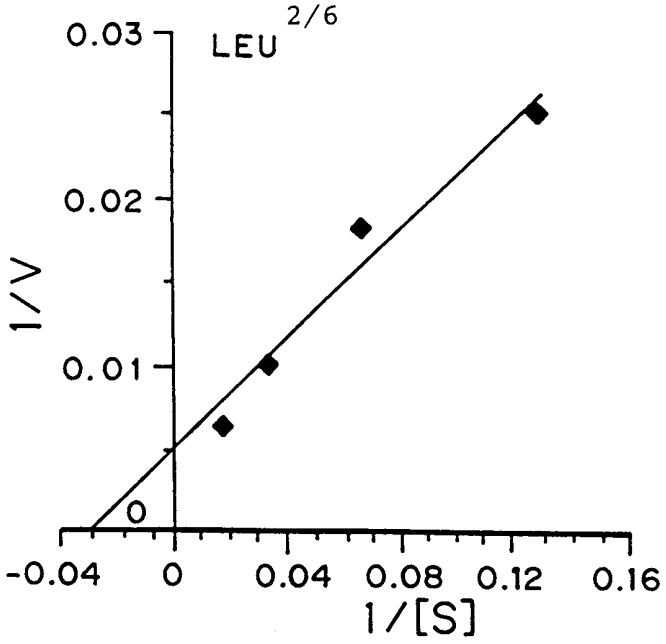


FIG. 2B

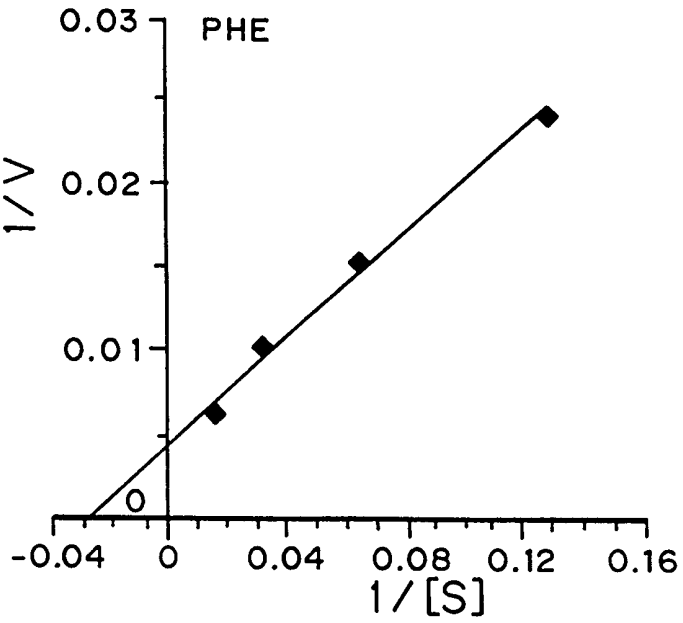
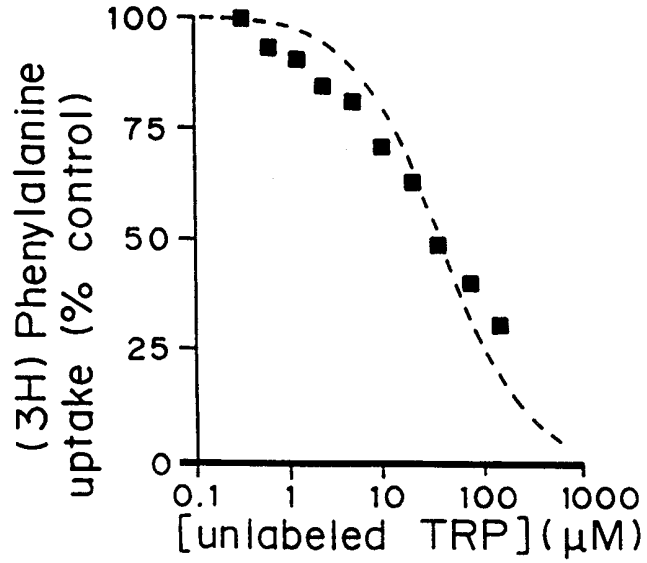


