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Interferons and indoleamine 2,3-dioxygenase: Role in antimicrobial and antitumor effects

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Summary. Indoleamine 2,3-dioxygenase (IDO) is an interferon (IFN)-induced protein that initiates the metabolism of tryptophan along the kynurenine pathway. Although IDO can be induced by IFN-γ in many cell types, only mononuclear phagocytes have been shown to be induced to decyclize tryptophan by all three IFN classes. Since tryptophan is an essential amino acid necessary for a variety of metabolic processes, depletion of available tryptophan may be an important mechanism for control of rapidly-dividing microbial pathogens and tumors. The purpose of this review is to present evidence that documents the effects of IFN-induced IDO on prokaryotic and eukaryotic pathogens, as well as on a variety of tumor cell lines.
Key words. Tryptophan; macrophage; monocyte; interleukin-2; cancer; *Chlamydia; Toxoplasma.*

Introduction

Since its initial characterization as a viral interfering substance[22], significant advances in describing the diverse effects of the interferon system (IFN) have occurred. No longer is IFN considered to affect only viruses; immunomodulatory, antimicrobial, and antitumor effects have been demonstrated using IFNs. Although the mechanisms of action for many of these effects have yet to be precisely determined, a number of proteins have been shown to be induced by interferons[58]. Presumably, as research continues, specific functions will be attributed to these proteins, and specific mechanisms of action will become better defined.

One IFN-induced protein for which a function has been demonstrated is indoleamine 2,3 dioxygenase [IDO; indoleamine:oxygen 2,3-oxidoreductase (decyclizing)]. IDO has been purified from both animal and human tissues, and has been characterized as a cytoplasmic, heme-containing, monomeric protein of approximately 40,000 molecular weight[36,49,63]. Unlike the liver-specific enzyme tryptophan 2,3-dioxygenase, a 168,000 molecular weight tetrameric enzyme with four heme-chromophores at its active site[19,24], IDO is ubiquitously distributed in normal[49,63,67] or malignant tissues[64,68], mononuclear phagocytes[12,13,34,59] and neoplastic cell lines[16,35,51,60]. IDO can be induced by cancer[68], viral infection[71], bacterial lipopolysaccharides (LPS)[13,65,69], IFN[9,11,12,16,34,35,51,52,59,60,64,66,70] and interleukin-2 (IL-2)[5,12,13]. In each system studied, induction of IDO has been shown to result in substantially increased tryptophan metabolism. Among the indoleamines against which IDO has been shown to possess activity are included D- and L-tryptophan, D- and L-5-hydroxytryptophan, tryptamine and serotonin[49]. However, IDO has been shown to be most active against the essential amino acid L-tryptophan. Its enzymatic activity involves the decyclization of tryptophan to *N*-formylkynurenine by oxidative cleavage of the pyrrole ring. Apparently, both molecular oxygen and superoxide anion can participate as the second substrate for this reaction[36,50,53]. The biological electron donors for the heme iron of IDO are proposed to be reduced flavin and pyridine nucleotides[36], as well as superoxide anion formed by coupling electrons from tetrahydrobiopterin[37], flavoenzymes, xanthine oxidase or glutathione reductase[20,21,53].

Although the exact physiologic function of IDO has not yet been completely defined, experimental evidence suggests that it may be involved in regulation of cellular growth and proliferation. Since tryptophan concentration either in the individual cell or in the whole organism is important for several metabolic processes, its regulation by IDO may have pleiotropic effects. Tryptophan is an essential amino acid and the least abundant of the amino acids required for mammalian cellular integrity. Its availability affects protein synthesis as well as protein degradation, genome replication, and organismal growth[2,7,15,27,28]. Restriction of available tryptophan due to degradation by IDO could lead to a condition in which cells become starved for tryptophan. Such a situation would more severely affect rapidly dividing cells such as microbial pathogens and tumor cells. However,

