

Dartmouth College

Dartmouth Digital Commons

Dartmouth Scholarship

Faculty Work

1994

Antiparasitic and Antiproliferative Effects of Indoleamine 2,3-dioxygenase Enzyme Expression in Human Fibroblasts.

Sohan L. Gupta
Hipple Cancer Research Center

Joseph M. Carlin
Miami University - Oxford

Padma Pyati
Hipple Cancer Research Center

Wei Dai
Hipple Cancer Research Center

Elmer R. Pfefferkorn
Dartmouth College

See next page for additional authors

Follow this and additional works at: <https://digitalcommons.dartmouth.edu/facoa>



Part of the [Medical Immunology Commons](#), and the [Medical Microbiology Commons](#)

Dartmouth Digital Commons Citation

Gupta, Sohan L.; Carlin, Joseph M.; Pyati, Padma; Dai, Wei; Pfefferkorn, Elmer R.; and Murphy, Martin J. Jr, "Antiparasitic and Antiproliferative Effects of Indoleamine 2,3-dioxygenase Enzyme Expression in Human Fibroblasts." (1994). *Dartmouth Scholarship*. 1001.
<https://digitalcommons.dartmouth.edu/facoa/1001>

This Article is brought to you for free and open access by the Faculty Work at Dartmouth Digital Commons. It has been accepted for inclusion in Dartmouth Scholarship by an authorized administrator of Dartmouth Digital Commons. For more information, please contact dartmouthdigitalcommons@groups.dartmouth.edu.

Authors

Sohan L. Gupta, Joseph M. Carlin, Padma Pyati, Wei Dai, Elmer R. Pfefferkorn, and Martin J. Murphy Jr

Antiparasitic and Antiproliferative Effects of Indoleamine 2,3-Dioxygenase Enzyme Expression in Human Fibroblasts

SOHAN L. GUPTA,^{1*} JOSEPH M. CARLIN,² PADMA PYATI,¹ WEI DAI,¹ ELMER R. PFEFFERKORN,³
AND MARTIN J. MURPHY, JR.¹

Hipple Cancer Research Center, Dayton, Ohio 45439¹; Department of Microbiology, Miami University,
Oxford, Ohio 45056²; and Department of Microbiology, Dartmouth Medical School,
Hanover, New Hampshire 03755³

Received 1 December 1993/Returned for modification 13 January 1994/Accepted 15 March 1994

Studies were carried out to evaluate the proposed role of indoleamine 2,3-dioxygenase (INDO) induction in the antimicrobial and antiproliferative effects of gamma interferon (IFN- γ) in human fibroblasts. The INDO cDNA coding region was cloned in the pMEP4 expression vector, containing the metallothionein (MTII) promoter in the sense (+ve) or the antisense (–ve) orientation. Human fibroblasts (GM637) stably transfected with the sense construct expressed INDO activity after treatment with CdCl₂ or ZnSO₄, but cells transfected with the antisense construct did not. The growth of *Chlamydia psittaci* was strongly inhibited in INDO +ve cells but not in INDO –ve cells after treatment with Cd²⁺ or Zn²⁺. The inhibition correlated with the level of INDO activity induced and could be reversed by the addition of excess tryptophan to the medium. The growth of *Toxoplasma gondii* was also strongly inhibited in INDO +ve cells but not in INDO –ve cells after treatment with Cd²⁺. Expression of Cd²⁺-induced INDO activity also inhibited thymidine incorporation and led to cytotoxicity in INDO +ve cells but not in INDO –ve cells. Thus, the induction of INDO activity by IFN- γ may be an important factor in the antimicrobial and antiproliferative effects of IFN- γ in human fibroblasts.