FIG. 2C



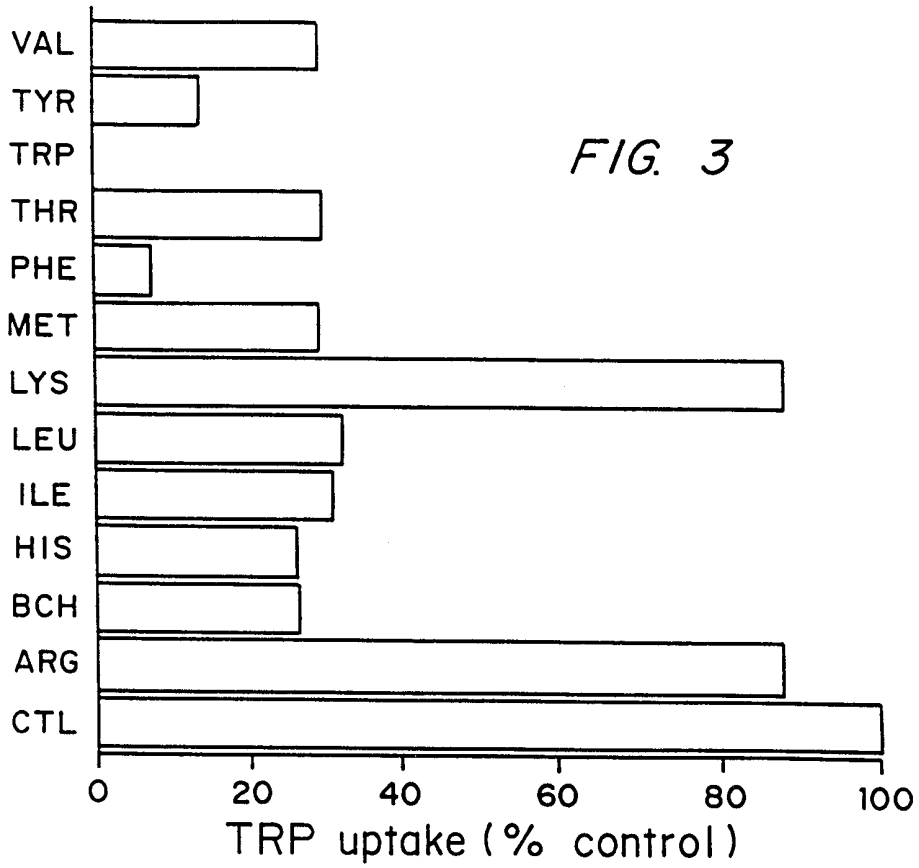

VAL
TYR
TRP
THR
PHE
MET
LYS
LEU
ILE
HIS
BCH
ARG
CTL
0
20
40
60
80
100
TRP uptake (% control)

FIG. 3

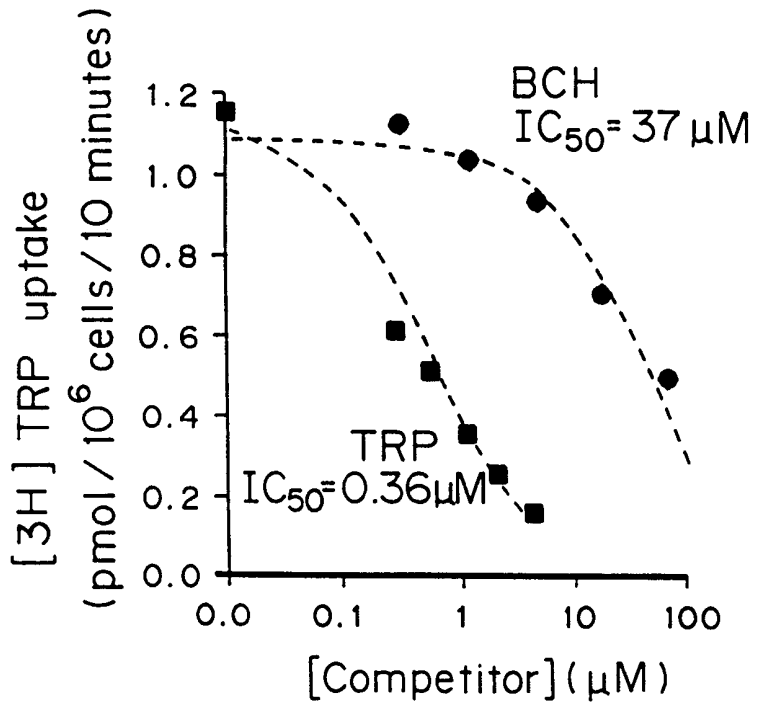

[3H] TRP uptake (pmol / 10^6 cells / 10 minutes)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
BCH
IC50= 37 µM
TRP
IC50=0.36µM
0.0
0.1
1
10
100
[Competitor] (µM)