tryptophan is also a precursor for several other important biomolecules. In one of the metabolic pathways, tryptophan can be hydroxylated and decarboxylated to become serotonin [15], a neurotransmitter and putative immunomodulator. It may be *N*-acetylated using acetyl CoA to form *N*-acetylserotonin (sepiapterin reductase inhibitor [23]), then O-methylated to become a pineal hormone, melatonin [62]. In a competing pathway, the pyrrole ring of tryptophan can be oxidatively cleaved at the 2–3 position by the catalytic action of tryptophan 2,3-dioxygenase to enter the complex kynurenine pathway, where some of the intermediate metabolites can be ultimately converted to a one carbon source for purine and pyrimidine synthesis [1, 55, 56], a tumor angiogenesis factor (nicotinamide [25]), electron transport coenzymes (NAD and NADP), and to glutaryl CoA, CO_2 and ammonia [4, 32, 43]. It is conceivable that unrestricted tryptophan decyclization could be responsible for some of the side effects associated with IFN administration in humans.

Induction of IDO

Early experiments concerned with the physiologic significance of IDO examined the function of IDO under various pathologic conditions. Intraperitoneal injection of LPS resulted in a 30–50-fold increase in IDO activity in murine lung tissue [65], while other inflammatory agents (glycogen and zymosan) did not significantly enhance IDO activity. Since both cycloheximide and actinomycin D have been shown to abolish the induction of IDO activity, de novo protein synthesis must be required for expression of its activity. Additional testing has demonstrated that viral infection could similarly stimulate tryptophan metabolism in murine lung tissue [71]. These observations have led investigators to examine whether IFN could substitute for LPS or viral infection in the induction of IDO. Using a naturally-derived murine IFN-α/β preparation, it was demonstrated that in vitro incubation of murine lung tissue with IFN resulted in a 10–15-fold increase in tryptophan decyclization [66].

Experimentation in human systems has stemmed from work in which the antimicrobial effect of IFN-γ on *Toxoplasma gondii* was ascribed to enhanced tryptophan degradation in the culture medium [39]. In studies designed to further characterize the enzyme responsible for the rapid breakdown of tryptophan in the culture medium, it was demonstrated that IDO activity in both normal fibroblasts as well as epithelial cell lines could be induced in response to IFN-γ but not to the type I interferons, IFN-α and IFN-β [9, 39, 42]. Induction of the enzyme was shown to be a function of IFN-γ concentration and could be abrogated by treatment of the cells with inhibitors of either RNA or protein synthesis. In IFN-γ-induced cells, tryptophan has been shown to undergo rapid decyclization and export. Within 90 min after IFN-induced cells were pulsed with radiolabeled tryptophan, radiolabeled *N*-formylkynurenine and kynurenine began to accumulate in the culture medium [10]. The second enzyme involved in the degradation of tryptophan along the kynurenine pathway, *N*-formylkynurenine formamidase, was shown to be constitutive with respect to IFN treatment; thus the rate at which the two tryptophan metabolites accumulated in the culture medium was dependent only on IDO activity [42].

The unique role of the mononuclear phagocyte

Since murine IFN-α/β mediated induction of IDO in the murine system [66], yet human IFN-γ but not human type I IFNs induced tryptophan decyclization in human cell lines, questions remained concerning the nature of the induction and the inducing agent in these two systems. To help clarify this area of research, a variety of cytokines including IFNs-α,β, and γ as well as interleukin-2 (IL-2) and tumor necrosis factor-α (TNF) were examined for their capacity to induce IDO activity in an immunocompetent cell population [12]. Peripheral blood mononuclear cells (PBMC) from normal human donors were treated with these agents for 48 h and changes in IDO activity were determined. All three classes of IFN as well as IL-2 induced both significant and comparable IDO activity in PBMCs.

