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Hypothesis

The end of the road for the tryptophan depletion concept in pregnancy and infection

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Abstract

We hypothesize that: (1) *L*-tryptophan (Trp) is greatly utilized and not depleted in pregnancy; (2) fetal tolerance is achieved in part through immunosuppressive kynurenine (Kyn) metabolites produced by the flux of plasma free (non-albumin-bound) Trp down the Kyn pathway; (3) the role of indoleamine 2,3-dioxygenase (IDO) in infection is not related to limitation of an essential amino acid, but is rather associated with stress responses and the production of Kyn metabolites that regulate the activities of antigen presenting cells and T-cells, as well as increased NAD^+ synthesis in IDO-expressing cells; (4) Trp depletion is not a host defence mechanism, but is a consequence of Trp utilization. We recommend that future studies in normal and abnormal pregnancies and in patients with infections or cancer should include measurements of plasma free Trp, determinants of Trp binding (albumin and non-esterified fatty acids), total Trp, determinants of activities of the Trp-degrading enzymes Trp 2,3-dioxygenase (TDO) (cortisol) and IDO (cytokines) and levels of Kyn metabolites. We also hypothesize that abnormal pregnancies and failure to combat infections or cancer may be associated with excessive Trp metabolism that can lead to pathological immunosuppression by excessive production of Kyn metabolites. Mounting evidence from many laboratories indicates that Trp metabolites are key regulators of immune cell behaviour, whereas Trp depletion is an indicator of extensive utilization of this key amino acid.

Key words: plasma free tryptophan, tryptophan utilization concept, immunosuppression, kynurenine metabolites.

INTRODUCTION

We hypothesize that depletion of the essential amino acid tryptophan (Trp) is not a regulatory or anti-pathogenic mechanism in pregnancy or infectious diseases, but rather that this key amino acid is utilized by cells of the immune system to regulate immune system responsiveness. The apparent decrease in plasma total (free + albumin-bound) Trp observed in pregnancy results from increased cellular uptake of free Trp and its utilization for a variety of physiological and host defence functions; a similar situation exists during infection. We also note that the flux of free Trp down the kynurenine (Kyn) pathway is the major determinant of Trp utilization and Kyn metabolite formation. In the following discussion, we shall briefly outline some of the origins of the Trp depletion concept, briefly review experimental evidence contradicting it, provide an updated view of the Trp status in pregnancy and infectious diseases, propose Trp utilization concepts in these conditions and outline experimental means of testing them.

ORIGINS OF THE TRYPTOPHAN DEPLETION CONCEPTS

Trp catabolism is greatly accelerated in response to interferon-γ (IFN-γ) released by leucocytes including T-cells. This is accomplished by strong induction of the extrahepatic enzyme indoleamine 2,3-dioxygenase (IDO), which initiates Trp degradation through the Kyn pathway in cells of the immune system. Much of the IDO induction and the resultant Kyn pathway metabolite formation take place within macrophages and dendritic cells, but other cells of the immune system also can catabolize

Abbreviations: 3-HAA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-γ; ISR, integrated stress response; KMO, kynurenine-3-monooxygenase; Kyn, kynurenine; PARP, poly ADP ribose polymerase; Polyl.PolyC, polyriboinosinic acid–polyribocytidylic acid; Quin, quinolinic acid; TDO, Trp 2,3-dioxygenase; Th1, T helper type 1; Trp, tryptophan.

[1] The opinions or assertions contained herein are the private views of the authors, and are not to be construed as reflecting the views of the Department of Defence, USA.

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Trp. The resultant drop in plasma Trp formed the basis of the depletion concepts, wherein proponents claimed that depletion of this essential amino acid underpinned the antibacterial, antiparasitic and antiviral effects of IFN-γ [1–3]. Perhaps the most cited publication on Trp depletion was that of Munn et al. [4] who proposed Trp starvation during pregnancy as a mechanism of T-cell suppression and defence against fetal rejection. These Trp depletion concepts (published in 1991 and 1998 respectively) stimulated much research over the past two and a half decades, which contributed greatly to our understanding of immune function and the role of IDO in health and disease [5]. However, paradoxically, this progress also culminated in disproving these concepts. Alternatively, Trp utilization concepts have been proposed, which focus on production of active Kyn metabolites and NAD^+ synthesis [6] and address specifically the Trp status and requirements in pregnancy [7,8].

