1-Tryptophan–L-Kynurenine Pathway Metabolism Accelerated by *Toxoplasma gondii* Infection Is Abolished in Gamma Interferon-Gene-Deficient Mice: Cross-Regulation between Inducible Nitric Oxide Synthase and Indoleamine-2,3-Dioxygenase

Suwako Fujigaki, Kuniai Saito,* Masao Takemura, Naoya Maekawa, Yasuhiro Yamada, Hisayasu Wada, and Mitsuru Seishima

*Department of Laboratory Medicine, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500-8705, Japan*

Received 13 April 2001/Returned for modification 4 June 2001/Accepted 31 October 2001

1-Tryptophan degradation by indoleamine 2,3-dioxygenase (IDO) might have an important role in gamma interferon (IFN-γ)-induced antimicrobial effects. In the present study, the effects of *Toxoplasma gondii* infection on IDO were investigated by using wild-type and IFN-γ-gene-deficient (knockout) (IFN-γ KO) mice. In wild-type C57BL/6J mice, enzyme activities and mRNA levels for IDO in both lungs and brain were markedly increased and lung 1-tryptophan concentrations were dramatically decreased following *T. gondii* infection. In contrast, these metabolite changes did not occur in *T. gondii*-infected IFN-γ KO mice or in uninfected IFN-γ KO mice. The levels of inducible nitric oxide synthase (iNOS) induction in infected IFN-γ KO mice were high in lungs and low in brain compared to those in infected wild-type mice. The extent of increased mRNA expression of *T. gondii* surface antigen gene 2 (SAG2) induced in lungs and brain by *T. gondii* infection was significantly enhanced in IFN-γ KO mice compared to wild-type mice on day 7 postinfection. Treatment with N-nitro-l-arginine methyl ester, an iNOS inhibitor, increased the levels of SAG2 mRNA in brain but not in lungs and of plasma 1-kynurenine after *T. gondii* infection. This in vivo study provides evidence that 1-tryptophan depletion caused by *T. gondii* is directly mediated by IFN-γ in the lungs, where iNOS is not induced by IFN-γ. This study suggests that there is an antitoxoplasma mechanism of cross-regulation between iNOS and IDO and that the expression of the main antiparasite effector mechanisms for iNOS and/or IDO may vary among tissues.

*Toxoplasma gondii*, an intracellular protozoan parasite, is a major pathogen of opportunistic infectious disease and causes severe encephalitis, pneumonia, and retinochoroiditis in infants, during pregnancy, and in immunocompromised hosts, such as patients with AIDS or patients treated with immunosuppressive drugs (25, 32, 43). Previous studies indicated that various cytokines have important roles in the regulation of suppressive drugs (25, 32, 43). Previous studies indicated that many studies have demonstrated that various cytokines have important roles in the regulation of suppressive drugs (25, 32, 43). Previous studies indicated that various cytokines have important roles in the regulation of suppressive drugs (25, 32, 43).
by toxoplasma infection was also evaluated by using IFN-γ KO mice.

MATERIALS AND METHODS

Materials. L-TRP, L-KYN, methylene blue, and ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals, of analytical grade, were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals. Wild-type female C57BL/6J mice (18 to 20 g) were obtained from Japan SLC Inc. (Hamamatsu, Japan). IFN-γ KO mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Animals were kept in a temperature-controlled facility at 25°C with a 12-h on-12-h off light cycle and allowed free access to food and water. All experiments were performed according to the guidelines for animal experiments set by Gifu University School of Medicine. Mice were killed at 0, 4, 7, 14, 21, and 28 days after infection by intraperitoneal access to food and water. All experiments were performed according to the guidelines for animal experiments set by Gifu University School of Medicine. Mice were killed at 0, 4, 7, 14, 21, and 28 days after infection by intraperitoneal injection of sodium pentobarbital (50 mg/kg of body weight). Blood was taken from the abdominal vena cava, and plasma was separated by low-speed centrifugation (1,000 × g, 10 min). Tissues were placed in polypropylene tubes and immediately frozen by immersion in liquid nitrogen. All samples were frozen at −80°C until analysis.