Since PBMCs contain T cells capable of generation of IFN-γ in response to IL-2 [18, 38], a panel of neutralizing antibodies were used to determine if IDO was induced directly or via in vitro generation of IFN-γ. As seen in figure 1, only IDO activity induced by IL-2 was blocked by antibody specific to a different cytokine. The effector cell present within the PBMC population has been identified as the peripheral blood monocyte [13, 34, 46]. In cell mixing experiments in which purified monocyte and lymphocyte populations were tested for their ability to

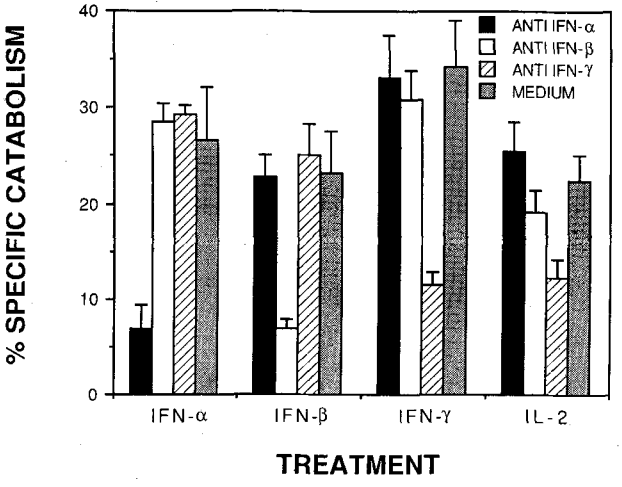

Figure 1. Effect of IFN class-specific antibodies on cytokine-induced IDO activity in PBMC cultures. Concentrations of cytokines used were: IFN-γ, 320 U/ml; all others, 1000 U/ml. Sufficient IFN-specific antibody was added to neutralize exogenously added IFN. Specific catabolism represents the mean percentage of tryptophan initially available in the culture medium that was specifically degraded by triplicate PBMC cultures $\pm$SD. Adapted with permission from Carlin et al.[12].

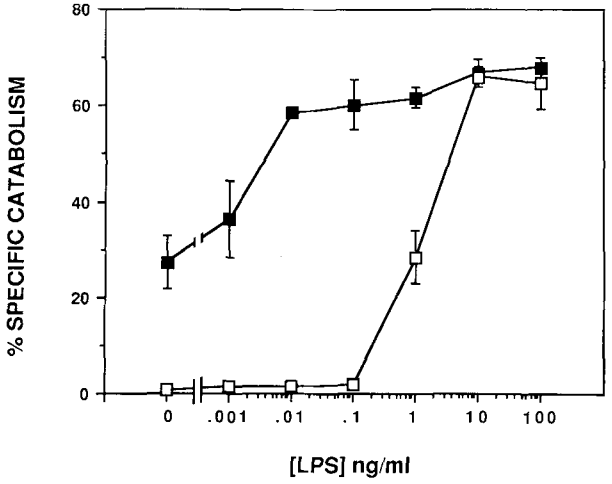

Figure 2. Effect of LPS concentration on induction of IDO activity in monocyte-derived macrophages. Specific catabolism represents the mean percentage of tryptophan available in the culture medium that was specifically degraded by the triplicate cell cultures during a 4-h period ±SD. IDO activity was measured in macrophages treated with medium alone (□) or with IFN-β, 20 ng/ml (■). Reprinted with permission from Carlin et al.[13].

degrade tryptophan in response to various cytokines, only the IFNs induced IDO activity and only in the monocyte population [13]. IL-2 required both lymphocytes and monocytes. Interaction was required between these populations, due to production of IFN-γ by T cells, with subsequent IFN-γ-mediated induction of IDO in monocytes.