IMMUNOSUPPRESSION BY KYNURENINE METABOLITES: A LANDMARK DISCOVERY

One major advance leading to these immune system Trp utilization concepts is the demonstration of the immunosuppressive properties of Kyn pathway metabolites, including 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA) and quinolinic acid (Quin) [reviewed in 5]. This development and a large body of experimental evidence at odds with the depletion concept led to the view that the IDO-mediated effects of IFN-γ are the combined results of Trp depletion and immunosuppression of specific aspects of host defences by Kyn metabolites [reviewed in 9]. However, as will be described below, Trp depletion *per se* plays no part in combating infection or maintaining pregnancy, but is rather associated with stress responses in both host and pathogen. The idea that Kyn metabolites might have immune modulatory functions was proposed from 1990 through 1997 [10–18], well before Munn et al. [4] published the Trp depletion hypothesis of immunosuppression in pregnancy. The seminal work of the Osamu Hayaishi group in Japan, which pioneered research into IDO induction by the immune system in the 1970s, deserves credit for initiating this field of study [reviewed in 19]. Following on from Hayaishi's work, many studies have linked Kyn metabolites and IDO to IFN-γ and immune system responses. Thus, picolinic acid was suggested to act as the second signal in the activation of IFN-γ-primed macrophages [10] and as a costimulus for the induction of reactive nitrogen intermediates in murine macrophages [11], and Quin was suggested as an immune system signalling agent when antibodies to Quin revealed its presence in high concentrations in macrophages and dendritic cells in the rat spleen [12]. Quin antibodies were then used to confirm the original observations and to demonstrate that Quin was produced predominantly in immune cells, and that large increases in Kyn pathway metabolism occur in macrophages, B-cells and dendritic cells in a variety of pathological conditions [13,15,17,20,21]. Inhibition of nitric oxide synthase expression and activity in macrophages by 3-HAA was reported in 1997 [18]. Thus, the concept that Kyn metabolites play a role in regulating immune function was well established prior to 1998 and could have been easily found by Munn et al. [4] with a simple literature search.

Many subsequent studies have confirmed and dramatically extended the role of Kyn metabolites as immunosuppressants. Thus, 3-HK and 3-HAA were shown to suppress allogeneic T-cell proliferation in an additive manner possibly by an apoptotic mechanism [22]. This mechanism was suggested as the basis of the ability of 3-HAA and Quin to undermine T helper type 1 (Th1) cells [23]. Prolonged survival of a murine model of cerebral malaria occurs when 3-HK synthesis is inhibited by a kynurenine hydroxylase (mono-oxygenase) inhibitor [24]. Apoptosis by Kyn metabolites is reported to be Th1-specific, thus shifting the Th1/Th2 balance towards the latter [23].

An equally important discovery is that of the ability of an excess of Trp to undermine T-cell suppression by Kyn metabolites [25], which may play a role in abnormal pregnancy and possibly also unsuccessful defense against infection [26]. A possible mechanism of this deleterious effect of excess Trp is overproduction of Kyn metabolites by the increased flux of Trp down the hepatic Kyn pathway through the high Trp affinity TDO. Under these conditions, IDO activity can be expected to be substrate-inhibited [27].

KYNURENINE METABOLITE FORMATION: RELATIVE ROLES OF THE TRYPTOPHAN FLUX AND TRYPTOPHAN-DEGRADING ENZYMES

The flux of Trp down the Kyn pathway is determined mainly by availability of plasma free Trp (see [8] for a discussion) and to lesser extents by activity of hepatic TDO under normal conditions and extrahepatic IDO under immune-related conditions. Normally, over 90% of dietary Trp is oxidized in the liver [8] and this is illustrated by the finding that deletion of the mouse TDO gene increases plasma total [Trp] by 9.3-fold [28]. The relative contribution of IDO1 gene deletion to plasma Trp availability was not assessed in a recent study [29], but this pathway is minimally active in the absence of an immune response [13,30]. Some researchers view Trp metabolism from the narrow window of IDO, which may give the impression to non-expert readers that IDO in general controls the Kyn pathway. This is especially the case when diagrams of the pathway include only IDO. Researchers should always include both IDO and TDO and point out the above differences in their contributions to Trp oxidation and utilization. Additionally, the complement of Kyn pathway enzymes expressed in different types of immune cells determines which Kyn pathway metabolites are produced, and therefore the degree and type of immunomodulation that results.