Gamma interferon (IFN- γ), a cytokine produced by activated T lymphocytes and natural killer cells, has pleiotropic effects; it plays a vital role in host self-defense against a variety of nonviral microbial pathogens, regulates immune responses, and inhibits cell growth (10, 37). Administration of neutralizing antibodies against IFN- γ abrogates the resistance of mice to various microbial pathogens (3, 4, 34, 40), indicating a primary role of IFN- γ in host defense against such pathogens. IFN- γ induces the synthesis of a number of gene products in cells (38). Several of the induced gene products have been implicated in the IFN- γ -mediated resistance to microbial infection. This includes the production of nitric oxide resulting from the induction of nitric oxide synthase (1, 14, 21, 24), activation of macrophages (22, 23), and starvation for tryptophan due to induction of the indoleamine 2,3-dioxygenase (INDO) enzyme (6, 7a, 26, 27, 33), which catalyzes the first step in tryptophan degradation. (The office of Human Gene Mapping nomenclature [Department of Human Genetics, University of Manitoba, Canada] brought to our attention that the term IDO had been assigned to indole 2,3-dioxygenase [EC 1.13.11.17]. Indoleamine 2,3-dioxygenase [EC 1.13.11.42] has therefore been abbreviated INDO.)

Since a variety of gene products are induced by IFN- γ , their relative importance in the biological effects of IFN- γ is difficult to determine. To assess the significance of INDO as a part of this host resistance mechanism, we cloned cDNA for human INDO (8) in an expression vector containing the metallothionein (MTII) promoter. Cells transfected with this construct provide a way to induce INDO activity independent of other IFN- γ -induced proteins. We have examined whether expression of INDO activity in cells stably transfected with the INDO

cDNA expression plasmid would inhibit the growth of *Chlamydia psittaci* and *Toxoplasma gondii* in the absence of IFN- γ , which would demonstrate whether the expression of INDO alone can block the replication of these pathogens. In addition, we have tested whether the expression of INDO in transfected cells would also result in cytostatic and cytotoxic effects, as observed after treatment with IFN- γ (9, 25, 30, 35, 36).

MATERIALS AND METHODS

Cloning of INDO cDNA in an inducible expression vector. A nearly full-length, ca. ~2-kb INDO cDNA was cloned originally in plasmid Bluescript SK– and named C42 (8). In order to express this cDNA in mammalian cells, it was first recloned in an expression vector. We chose vector pMEP4 (Invitrogen, San Diego, Calif.), based on the Epstein-Barr virus, because of the presence of the inducible metallothionein (MTII) promoter and its ability to replicate to a high copy number. The INDO cDNA contained more than 600 nucleotides of 5' untranslated sequence. Therefore, in order to obtain optimal expression of INDO enzyme, we decided to clone (i) the full-length cDNA, including >600 bp of untranslated 5' sequence, and (ii) the coding region after deletion of most of this untranslated sequence. In both cases, the cDNA was cloned in the sense orientation as well as the antisense orientation with respect to the MTII promoter. The 2-kb insert was obtained by digestion of plasmid C42 with *Kpn*I plus *Not*I enzymes and cloned into the pMEP4 vector digested with the same enzymes to insert the INDO cDNA in the sense orientation (see Fig. 1). For cloning in the antisense orientation, the 2-kb cDNA was obtained by digestion of plasmid C42 with *Xho*I plus *Sma*I enzymes and ligated to pMEP4 digested with *Xho*I plus *Pvu*II enzymes. After transformation of *Escherichia coli* DH1 cells with each of the two constructs, several colonies were isolated, and plasmid minipreps were prepared and analyzed for the

* Corresponding author. Mailing address: Hipple Cancer Research Center, 4100 S. Kettering Blvd., Dayton, OH 45439. Phone: (513) 293-8508. Fax: (513) 293-7652.