T. gondii infection. Parasites were harvested from the brains of mice chronically infected with T. gondii cysts (Fukaya strain). Brain tissue was dispersed in saline. The final concentration of the infectious agent was adjusted to a dose of 20 cysts per 0.2 ml, which was injected intraperitoneally into mice.

Inhibitor studies. N-Nitro-L-arginine methyl ester (L-NAME) and 6-chloro-L-tryptophan were dissolved in 0.5% carboxymethyl cellulose sodium salt. L-DL-tryptophan were dissolved in 0.5% carboxymethyl cellulose sodium salt. The final concentration of the infectious agent was adjusted to a dose of 20 cysts per 0.2 ml, which was injected intraperitoneally into mice.

Determination of L-TRP and L-KYN concentrations. Plasma was mixed with 3% perchloric acid, and tissue samples were sonicated in 4 volumes of 3% perchloric acid, and tissue samples were sonicated in 4 volumes of 3% perchloric acid. L-TRP in tissues. The mobile phase consisted of 2.5% acetonitrile in 0.1 M sodium acetate (pH 3.9) and was filtered through a 0.45-m-pore-size HA-type filter obtained from Millipore Corp. (Bedford, Mass.). The flow rate was maintained at 0.75 ml/min throughout the chromatographic run.

Enzyme assay. IDO activity was measured as described previously (11). Briefly, tissues were homogenized with a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) in 1.5 volumes of ice-cold 0.14 M KCl-20 mM potassium phosphate buffer (pH 7.0). The homogenate samples were centrifuged at 10,000 × g for 10 min. An aliquot of supernatant was taken for the measurement of IDO activity. The reaction mixture contained 50 μl of enzyme preparation and 50 μl of substrate solution. The composition of the substrate solution was 100 mM potassium phosphate buffer (pH 6.5), 50 μM methylene blue, 20 μg of catalase, 50 mM ascorbate, and 0.4 mM l-TRP. After incubation of the reaction mixture at 37°C, samples were acidified with 3% perchloric acid and centrifuged at 7,000 × g and 4°C for 10 min. The concentrations of the enzymatic products were measured by using HPLC. Enzyme activity was expressed as the product content per hour per milligram of tissue protein.

Table 1. Primer sequences for RT-PCR

<table>
<thead>
<tr>
<th>Mouse molecule</th>
<th>Primera</th>
<th>Sequence</th>
<th>PCR product (bp)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDO</td>
<td>F</td>
<td>5′-CAGTGACGCGGACGACTGAGA</td>
<td>400</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5′-TCCATGCTTTACTCGTTGACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>F</td>
<td>5′-AAGCTGATGTGACATCGACCCGT</td>
<td>598</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5′-GCACTGTTGAACGCCACGTCGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAG2</td>
<td>F</td>
<td>5′-ATGAGTGTCTTCAAAAGCAGGCTA</td>
<td>560</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5′-TTACAAACGATGACCAAACTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>F</td>
<td>5′-ATGGATGACGATATCGCT</td>
<td>569</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5′-ATGAGGTAGTCTTGCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F</td>
<td>5′-ACGCCCTTTACATGACCTCACT</td>
<td>320</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5′-ATAATCTTCGTTGTCACCCCAT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a F, forward; R, reverse.

Statistical analyses. Results were expressed as the mean and standard error of the mean (SEM) or as a percentage of the control value. Intergroup comparisons were made by using one-way analysis of variance, followed by the Mann-Whitney test or Scheffe post hoc test. A value of less than 0.05 was considered statistically significant.