There are apparent differences in the mechanisms by which various IFNs induce IDO activity. In a comparison of IFN-α and IFN-γ-induced IDO activity, IFN-γ at one fifth the unit concentration of IFN-α induced seven times the amount of tryptophan decyclization [34]. In addition, differential effects of antiinflammatory agents on IDO induction have been observed between various IFNs. While acetaminophen, 3-deazaadenosine, indomethacin, and dexamethasone all inhibited IFN-α-induced IDO, only acetaminophen and 3-deazaadenosine inhibited IFN-γ-induced IDO [34]. And contrary to what was observed with IFN-α, dexamethasone enhanced induction of IDO by IFN-γ suggesting that different induction mechanisms are being affected by these agents. In a study of IDO activity induced in monocyte-derived macrophages, the presence of LPS in the culture medium was shown to modulate the induction of IDO by IFN-β, but not IFN-γ [13]. Although IFN-β induced some IDO activity in the absence of exogenous LPS, as little as 10 pg/ml of LPS allowed induction of IDO by IFN-β to a level equal to that observed with IFN-γ (fig. 2). A recent investigation concerning the regulation of IDO synthesis in response to IFN has demonstrated that induction of IDO activity involves the de novo synthesis of the IDO molecule, and has provided evidence that synthesis of an intermediary protein is required in the induction of IDO by IFN [46]. Conceivably, IFN-γ could supply all of the necessary signals for production of both the intermediary and IDO, while stimulation of mononuclear phagocytes with IFN-α and IFN-β could provide only one signal and requires a second stimulus for maximum induction of tryptophan decyclizing activity. However, in non-myeloid cells, even the combination of type I IFNs with LPS has been shown to be insufficient to induce IDO. Thus, mononuclear phagocytes appear to be unique in their ability to respond to all classes of IFN with enhanced tryptophan decyclization.

Antimicrobial effects of IDO

IFN-γ is known to mediate the inhibition of a variety of intracellular pathogens [30, 41, 47, 57]. Although IFN-γ-activated mononuclear phagocytes have been shown to possess enhanced respiratory activity which has been associated with the destruction of intracellular pathogens [29, 31], oxygen-independent mechanisms (unrelated to respiratory burst phenomenon) have also been implicated in the antimicrobial effects of IFN [8, 10, 45].

One oxygen-independent mechanism by which IFN-γ has been shown to restrict the replication of microbial pathogens is via the induction of IDO. The initial observation of the effect of IDO on microbial replication was made using the IFN-γ-sensitive, obligate intracellular eukaryotic parasite, *T. gondii*. Although increasing IFN-γ concentrations led to complete inhibition of *T. gondii* growth in human fibroblasts [41], it was found that the antitoxoplasma effect of IFN-γ was related to the type of medium used in culturing the host fibroblasts [42]. Analysis of the medium demonstrated that the concentration of tryptophan was the sole difference in medium components to which the antimicrobial modulatory activity could be attributed. As the concentration of tryptophan in the medium was increased, the antitoxoplasma effect of IFN-γ was diminished. Chromatographic analysis of culture supernatants of [^{14}C]tryptophan-spiked, IFN-γ-treated cultures revealed the appearance of two radiolabeled tryptophan metabolites which co-chromatographed with *N*-formylkynurenine and kynurenine. IFN-α and IFN-β had no effect on *T. gondii* growth in these cells and failed to induced decyclization of tryptophan. Additional experiments were performed to determine the mechanism by which IDO activity inhibited *T. gondii* growth [40]. To determine if the tryptophan metabolites were toxic to *T. gondii*, cultures were supplemented with *N*-formylkynurenine and kynurenine. The metabolites were found to be inhibitory to *T. gondii* growth only when their concentrations were raised to 32–64 times the concentration of tryptophan in the culture medium. In contrast, reduction of tryptophan in the medium did lead to restricted parasite growth. It was concluded that the effect of IFN-γ-induced IDO was primarily microbistatic and due to starvation for tryptophan.

Similar studies were performed using the obligate intracellular prokaryote *Chlamydia psittaci* [10]. As had been observed with *T. gondii*, the replication of *C. psittaci*