Whereas the role of IDO in Trp metabolism during infection is well established, the potential role of TDO has received little attention, because of the reported inhibition of its activity by agents that stimulate the release of IFN-γ, such as endotoxin (lipopolysaccharide or LPS) [31] and polyriboinosinic acid–polyribocytidylic acid (PolyI.PolyC) [31,32]. IFN-γ, however, does not inhibit TDO [31] and inhibition by PolyI.PolyC

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may involve inhibition of protein synthesis [32]. Additionally, PolyI.PolyC [31] and LPS [33] deprive TDO of its haem cofactor by inducing haem oxygenase. TDO inhibition by LPS may also involve inhibition of protein synthesis [34]. However, both PolyI.PolyC and LPS induce an early activation of TDO [32,35,36] involving increased haem saturation of the apoenzyme. With the former, dissociation of haem from cytochrome P450 has been implicated [32], whereas with LPS, enhanced lipolysis leading to release of albumin-bound Trp was demonstrated [35], most likely due to increased release of adrenal medullary catecholamines by reflex activation of the sympathetic nervous system secondarily to vasodilatation (see [35] for references). Indeed, [NEFA] is elevated by 2.2–17-fold in rabbit serum by three different bacterial infections (*anthrax*, *tularemia* and *pneumococcus*) [37] and in human serum by Dengue viral infection [38]. As well as catecholamines, which activate TDO via Trp, infection is also associated with increased release of cortisol, e.g. in Dengue [38] and *Toxoplasma gondii* [36] infections in humans. TDO activity can therefore be assumed to be enhanced in infection by both Trp and cortisol, both of which increase TDO activity additively.

TDO and IDO activities are rate limiting for Trp entry into the hepatic and extrahepatic Kyn pathways, but the overall flux through these pathways is a more important determinant of the downstream effects on immune system behaviour. An increase in this flux in infection is strongly suggested by the observed increase in [NEFA] described above and the decrease in serum albumin, e.g. in viral infection [39]. In cancer anorexia, an abnormally high free [Trp] is observed [40], which may be due to the known NEFA elevation [41]. A low albumin in cancer is associated with poor prognosis [42]. Plasma free [Trp] can therefore be expected to rise in infections of various types and, as stated above, an excessive rise could be counterproductive.

TIME TO ABANDON THE TRYPTOPHAN DEPLETION CONCEPT IN INFECTION

The concept of Trp deprivation as a host defence mechanism that limits the growth of pathogens continues to be cited to this day [43], despite substantial reasons for doubting that this mechanism operates *in vivo* [reviewed in 6]. Plants, bacteria, fungi and even some parasites use the shikimate pathway to synthesize aromatic amino acids, including Trp, among other key metabolites. Bacteria up-regulate Trp-related biosynthetic pathways in the presence of low Trp concentrations and therefore do not suffer from low levels or require external sources [44]. Host cells, however, have an absolute requirement for Trp supplied from dietary sources. Virtually all studies on Trp depletion and deprivation were done in cell culture, and therefore do not represent the *in vivo* conditions associated with infection. Factors differentiating *in vitro* from *in vivo* conditions include nutrients derived from the blood supply, the presence of multiple cell types, including cells of the immune system, complex structural/functional arrangements in tissues, the presence of an extracellular matrix, hormonal, cytokine and paracrine interactions, neural innervation of the tissue and lymphatic drainage, among many other factors that make extrapolation from cell culture to whole organism unconvincing.

Trp depletion as an operational biostatic defence tied to limiting the availability of a critical amino acid has never been verified *in vivo*. However, the concept has survived in a modified form in studies of obligate intracellular parasites such as members of the *Chlamydiae*. As obligate intracellular parasitic bacteria, *Chlamydia* species have reduced genomes and are therefore far more reliant on host resources such as Trp than free living pathogens [45]. As intracellular parasites, *Chlamydiae* have lost some or most of the enzymes for Trp synthesis, and therefore rely on host supplies. IDO-mediated Trp concentration reduction *in vitro* initiates a pathogen translational suite shift that leads to a persistent, non-proliferative state [46,47]. In response to Trp depletion, *Chlamydia* alters protein expression levels from proteins with high Trp content that facilitate growth and reproduction to a suite of low Trp-containing proteins that facilitate maintenance of a persistent, non-proliferative state. Like other intracellular parasites, the *Chlamydiae* are particularly sensitive to host nutrient levels in general, including levels of amino acids other than Trp [48]. It is notable that the original observations on IDO-mediated Trp depletion as a postulated biostatic defence by Pfefferkorn et al. in the 1980s were made *in vitro* on *T. gondii*, a protozoan that is also an obligate intracellular parasite [2,49].