RESULTS

Temporal profiles of IDO induction in wild-type mice infected with T. gondii. We investigated the time course of changes in plasma L-KYN concentrations and the levels of IDO activity and mRNA expression in the lungs and brain in wild-type mice infected with T. gondii (Fig. 1 and 2). Plasma L-KYN levels were significantly increased at least as early as day 7 following T. gondii infection (1.42 ± 0.09 versus 4.07 ± 0.33 μM, day 0 versus day 7, respectively; P < 0.05), with a maximal elevation on day 14 (3.24-fold), and were decreased on day 28 following T. gondii infection. Lung IDO activity was markedly increased (179-fold) on day 14 (from 450 ± 79 to 80,900 ± 29,500 pmol/h/mg of protein), and this level was maintained until day 28. Brain IDO activity was also increased...
(198-fold) on day 14 (from 25.5 ± 5.1 to 5,049 ± 1,936 pmol/h/mg of protein) and was maintained at that level until day 28. Furthermore, the lung IDO mRNA expression level was rapidly increased (2.13-fold) on day 4 and gradually increased until day 28. The brain IDO mRNA expression level was markedly increased on day 14, and this level was maintained until day 28.
Effects of IFN-γ/H9253 KO on degradation of L-TRP and accumulation of L-KYN in the lungs and brain following T. gondii infection. In the lungs, there were marked increases in L-KYN concentrations and almost complete L-TRP depletion in wild-type mice 7 days after T. gondii infection. In the brain, L-TRP concentrations tended to decrease, but the decrease was not statistically significant, and L-KYN concentrations were markedly increased on day 7 after T. gondii infection. In contrast, infected IFN-γ/H9253 KO mice did not have these metabolic changes in the lungs or brain (Fig. 3).

Differential effects of IFN-γ deficiency on IDO and iNOS induction in T. gondii-infected mice. All IFN-γ KO mice died 9 to 11 days after T. gondii infection, whereas wild-type mice all survived until day 28 (data not shown). On day 7 after T. gondii infection, the level of SAG2 mRNA expression in IFN-γ KO mice was significantly higher than that in wild-type mice for both the lungs and brain (Fig. 4E).

To investigate whether IDO and iNOS could be induced in IFN-γ KO mice infected with T. gondii as well as in wild-type mice, IDO and iNOS mRNA expression levels in the lungs and brain in IFN-γ KO mice were compared with those in wild-type mice. The markedly enhanced IDO mRNA expression seen in wild-type mice on day 7 after infection was not seen in IFN-γ KO mice for both the lungs and brain (Fig. 4A and B). In contrast, iNOS mRNA expression in the lungs after infection in IFN-γ KO mice was significantly higher than that in infected wild-type mice, although a significant increase in iNOS mRNA expression in the brain after infection was not observed in IFN-γ KO mice relative to wild-type mice (Fig. 4C and D).

Effect of an inhibitor of iNOS on SAG2 expression in wild-
FIG. 4. Effects of IFN-γ gene deficiency on the induction of IDO and iNOS and tachyzoite SAG2 mRNA expression by *T. gondii* infection. The expression of IDO mRNA (A and B), iNOS mRNA (C and D), and SAG2 mRNA (E) was analyzed by using RT-PCR with total RNA from the lungs and brain of wild-type and IFN-γ KO mice on days 0 and 7 following intraperitoneal injection of *T. gondii*. Each bar represents the mean and SEM for results from three to six samples. *, *P* < 0.05 (for comparisons with day 0 postinfection). **, *P* < 0.005 (for comparisons with day 0 postinfection). (F) Representative gel photograph.
type mice after infection. L-NAME inhibited the increase in the plasma nitrate concentration and enhanced the increase in the plasma l-KYN concentration after T. gondii infection. On the other hand, L-NAME markedly enhanced the level of SAG2 mRNA expression on day 7 in the brain but not in the lungs (Fig. 5).

DISCUSSION

The present in vivo study demonstrates for the first time that IDO activity depends on IFN-γ synthesis following T. gondii infection and that antitoxoplasma activities induced by iNOS and/or IDO are regulated in a tissue-specific manner.