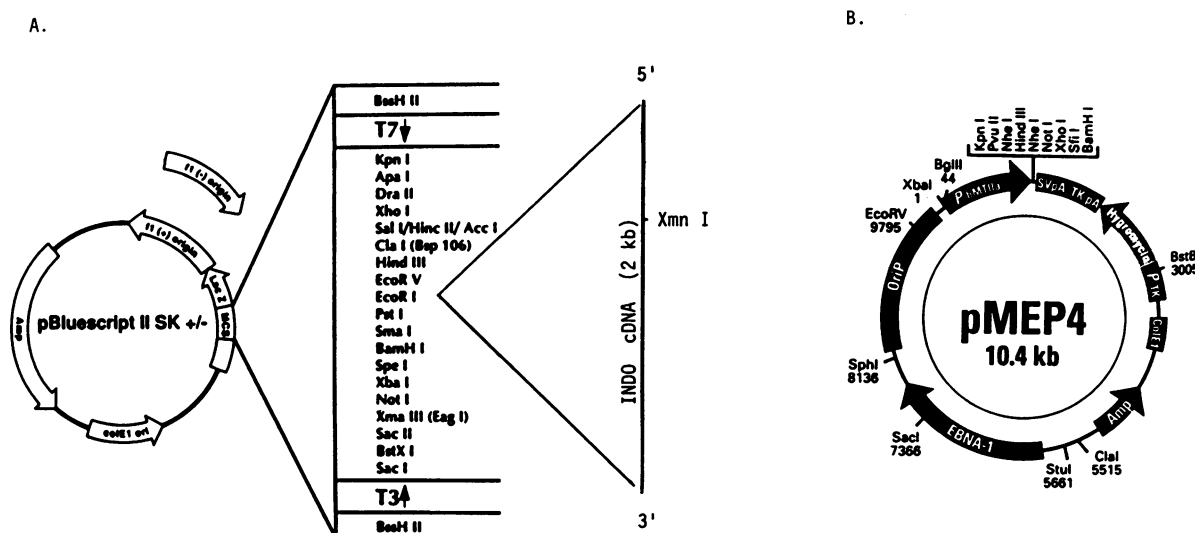


FIG. 1. INDO cDNA (ca. ~2 kb) cloned into Bluescript SK- at the *EcoRI* site (A) was isolated either in its entirety or as a 1.4-kb subfragment after deletion of 592 bp of 5' untranslated sequence as described in Materials and Methods and cloned into the pMEP4 vector (B) containing the metallothionein (MT II) promoter in both the sense and antisense orientations. (The Bluescript vector map has been reproduced with the permission of Stratagene, and the pMEP4 map has been reproduced with the permission of Invitrogen.) MCS, multiple cloning site.

presence of the insert and its orientation with respect to the MTII promoter by restriction analysis. Separately, a ca. ~1.4-kb INDO cDNA fragment containing the entire coding sequence but depleted of much of the untranslated 5' sequence was isolated by making use of an *XmnI* site at position 592 from the 5' end of the 2-kb INDO cDNA insert. Plasmid C42 was digested with *XmnI* and then with *XhoI* and *SmaI* enzymes. This allowed the isolation of a 1.4-kb fragment with both ends blunt (*XmnI* and *SmaI*). (The *XhoI* digestion was included because the vector also has an *XmnI* site which yielded another fragment of approximately the same size that could be cleaved by *XhoI* to facilitate the isolation of the INDO 1.4-kb fragment.) The 1.4-kb fragment isolated after agarose gel electrophoresis was ligated to the pMEP4 vector digested with *PvuII* enzyme. After transformation of DH1 cells, plasmid minipreps were obtained from several colonies and analyzed to determine the presence of the 1.4-kb insert and its orientation with respect to the MTII promoter by restriction analysis. Clones containing the 2-kb cDNA as well as the 1.4-kb cDNA insert in both the sense and antisense orientations with respect to the MTII promoter were obtained and grown at a preparative scale, and the plasmids were purified by previously described procedures (31).

Stable transfection of human fibroblasts with INDO cDNA cloned in pMEP4 expression vector. Transformed human fibroblast cell line GM637 was obtained from the Coriell Institute for Medical Research, Camden, N.J., and maintained in Eagle's minimal essential medium (MEM) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum (FBS). Individual cultures of GM637 cells were transfected with plasmid preparations containing either the 2-kb INDO cDNA or the 1.4-kb fragment containing the INDO coding region in either the sense or the antisense orientation. The calcium phosphate coprecipitation method was used for transfection of cells (2), and the transformants were selected with hygromycin (250 U/ml), as the vector contains the hygromycin resistance gene. Cells stably transfected with each of the four plasmid constructs were obtained and maintained in the presence of hygromycin (250 U/ml).