Studies where Trp levels were actually determined *in vivo* were often done in whole tissue homogenates, e.g. whole brain and whole spleen in cerebral and non-cerebral malaria infections in mice [50]. In one investigation of *T. gondii* infection in mouse lung, dramatically reduced levels of Trp were observed in homogenates of whole lung 7 days after onset of infection, with a large concomitant increase in Kyn levels [51]. These studies show that it is possible to address questions surrounding Trp depletion *in vivo*, which could be extended for example by the use of microdialysis, computational metabolomics or other methods to determine the levels of Trp and Kyn metabolites within cells and tissues during infection.

THE TRYPTOPHAN DEPLETION CONCEPT IN PREGNANCY: ALSO AN UNTENABLE CONCEPT

Immunosuppression via Trp depletion would not guarantee a successful pregnancy, as the Trp requirements in pregnancy are manifold: increased protein synthesis by mother and fetus, fetal growth and development, serotonin for signalling pathways, kynurenic acid for neuronal protection, immunosuppressive kynurenines for fetal tolerance and Quin for NAD^+ synthesis. None of these goals could be achieved optimally if Trp was depleted to the point where pathogens would be starved. Munn et al. [4] did not consider the Trp status in pregnancy or the unusual Trp-metabolic status and immune vulnerability of the C57BL/6J mouse strain used (see [7,8]), nor could they have predicted that the IDO inhibitor 1-methyltryptophan used to inhibit IDO would subsequently be shown to inhibit placental Trp transport [52] and up-regulate

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IDO1 [53]. A Trp utilization concept, as an alternative to the notion of depletion, satisfying these physiological requirements in pregnancy has been proposed [7,8].

The depletion concept fails mainly because it did not consider the elevation of plasma free (non-albumin-bound) [Trp] in pregnancy, which has been known for some considerable time to occur in human and rat pregnancy [7,8]. Munn et al. [4] relied only on the previously reported decrease in total [Trp], which does not reflect IDO induction, but is the result of increased tissue Trp uptake due to an increased release of albumin-bound Trp by a combination of albumin depletion and NEFA elevation, and the rapid equilibration between the free and bound fractions. The higher [Trp] in placenta, umbilical cord and fetus reflect increased uptake and transport of maternal Trp, rather than its depletion [7,8].

Not all researchers measure plasma free Trp in pregnancy (or infection) studies [8], mainly because many studies on Trp metabolism are conducted *in vitro* using peripheral blood cells in culture or isolated tissues, wherein such measurement could not be accurately interpreted because of the presence of proteins in culture media. Further, the situation *in vivo* is distinctly different, with food availability and Trp release from tissues providing ready supplies for Kyn pathway metabolism. Future studies in whole animals *in vivo* or humans should always include both free and total plasma Trp, not only for accurate interpretation of changes in Trp disposition, but also to establish the baseline Trp status and its biological determinants [54].

Progress in IDO research culminating in discovering the immunosuppressive properties of Kyn metabolites could have been hastened if Munn et al. [4] reviewed the literature on the Trp status in pregnancy, or if one of the manuscript's reviewers had been knowledgeable in the relevant information. Depletion would not have been proposed, the hypothesis would have been modified, and researchers could have focused directly on alternative mechanisms. Here is a lesson for peer reviewing, where the expertise of the reviewers is paramount in a successful manuscript vetting. Open peer reviewing has been proposed [55] in the hope of dramatically reducing the incidence of poor attribution, and errors of commission and omission. However, this open-system is also not without disadvantages and may not suit certain journals. Until these disadvantages are ironed out, some journal editors will need to strengthen the current peer review process by paying greater attention to the choice of expert reviewers.