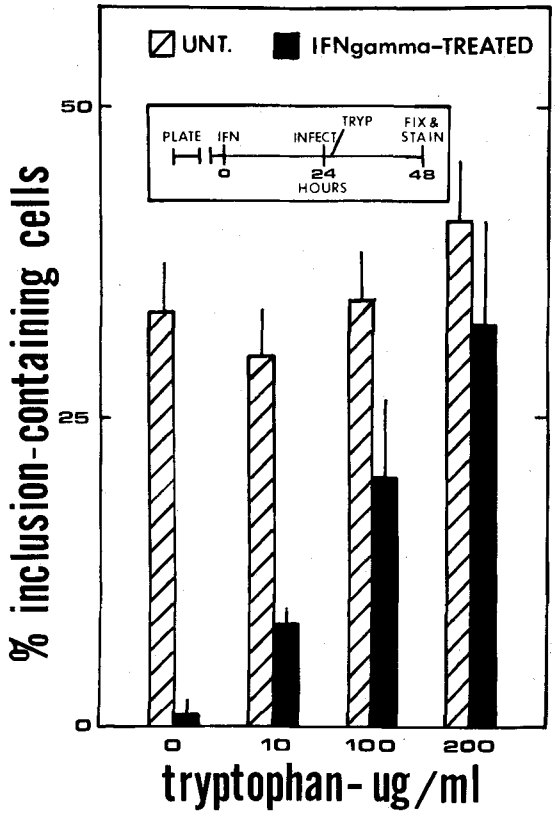

Figure 3. Effect of tryptophan (TRYP) on *C. psittaci* growth in IFN-γ-treated T24 cells. Cells were plated, treated, infected, and processed as described in the inset. The indicated amounts of tryptophan were added 60 min after infection. Data is presented as the mean of triplicate determinations ±SD. Solid bars represent cells that received 20 ng/ml of IFN-γ. Hatched bars represent wells that received medium alone. Reprinted with permission from Byrne et al.[10].

within human epithelial cells was inhibited in proportion to the concentration of IFN-γ present. When tryptophan was added to IFN-γ-treated cells just after infection with *C. psittaci*, reversal of the inhibition occurred in proportion to the concentration of tryptophan added (fig. 3). In cultures supplemented with 1 mM tryptophan, growth of chlamydiae in IFN-γ-treated cells was not significantly different from growth in cells treated with medium alone. When culture supernatants were subjected to reversed-phase high performance liquid chromatography (HPLC), an IFN concentration-dependent effect on tryptophan was observed. With increasing IFN-γ concentration, proportionally, less tryptophan was present, and two new peaks co-chromatographing with *N*-formylkynurenine and kynurenine were identified.

IDO-mediated inhibition of intracellular replication has also been demonstrated for the other chlamydial species, *C. trachomatis*[48]. As had been previously demonstrated with *T. gondii*, progressively more IFN-γ was required to inhibit intracellular chlamydial growth as the concentration of tryptophan in the culture medium was raised. However, when the cells used to culture chlamydiae were pretreated with a relatively high concentration of IFN-γ (1000 U/ml), the inhibitory effect could not be completely reversed with tryptophan. These results may be explained by either the possibility that, at high IFN-γ concentrations, damage to the phagosome which encloses the chlamydiae may permit phagolysosomal fusion with the subsequent destruction of the parasite, or that an irreversible effect on host cell metabolism results in inhibition of *C. trachomatis* replication.

In reversing IDO-mediated inhibition of *C. trachomatis* replication by excess tryptophan, the time at which tryptophan was added to IFN-γ-treated host cells influenced the outcome of the infection. When tryptophan was added up to 24 h after infection, the antimicrobial effect could be completely reversed. However, when tryptophan supplementation was delayed until 48 and 72 h post-infection, recovery was limited to 30% and 1%, respectively, of optimal yield. The effect of long-term IDO-mediated tryptophan deprivation on recovery of *C. psittaci* was also examined (manuscript submitted for publication). As reported for *C. trachomatis*, delay in tryptophan addition to IFN-γ-treated, chlamydiae-infected cells resulted in a progressively lower recovery of chlamydiae. Depletion of other essential nutrients in addition to tryptophan during the prolonged incubation did not influence chlamydial recovery. Replenishment of culture medium and supplementation with increased concentrations of amino acids and vitamins failed to restore the ability of tryptophan to reverse the antimicrobial effect of IFN-γ. However, while prolonged tryptophan deprivation resulted in diminished chlamydial recovery subsequent to tryptophan addition, the same cells could fully support a fresh infection following tryptophan supplementation. This indicated that while, in the short term, IFN-induced IDO had a microbistatic effect, the extended effect of IDO activity of chlamydiae was microbicidal.