Stocks of *C. psittaci* and *T. gondii*. Stocks of *C. psittaci* 6BC were propagated in confluent L929 mouse cell monolayers in M199 medium supplemented with 10% FBS, 10 µg of gentamicin sulfate per ml, 100 µg of streptomycin sulfate per ml, and 2 µg of cycloheximide per ml at 37°C in 5% CO₂ in air. The cells were infected with 10 times the 50% infectious dose (ID₅₀) of 6BC, incubated for 2 days, and removed from the tissue culture plate with trypsin. Infected cells were then disrupted by sonication, and elementary bodies were partially purified by differential centrifugation, resuspended in phosphate-buffered saline (PBS) containing 0.2 M sucrose and 2% FBS, distributed into cryovials, and frozen at -70°C until needed. Infectivity titers were quantified by the ID₅₀ method in L cells (16). A cloned line of the RH strain of *T. gondii* was maintained in normal human fibroblast cultures as described previously (29).

Replication of *C. psittaci* and *T. gondii* in transfected cells. For experiments on *C. psittaci* replication, cells transfected with the sense or antisense INDO construct were cultivated in 24-well plates on cover slips in triplicate wells (2 × 10⁵ cells per well) in Eagle's MEM supplemented with 10% FBS, 100 µg of gentamicin sulfate per ml, and 10 µg of streptomycin sulfate per ml. Some wells received ZnSO₄ or CdCl₂ at final concentrations of 75 and 5 µM, respectively. In experiments measuring the effect of metal ion concentration on INDO induction and inhibition of chlamydiae, half-log dilutions of the metal ion inducers were used. After 24 h of incubation, cells were infected with 0.3 ID₅₀s of *C. psittaci* in 50 µl of Hanks' balanced salt solution (HBSS), which resulted in infection of between 30 and 50% of the cells. Incubation was continued for an additional 24 h to permit sufficient development of chlamydial inclusions. After incubation, cover slips from these wells were washed with PBS, fixed with methanol, stained with 5% Giemsa, and mounted on slides. The percentage of *C. psittaci* inclusion-containing cells was determined by light microscopy (5).

For experiments on *T. gondii* replication, transfected cells were grown in Eagle's MEM supplemented with 10% FBS, antibiotics, and 250 U of hygromycin per ml. Cells were washed

three times with PBS, trypsinized, and pelleted by centrifugation. The cells were suspended in medium containing 5% FBS and seeded in 0.5-cm² wells at a density calculated to yield a confluent monolayer without further cell division. The next day, the cultures were incubated in medium either without or with CdCl₂ added. After 2 days, both treated and control cultures were supplemented with EDTA (45 μ M) to chelate Cd²⁺ ions. The addition of EDTA was essential to block the inhibitory effect of Cd²⁺ on the growth of the parasite. The cultures were then infected with 1.5×10^4 *T. gondii*, and the growth of the parasite was measured 2 days later by adding 0.6 μ Ci of [5,6-³H]uracil (specific activity, 20 Ci/mmol; Moravsek Biochemicals) and measuring the acid-precipitable radioactivity 4 h later, as described previously (28).

Effect of exogenous tryptophan on INDO-mediated inhibition of *C. psittaci* replication. Transfected cell cultures were treated with inducers or medium alone and infected with *C. psittaci* as described above. After a 2-h incubation, serial log dilutions of tryptophan in HBSS or HBSS alone was added to the culture medium. After an additional 24 h., cover slips were fixed and stained, and chlamydial growth was determined.

Determination of INDO activity in transfected cells. INDO activity was determined in transfected cell cultures set up in parallel to cultures infected with *C. psittaci*. After 44 h of incubation, the culture medium was aspirated and replaced with 400 μ l of 50 μ M tryptophan in HBSS containing 1 μ Ci of [5-³H]tryptophan (specific activity, 20 to 30 Ci/mmol; New England Nuclear) per ml. Some wells received radiolabeled medium without cells to determine the amount of nonspecific tryptophan decyclization that occurred. After 4 h of incubation, supernatants were collected and frozen at -20°C until analyzed for accumulated tryptophan metabolites. INDO activity was assessed by a modification of a reversed-phase high-performance liquid chromatographic (HPLC) technique (39). Briefly, 50- μ l aliquots of culture supernatants were injected into a μ Bondapak C₁₈ column (30 cm by 3.9-mm inner diameter) (Millipore Waters, Milford, Mass.) and eluted with 10% methanol in 1 mM KH₂PO₄ buffer, pH 4.0, at a flow rate of 1.6 ml/min. Radioactivity was measured by integration of peak areas with a fully automated HPLC (Isco, Lincoln, Neb.) equipped with a radioisotope detector (Radiomatic Instruments, Tampa, Fla.). The percentage of specific tryptophan catabolism was calculated by the following equation:

$$\% \text{ specific catabolism} = \frac{\text{CPM}_{\text{test}} - \text{CPM}_{\text{spontaneous}}}{\text{CPM}_{\text{total}} - \text{CPM}_{\text{spontaneous}}} \times 100 \quad (1)$$

where CPM_{test} is the counts per minute (CPM) present in *N*-formylkynurenine and kynurenine-containing fractions, CPM_{spontaneous} represents the CPM of nonspecific tryptophan degradation products, and CPM_{total} is the sum of CPM from all fractions (7a).

Effect of INDO expression on cell growth. Cells transfected with the 1.4-kb INDO cDNA coding region in either the sense or antisense orientation were seeded in 24-well tissue culture plates (4×10^4 cells per well). The next day, duplicate wells were treated with either IFN- γ (250 U/ml) or Cd²⁺ (5 μ M) or left untreated (controls). To measure the effect on cell growth, cells were labeled with [³H]thymidine (3 μ Ci per well) for 2 h on day 3 or day 5 from the start of treatment. The medium was then removed, and the cells were washed with cold PBS and solubilized in 0.2 ml of 1 N NaOH. Aliquots of each sample were used for determination of trichloroacetic acid-precipitable counts in duplicate.

RESULTS

Stable transfection of human fibroblasts with INDO cDNA cloned in pMEP4 expression vector. Cultures of GM637 cells were transfected with plasmid preparations containing either the 2-kb INDO cDNA or the 1.4-kb fragment containing the INDO coding region cloned in the pMEP4 vector in either the sense or antisense orientation with respect to the MTII promoter, as described in Materials and Methods. In parallel, cells were transfected with pMEP4 vector alone, and the transformants were selected with hygromycin (250 U/ml), as the vector contains the hygromycin resistance gene. Cells stably transfected with each of the four plasmid constructs were obtained and maintained in the presence of hygromycin (250 U/ml).

Cultures of stably transfected cells were tested for the expression of INDO activity after treatment with CdCl₂ (0 to 10 μ M) or ZnSO₄ (0 to 75 μ M) or with IFN- γ (250 U/ml) for 24 h. Cells were then washed, and the medium was replaced with Earle's basal salt solution containing tryptophan (100 μ M) and 10% FBS. After incubation for a further 24 h, the medium was collected and assayed for kynurenine by a colorimetric assay as described before (11). We found that INDO activity was strongly induced in cells transfected with the plasmid containing the 1.4-kb INDO coding region in the sense orientation after treatment with Cd²⁺ or Zn²⁺ in a concentration-dependent manner. Stronger induction was obtained with Cd²⁺, reaching a maximum at 5 to 10 μ M, which was comparable to the level of INDO activity induced (from the cellular INDO gene) after treatment with 250 U of IFN- γ per ml (Fig. 2A). Cells transfected with the plasmid containing the 1.4-kb INDO coding region in the antisense orientation showed no detectable INDO activity after treatment with Cd²⁺, as expected for the antisense construct, but responded normally to IFN- γ (Fig. 2A).

By Northern (RNA blot) analysis, INDO-specific RNA transcripts were found in cells transfected with either the sense or antisense construct after treatment with CdCl₂ (Fig. 2B). The Cd²⁺-induced INDO transcripts from the transfected plasmid constructs (Fig. 2B, lanes 3 and 6) were slightly longer than the IFN- γ -induced INDO transcripts from the cellular gene (Fig. 2B, lanes 2 and 5), apparently because of the contribution from the vector. The level of Cd²⁺-induced INDO transcripts was lower in cells transfected with the antisense construct (Fig. 2B, lane 6) than in cells transfected with the sense construct (lane 3). This could be due to a shorter half-life of the antisense transcripts. The cells transfected with the plasmid containing the entire 2-kb INDO cDNA did not express any measurable INDO activity when treated with Cd²⁺, although RNA transcripts were found in Northern blots (data not shown). We believe that this is due to the rather long untranslated 5' sequence in the 2-kb clone, which contains termination codons in each reading frame (8). Therefore, the studies reported here were carried out with cells transfected with the 1.4-kb INDO cDNA constructs. The cells transfected with the vector alone showed no Cd²⁺-induced INDO activity but responded normally to IFN- γ (not shown), as expected.