TRYPTOPHAN UTILIZATION IN PREGNANCY AND INFECTION: FUTURE PERSPECTIVES

From the above account, it seems reasonable to suggest that fetal tolerance and combating infection depend on Trp utilization, rather than starvation or depletion. Utilization meets all the physiological requirements in pregnancy and infection. The increased requirements for protein synthesis by the pregnant mother and her fetus necessitate an increased availability of maternal Trp. This is met by the elevation of maternal plasma free Trp by albumin depletion and NEFA elevation. The free Trp elevation then determines the flux of Trp down the Kyn pathway [8] to produce immunosuppressive Kyn metabolites that regulate T-cell responses via a number of mechanisms in order to balance immunity and tolerance [56] and ensure adequate NAD^+ synthesis from Quin to support metabolism and the poly ADP ribose polymerase (PARP) reaction [6,57]. An increase in maternal plasma free Trp is therefore paramount and this parameter should be measured in future studies, in conjunction with determinants of Trp binding (albumin and NEFA), total Trp, determinants of IDO (cytokines) and TDO (cortisol) activities, and kynurenine and its main immunosuppressive metabolites, 3-HK, 3-HAA and Quin. In addition to maternal plasma, these parameters should, where appropriate, be determined in cord blood, placenta and fetal tissues in normal and abnormal pregnancies. A potential increase in plasma free [Trp] is also likely in infections with various pathogens and cancers and the above parameters should also be measured in plasma and normal and diseased tissue from patients suffering from these conditions. The potential negative effect of an excess of Trp on pregnancy outcome and in combating infection should be explored under these unfavourable conditions by assessing Trp availability against levels of Kyn metabolites and extents of immunosuppression. Changes in Trp could also be assessed by microdialysis or similar techniques in experimental studies *in vivo* during immune responses to various pathogens and cancers. If, as expected, excess Trp is proven to cause harm under certain circumstances, corrective strategies could be adopted, e.g. reduced protein intake or Trp sequestration through albumin binding.

One topic of IDO-mediated Trp metabolite formation frequently overlooked is the substantial species differences in Kyn metabolism and responsiveness [1,6,46]. Results in one species may not hold up in another, so data on immune system responsiveness to Kyn metabolites must be viewed with caution when applied to other species. With this in mind, recent work on knockout mice lacking the gene for kynurenine-3-monooxygenase (KMO), and wild type mice administered inhibitors of this enzyme, suggest that some Kyn metabolites may also enhance the immune response, whereas others suppress it [58]. Acute experimental pancreatitis in mice leads to multiple organ failure due to excessive inflammatory responses. KMO knockout and KMO enzyme inhibition in mice subjected to acute pancreatitis prevent the inflammatory response and organ failure [58]. These findings implicate 3-HK in mediating the inflammatory response in acute pancreatitis in mice. However, it is not known if this finding will hold up in other species.

The distinction between Trp depletion and Trp utilization is conceptual. When precursors are used to synthesize active biomolecules it is never conceptualized as precursor depletion, but rather it constitutes substrate utilization. The same is true for up-regulated Trp metabolism in the immune system in response to various physiological and pathological signals. Increased Trp utilization via IDO is a host defence response directed at increasing Kyn pathway metabolites and NAD^+, and possibly also as a mechanism for regulating cellular redox state, not a mechanism for removing Trp from the blood supply or tissues. The experimental paradigm of manipulating Trp levels in cell culture continues to stimulate research on the actions of IDO. However, the

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conceptualization of Trp depletion during an immune challenge has shifted in recent years to focus on downstream host cell responses to lowered intracellular Trp availability. Diverse cellular stressors, including lowered intracellular amino acid availability, initiate the integrated stress response (ISR) leading to an adaptive shift in translation initiation and protein expression. For example, Liu et al. showed that macrophages in cell culture responded to lowered Trp availability by activating the ISR and enhancing LPS-induced IL-6 production through activation of a protein kinase signalling cascade [59]. This suggests that rather than starving host cells or pathogens, IDO-induced intracellular Trp depletion acts through nutrient sensing pathways to enhance macrophage activation and cytokine production as part of a general metabolic stress response. It is quite possible that *in vitro* Trp depletion studies in general have simply reflected a triggering of the ISR, which has profound effects on protein expression and cellular responses.

We propose that the core Trp utilization concepts are: (1) the role of the Kyn pathway rate-limiting enzymes TDO and IDO in regulating hepatic and extrahepatic Kyn metabolite formation respectively; (2) plasma free Trp availability and Kyn pathway enzyme expression for production of Kyn metabolites to achieve immune regulation and adequate NAD^+ synthesis in immune cells; (3) the protective role of IDO in defending hosts against oxidative damage; and (4) a potential counterproductive effect of excessive Trp availability from dietary sources or internal stores leading to overproduction of Kyn metabolites induced by a heightened IDO activity during an immune response. We hope that Trp utilization concepts will continue to stimulate further productive research that can ultimately lead to suitable and effective intervention strategies to combat infectious diseases and cancer and ensure a safe pregnancy outcome.

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