Recent experiments have extended the range of cell types in which IFN-induced IDO is capable of inhibiting *C. psittaci* to monocyte-derived macrophages (manuscript submitted for publication). Not only did IFN-γ inhibit intracellular chlamydial growth in a concentration-dependent manner, but also IFN-β inhibited growth when combined with LPS. The role of IDO in IFN-mediated inhibition was confirmed by the fact that excess tryptophan substantially reversed the antimicrobial effect of IFN treatment, and that tryptophan was rapidly degraded in these IFN-treated cultures. However, complete restoration of chlamydial growth by exogenous tryptophan was not achieved; apparently other IFN-induced activities unrelated to tryptophan also contributed to the antimicrobial effects of IFNs.

Evidence has been presented which clearly indicates that decyclization of tryptophan is not the only mechanism by which IFN-γ exerts an antimicrobial effect. Although IFN-induced IDO activity in mouse tissue has been documented[66], it has been difficult to demonstrate in murine cell lines[57], (personal observations). Murine IFN-γ has been shown to be a potent antichlamydial agent in a

mouse fibroblast cell line, yet inhibition of *C. trachomatis* by murine IFN-γ has been shown to be independent of tryptophan concentration [17]. In a study of the role of amino acid deprivation in IFN-γ-induced suppression of the growth of another obligate intracellular prokaryote, *Rickettsia prowazekii*, it was concluded that neither tryptophan depletion nor depletion of other amino acids contributed to the inhibitory effect of murine IFN-γ on rickettsial growth [57]. Furthermore, despite demonstrated induction of IDO activity in a human cell line, excess tryptophan was unable to reverse the anti-rickettsial effect of human IFN-γ. And finally, no role has been demonstrated for IDO in the IFN-γ-mediated inhibition of development of intrahepatocytic malaria parasites [47]. Thus, while in certain host cell/pathogen systems, IFN-induced tryptophan decyclization may be an important factor in the antimicrobial effect of IFN, it is not the only activity important in inhibition of intracellular pathogens.

Antitumor effects of IDO

In this section, evidence suggesting a molecular mechanism for the antitumor activity of IFN, mediated through induction of IDO and subsequent reduced availability of tryptophan, is reviewed. It has been proposed that a substantial increase in tryptophan metabolism by virtue of a cell's own capacity to express IDO results in deprivation of the amino acid from surrounding cells, tissues and extracellular fluids, leading to tumor growth inhibition. Recently, the antiproliferative action of IFN-γ on normal and neoplastic cells through induction of IDO has been shown in at least three independent research laboratories in the United States and Japan [16, 35, 51]. The antiproliferative effect of IFN-γ was found to be cell – as well as time – (length of treatment) dependent. Those cells bearing a capacity to express IDO in response to the IFN-γ treatment exhibited greater inhibition of cellular proliferation than those without the dioxygenase activity. Tryptophan supplementation of the culture medium during the initial 4 days of an 8-day experiment effectively neutralized or modulated the antiproliferative effect of IFN in those cells bearing a relatively stable post-transcriptional apparatus (oral carcinoma: KB). However, those cells (colon adenocarcinoma: WiDr) sensitive to a transcriptional inhibitor (actinomycin D) as well as to IFN-γ, or KB cells after 8 days of prolonged treatment, failed to proliferate even at higher levels of tryptophan supplementation (400–800 μM) [35]. Although this concentration of tryptophan might result in alteration of cellular metabolism through the amino acid imbalance, a clear amino acid toxicity was observed at tryptophan concentrations greater than 1–2 mM. Cellular growth was suppressed at this concentration in the absence of IFN [16]. These results suggest that modulation of the antiproliferative action of the IFN/IDO system is, no doubt, a function of multiple factors such as tryptophan availability (e.g., the amino acid concentration and competition for a transport system with other aromatic or large neutral amino acids), and the stability of DNA transcriptional and post-transcriptional processes.