FIG. 4A

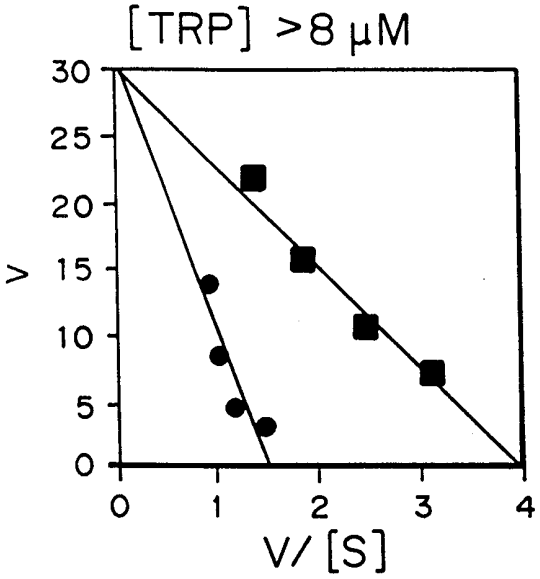


FIG. 4B

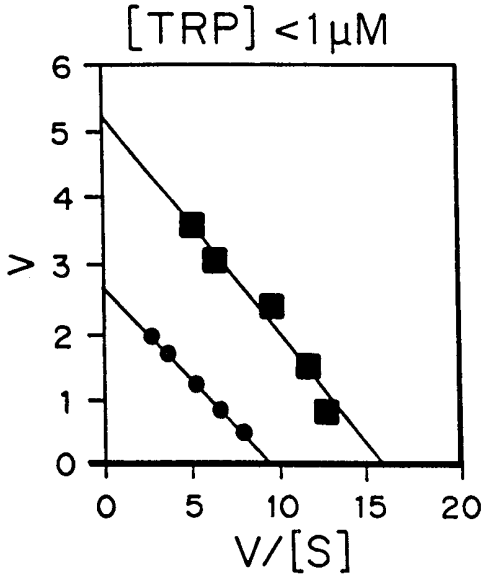


FIG. 4C

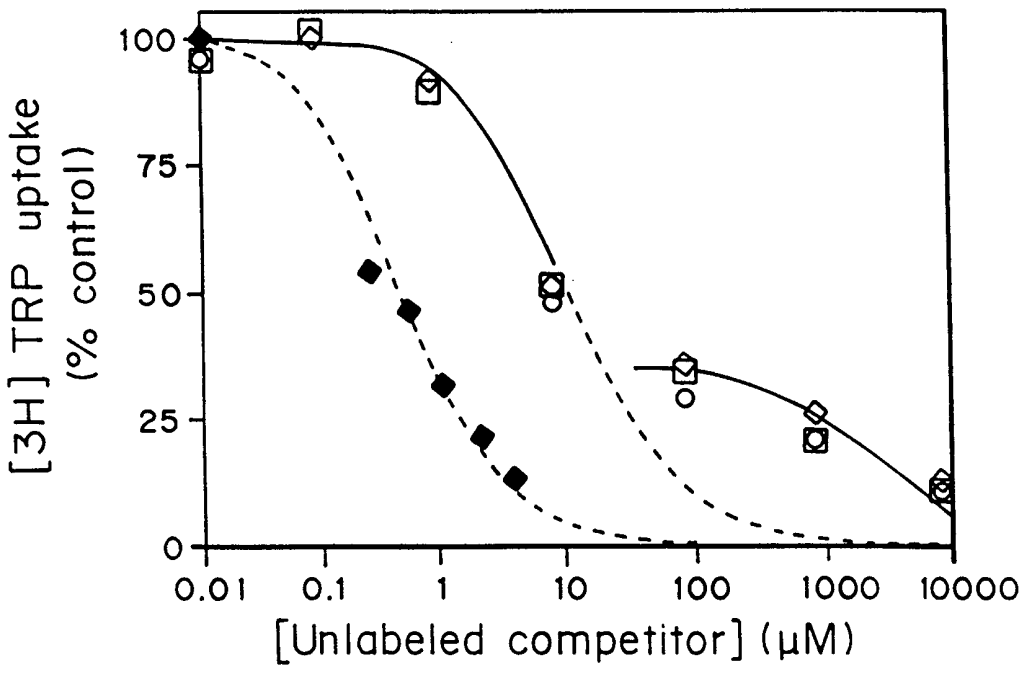