IFN-γ acts as an antimicrobial agent by activating macrophages, lymphocytes, and other cells following T. gondii infection. Some in vitro studies (8, 29, 30, 31) indicate that IFN-γ-induced antitoxoplasma activities are involved in IDO-dependent mechanisms by IDO induction, which is mainly induced by IFN-γ. IDO converts L-TRP to N-formylkynurenine, and strong IDO induction results in L-TRP depletion. Indeed, especially in human fibroblasts, epithelial cells, and glioblastoma cells, IDO induction by IFN-γ results in L-TRP depletion and inhibition of parasite growth. On the other hand, a recent study indicates that reactive nitrogen intermediates, including NO produced by IFN-γ, are important factors for antitoxoplasma activities (16). In murine macrophages and microglia cells, NO production is the main antiparasite effector mechanism, whereas iNOS induction is not involved in defense against toxoplasma in human macrophages (1, 23, 24). A previous study demonstrated that acute T. gondii replication was enhanced in the brain but not in the peritoneal cavity in iNOS-deficient mice (38). Thus, the mechanisms of IFN-γ-induced antitoxoplasma activities are complex. The expression of the main antiparasite effector mechanisms for iNOS and/or IDO seems to depend on the cell type, tissue, and species.

In the present study, IFN-γ KO mice had significantly shorter survival than wild-type mice in the early T. gondii infection period (data not shown), consistent with the results of a previous study (22). Furthermore, the levels of SAG2 mRNA expression in the lungs and brain were markedly increased in IFN-γ KO mice compared to wild-type mice (Fig. 4E). In wild-type mice, IDO activities in the lungs and brain were markedly increased by T. gondii infection (Fig. 1B and C). Similar results were obtained with IDO mRNA (Fig. 2). The increased levels of lung IDO activity were maintained for at least 28 days, whereas plasma l-KYN levels increased to a maximum on day 14 and declined thereafter (Fig. 1). Previous studies indicated that l-KYN accumulates in the lungs, where IDO is induced the most, and might enter the circulation to reach the liver or to undergo further metabolism (35, 40). We speculate that the lungs and other systemic tissues produce l-KYN in response to toxoplasma infection and that elevated plasma l-KYN levels result from increased tissue production. Therefore, decreased plasma l-KYN levels might reflect decreased l-KYN accumulation in the lungs and other systemic tissues due to tissue substrate depletion by significant IDO induction on day 14 following toxoplasma infection. l-TRP in the lungs was almost completely depleted by toxoplasma infection, and brain l-TRP levels tended to decrease. These results were due to the relatively low levels of IDO

FIG. 5. Effects of an iNOS inhibitor on the plasma l-KYN and nitrate concentrations and tachyzoite SAG2 mRNA expression in T. gondii infection. Plasma l-KYN (A) and nitrate (NOx) (B) concentrations and tachyzoite SAG2 mRNA expression (C) in uninfected and infected mice treated with saline (Control) or L-NAME on day 7 following intraperitoneal injection of T. gondii were examined. Each bar represents the mean and SEM for results from four to six samples. *, P < 0.05 (for comparisons with controls). **, P < 0.005 (for comparisons with controls).
activity in the brain. Indeed, absolute values of IDO activity in the lungs are at least 10 times higher than those in the brain following toxoplasma infection. It is possible, however, that L-TRP degradation in the brain was caused at the local level. Although the localization of IDO was not demonstrated in the present study, increases in IDO activity in the lungs and brain following toxoplasma infection might arise from both resident cells and secondary infiltrated cells. Indeed, previous observations indicated that IDO immunoreactivity is markedly increased in the spinal cord of macaques with poliovirus infection, in areas of intense inflammatory response in which macrophage or microglia cell proliferation has occurred (18, 37).

In IFN-γ KO mice, in which enhanced T. gondii replication was found, the IDO induction, L-TRP depletion, and L-KYN accumulation caused by toxoplasma infection in the lungs of wild-type mice were completely absent (Fig. 3 and 4A). In contrast, lung iNOS induction was more prominent in infected IFN-γ KO mice than in infected wild-type mice (Fig. 4C). These results demonstrate that iNOS induction is not associated with an IFN-γ-mediated mechanism for resistance against T. gondii in the lungs. Therefore, we speculated that L-TRP depletion caused by IDO induction and/or other activities is more important than iNOS induction for antitoxoplasma mechanisms in the lungs. On the other hand, in the brain, L-TRP depletion did not occur in spite of IDO induction, and L-KYN accumulation occurred in wild-type mice following toxoplasma infection (Fig. 3). Furthermore, iNOS mRNA expression in the brain was enhanced in infected wild-type mice but not in infected IFN-γ KO mice (Fig. 4D). A previous study demonstrated that acute T. gondii replication was not suppressed in the brain in iNOS-deficient mice (38). Therefore, it is likely that the antitoxoplasma activity caused by iNOS induction in the brain is more dominant than that caused by L-TRP depletion and other factors, such as phagocytic NADPH oxidase. These results obtained in the lungs and brain suggest that the IFN-γ-induced mechanisms of resistance against T. gondii are different among tissues.