Inhibition of *C. psittaci* replication upon expression of INDO activity. To determine whether the expression of INDO activity in transfected cells would have an effect on *C. psittaci* infection, cells transfected with either the sense or antisense INDO construct were treated with CdCl₂ (5 μ M) or ZnSO₄ (75 μ M) for 24 h and then infected with 0.3 ID₅₀ of *C. psittaci* as described in Materials and Methods. After incubation for a further 24 h, cells were fixed and scored for percentage of inclusion-containing cells by light microscopy (5). The results

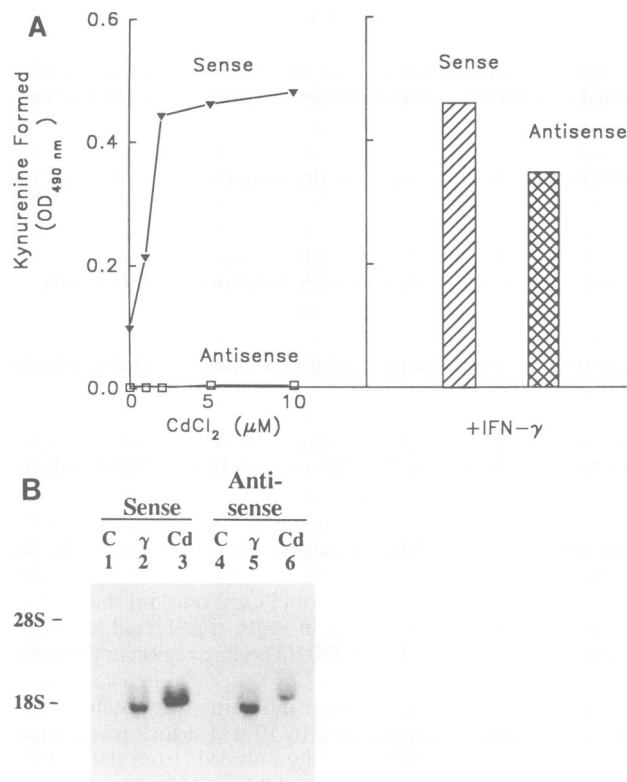


FIG. 2. Expression of INDO cDNA in cells transfected with the sense and antisense constructs. (A) Transfected cells were grown to confluence in 24-well plates and then incubated either with IFN- γ (250 U/ml; right panel) or with increasing concentrations of CdCl₂ (0 to 10 μ M; left panel) for 24 h. The cells were then washed with PBS and incubated with 100 μ M L-tryptophan in Earle's basal salt solution containing 10% FBS for 24 h at 37°C. The medium was collected, treated with 5% trichloroacetic acid at 50°C for 30 min, and centrifuged. The supernatant was assayed for L-kynurenine by adding an equal volume of 0.4% Ehrlich's reagent in glacial acetic acid and measuring the color developed by the A_{490} (11, 35). (B) Cells transfected with the sense or antisense construct were grown in 100-mm plates and treated with either IFN- γ (250 U/ml) or CdCl₂ (5 μ M) for 24 h. Cells were washed with PBS, and total RNA was isolated by the acid-phenol-chloroform extraction procedure (7b) and precipitated with an equal volume of isopropanol. The precipitate was extracted with 4 M LiCl₂-1.5 M guanidine isothiocyanate-25 mM sodium citrate (pH 7.0)-0.1 M 2-mercaptoethanol at 0 to 4°C for 2 h, washed with 75% ethanol, and then dissolved in 0.1% sodium dodecyl sulfate. A 15- μ g amount of each sample was fractionated by electrophoresis in 1% agarose and transferred to a nitrocellulose membrane. The membrane was baked and prehybridized and then hybridized with the ³²P-labeled INDO cDNA probe. The membrane was washed and autoradiographed as described before (7). The positions of the 28S and 18S rRNA markers are indicated.