FIG. 5

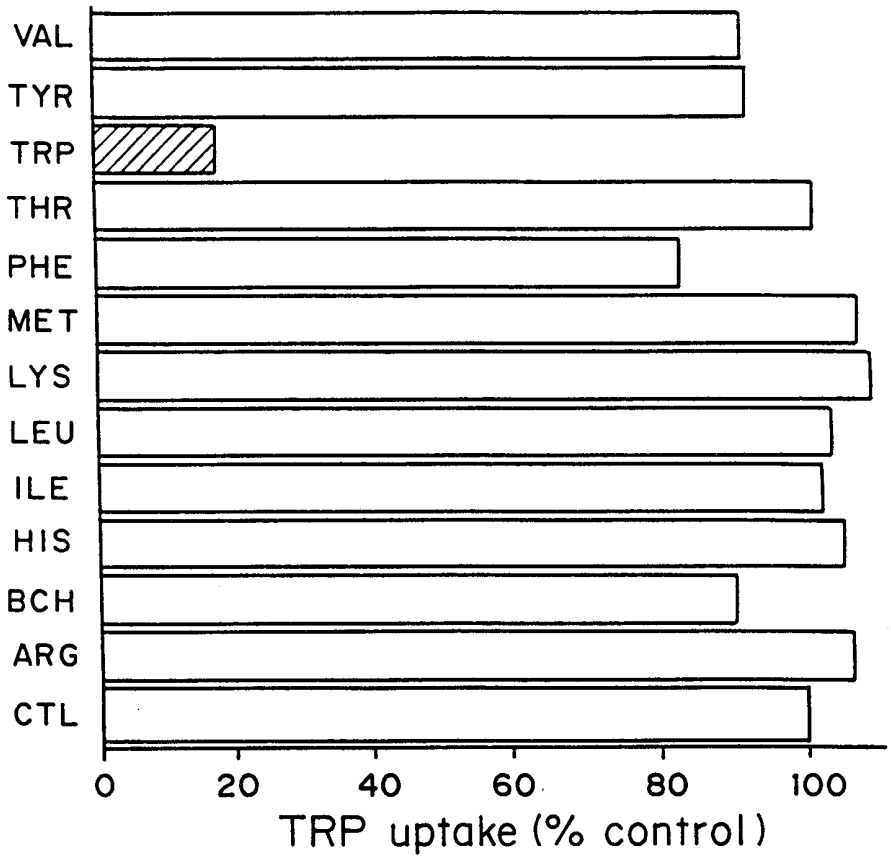

VAL
TYR
TRP
THR
PHE
MET
LYS
LEU
ILE
HIS
BCH
ARG
CTL
0
20
40
60
80
100
TRP uptake (% control)