The present study also assessed the effects of 6-chloro-DL-tryptophan, an IDO inhibitor, and L-NAME, an iNOS inhibitor, on T. gondii replication. 6-Chloro-DL-tryptophan was not adequate as an IDO inhibitor when daily oral administration (5 mg/mouse) was performed in this study. In fact, 6-chloro-DL-tryptophan slightly attenuated the increases in lung and brain IDO activities (data not shown) (36). On the other hand, inhibition of iNOS induction increased plasma L-KYN concentrations. Furthermore, L-NAME did not have any effect on T. gondii replication in the lungs, although the level of T. gondii replication was increased by L-NAME in the brain. The present result of increased brain T. gondii replication in L-NAME-treated T. gondii-infected mice was in agreement with the result of the above-mentioned study with iNOS-deficient mice (38).

A previous in vitro study demonstrated that the levels of IFN-γ-induced IDO activity and IFN-γ-induced antitoxoplasma activities were significantly increased by treatment with an iNOS inhibitor (9). It is also known that NO can inhibit the activity of IDO in vitro (41). In acute toxoplasma infection, NO may prevent total depletion of tryptophan and/or other indoleamines, which are important in the functions of the host cell, and may also prevent the accumulation of potentially toxic metabolites in the kynurenine pathway. These data suggest that there is cross-regulation between iNOS and IDO.

It has not yet been established whether the physiologic role of IDO regulation by IFN-γ is beneficial or detrimental. Several studies suggest that IDO induction by IFN-γ exerts antimicrobial and antiproliferative effects, possibly by depletion of L-TRP. Indeed, transfected murine cells expressing the IDO gene inhibit toxoplasma replication following IDO induction (15). However, local accumulation of kynurenine metabolites, in particular, quinolinic acid, following IDO induction may also represent a potentially detrimental event. In fact, quinolinic acid is a potent excitotoxin, and its overproduction has been linked to neuronal damage occurring in brain inflammation (17), initiation of lipid peroxidation (33), and other conditions (3, 42). Indeed, quinolinic acid concentration was significantly increased by T. gondii infection (S. Fujigaki and K. Saito, unpublished observation). Therefore, it is possible that increased levels of quinolinic acid and other kynurene pathway metabolites play a detrimental role following T. gondii infection. Although it is beyond the scope of the present study to determine whether the effects of these responses are toxic or beneficial, L-TRP depletion caused by strong IDO induction does occur in certain tissues following toxoplasma infection in an in vivo animal model.

In summary, the present study demonstrates that both lung and brain IDO activities increase as a result of toxoplasma infection and that L-TRP can be depleted in the lungs under such pathological conditions. In addition, the results obtained from IFN-γ KO mice suggest that IFN-γ is one of the most important cytokines for the induction of IDO and for antitoxoplasma activities in vivo. This study suggests that there is an antitoxoplasma mechanism of cross-regulation between iNOS and IDO and that the expression of the main antiparasite effector mechanisms for iNOS and/or IDO may vary among tissues. Further in vivo studies on the role of IDO and iNOS induction and on the consequences of increases in kynurenine pathway metabolites following toxoplasma infection are warranted.

ACKNOWLEDGMENTS

We thank A. Makioka for providing T. gondii. We also thank John Cole for proofreading the English of the manuscript.

This study was supported in part by grant-in-aid 13680840 from the Ministry of Education, Science and Culture and by grants (Research on HIV/AIDS, Health Sciences Research) from the Ministry of Health and Welfare to K. Saito.

REFERENCES