A mechanism of the antitumor effect of IFN-γ through induction of IDO, resulting in a substantial increase in tryptophan decyclization, appears to be merely an alteration in cellular metabolism; however, once the antineoplastic sequence is activated by the deprivation of tryptophan, more complex molecular events may be initiated which relate directly to the fate of the cell. For example, many studies have shown that deprivation of other amino acids such as isoleucine, glutamine [26, 54], asparagine [14], and histidine [44] also are effective in influencing genome replication and cellular growth. Chinese hamster cells, mouse L cells, and Syrian hamster BHK 21 cells grown in isoleucine and/or glutamine deficient medium accumulated in a state of G1 arrest (i.e., cells initially in S, G2, and M continue the forward-cell cycle and accumulated at G1 state) and were unable to initiate DNA synthesis. Similar results also were observed with murine L1210 leukemia or LM fibroblast cells when they were cultured in tryptophan-deficient medium. In those studies, inhibition of DNA synthesis was much more sensitive to the treatment than was protein synthesis, including synthesis of histones [7, 61]. Moreover, it was shown that exogenously-administered L-asparaginase/L-glutaminase isolated from *Pseudomonas* was effective in suppressing the growth of subcutaneously or intraperitoneally implanted murine leukemias (L1210, EARAD/1, and C1498), ascites tumors (Taper liver), and solid tumors (Walker 256 carcinosarcoma and B16 melanoma). Intraperitoneal administration of a tryptophan side chain-oxidizing enzyme, indolyl-3-alkane alpha-hydroxylase, significantly lowered the tryptophan concentration in plasma as well as in other tissues, and also was shown to inhibit growth of implanted Meth A sarcoma, Ehrlich carcinoma, and Taper liver tumor. It is interesting to note that implantation of Meth A sarcoma in allogeneic but not in syngeneic mice elicited the induction of IDO in malignant cells or tumor infiltrating macrophages; elevated IDO levels were associated with significant suppression of tumor growth [68]. Administration of exogenous histidase also was shown to be effective against Ehrlich carcinoma and Meth A sarcoma [44]. Treatment of mice with guinea pig serum-L-asparaginase was effective in suppression of leukemias [3, 6]. Although there was a development of hypersensitivity to the enzyme preparation in some patients, the administration of the *Escherichia coli* enzyme, L-asparaginase, was shown to be effective against leukemias in humans [33].

These studies have clearly demonstrated that amino acid deprivation induced in normal or neoplastic cells in vitro by simple removal of an amino acid from the culture medium, and from the tissues or plasma in vivo by the administration of exogenous enzymes, are indeed an ef-

fective antineoplastic treatment against some types of murine and human tumors. It is important that the treatment of humans with L-asparaginase isolated from bacteria was effective against leukemias; however, development of a hypersensitivity to the exogenous protein preparation limited its application. Administration of IDO may also prove to be an effective antineoplastic treatment, and one with less potential hypersensitive reactivity, since IDO of human origin could be used. However, induction of endogenous IDO, and consequential deprivation of tryptophan in cells, tissues and extracellular fluids by IFN or by other biologic response modifiers may be a more physiological and efficacious approach.

Conclusion

Today, knowledge of the molecular mechanisms of the antimicrobial and antineoplastic actions of the IFN system is in a rudimentary state, and the physiological role of IDO in protective immunity has not yet been fully explored. The use of nucleic acid and antibody probes specific to IDO would be helpful in determining the usefulness of IDO as a monitor of the biologic effects of IFN in immunotherapy and immunoprophylaxis, in determining the specificity of IDO induction in normal and malignant tissues, and in answering questions concerning the function of IDO under a number of pathologic as well as normal conditions. Evidence has been presented that IFN-induced IDO may be partly responsible for some of the antimicrobial and antitumor effects of IFN. The exhibited efficacy of the antineoplastic action of the IFN/IDO system in vitro and the administration of tryptophan sidechain-oxidizing enzyme in mice provide a hope for future cancer treatment. Induction of IDO by IFN in immunosuppressed patients may prove useful in treating opportunistic infections. Clearly, more investigation will be required to understand the full potential of this aspect of IFN action.

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