FIG. 6

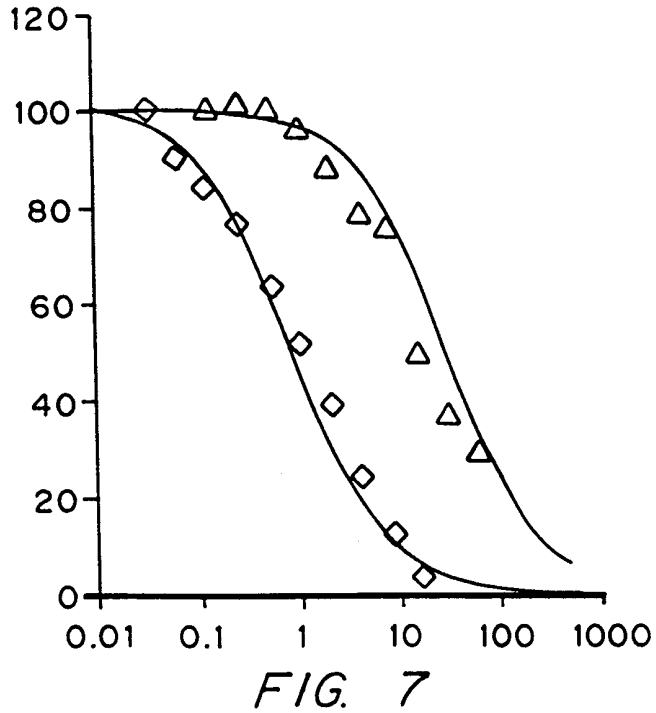

120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000

FIG. 7

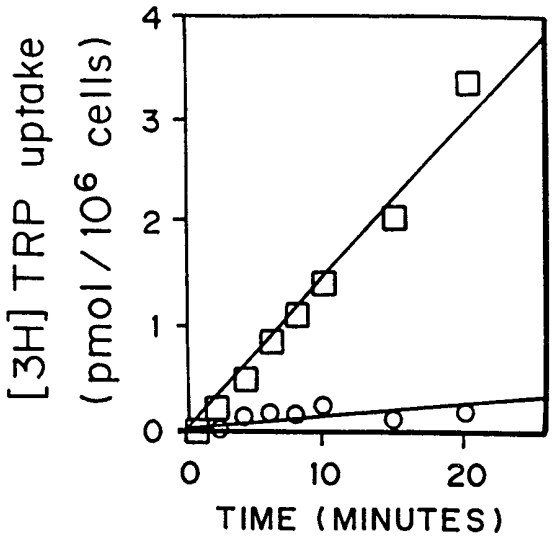


FIG. 8

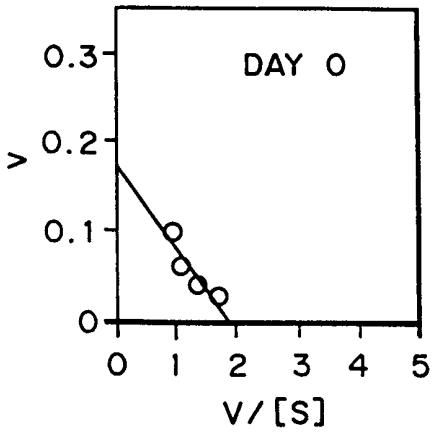


FIG. 9A

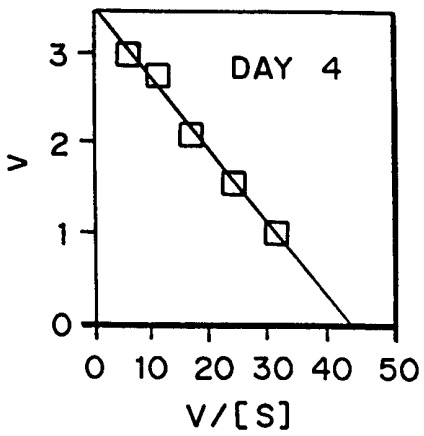


FIG. 9B

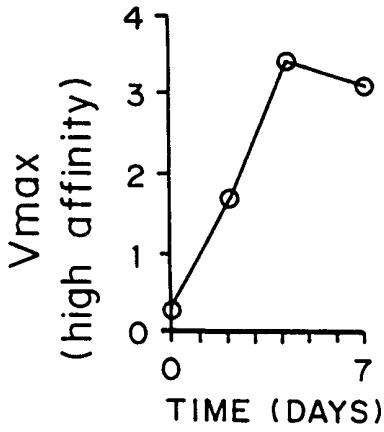


FIG. 9C

INTERNATIONAL SEARCH REPORT

Inte .onal Application No
PCT/US 98/25841

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SEYMOUR R AND MUNN D H: "Identification and characterization of a novel, high-affinity tryptophan-selective transport system in human macrophages" BLOOD, vol. 90, no. 10, supplement 1(Part 1 of 2), 15 November 1997, page 448a XP002097470 See abstract 1987(46-IV) --- -/--	1

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 1999	06/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

4

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/US 98/25841

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document. with indication,where appropnate. of the relevant passages	Relevant to claim No.
X	ATTWOOD J T T: "The role of tryptophan depletion in T cell suppression by macrophages." IMMUNOLOGY, vol. 92, no. supplement 1, December 1997, page 7 XP002097471 See Abstract 2.7	1
O,X	& 5TH ANNUAL CONGRESS OF THE BRITISH SOCIETY FOR IMMUNOLOGY, 2-5 DECEMBER 1997 BRIGHTON, UK; PRESENTED ON TUESDAY, 2 DECEMBER, SESSION 2: TOLERANCE.,	1

4