showed that treatment with Cd²⁺ or Zn²⁺ strongly inhibited the growth of *C. psittaci* in cells transfected with the sense construct but not in cells transfected with the antisense construct (Fig. 3). In the absence of heavy metal ions, both cell types supported *C. psittaci* growth to a comparable extent. In parallel cultures, tryptophan catabolism was assayed by HPLC (39) as described in Materials and Methods. After treatment with Cd²⁺ or Zn²⁺, a large part of the tryptophan in the culture medium was catabolized in cells transfected with the INDO sense construct but not in cells transfected with the antisense construct (Fig. 3, bottom).

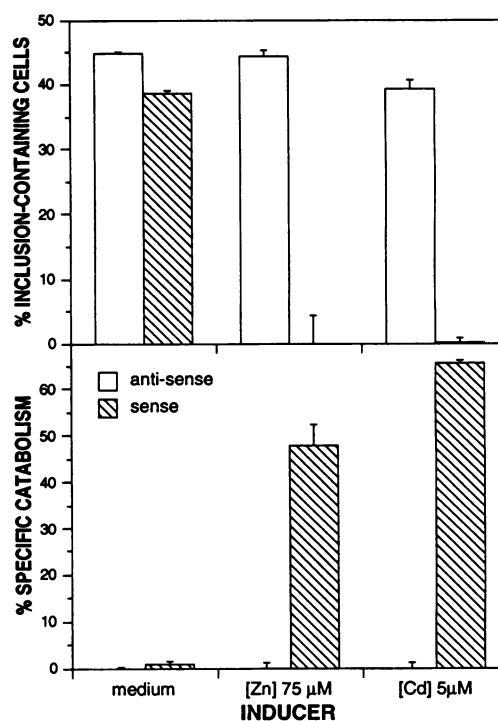


FIG. 3. Effect of Zn²⁺ and Cd²⁺ on the induction of INDO activity (lower panel) and the inhibition of *C. psittaci* (upper panel) in human GM637 fibroblasts transfected with INDO cDNA cloned in the pMEP4 expression vector in either the sense orientation (hatched bars) or the antisense orientation (open bars). Infection with chlamydiae is expressed as percentage of inclusion-containing cells \pm standard deviation (SD), and INDO activity is represented as percent specific catabolism \pm SD. See Materials and Methods for details.

The effects of the Cd²⁺ and Zn²⁺ concentration on the induction of INDO activity and the growth of chlamydiae in cells transfected with the INDO sense construct are shown in Fig. 4. The results showed that the level of INDO activity induced (as measured by tryptophan catabolism) was dependent on the Cd²⁺ or Zn²⁺ concentration and that the inhibition of chlamydial inclusions upon treatment with increasing concentrations of CdCl₂ or ZnSO₄ correlated closely with tryptophan degradation. Complete inhibition was obtained at 5 μ M Cd²⁺ and 75 μ M Zn²⁺. In order to determine whether this inhibition of chlamydial growth was indeed due to the depletion of tryptophan, we tested whether it would be reversed by the addition of excess tryptophan. The inhibitory effect observed after treatment of INDO sense-transfected cells with Cd²⁺ (5 μ M) or Zn²⁺ (75 μ M) was completely overcome by the addition of 100 μ M L-tryptophan (Fig. 5), indicating that induction of the transfected INDO gene construct caused depletion of tryptophan and thus inhibited chlamydial growth in these cells after treatment with Cd²⁺ or Zn²⁺.

Inhibition of *T. gondii* replication in INDO-transfected cells. The replication of *T. gondii* in human fibroblasts is strongly inhibited by treatment with IFN- γ , which has been attributed to tryptophan depletion due to induction of the INDO enzyme (26, 27). Since IFN- γ induces a number of gene products, we tested whether the expression of INDO enzyme alone in transfected cells would block the replication of *T. gondii* in the absence of IFN- γ . Cells transfected with the sense or antisense INDO construct were incubated with or without CdCl₂ (10 μ M) for 2 days. Then, EDTA was added to 45 μ M (final

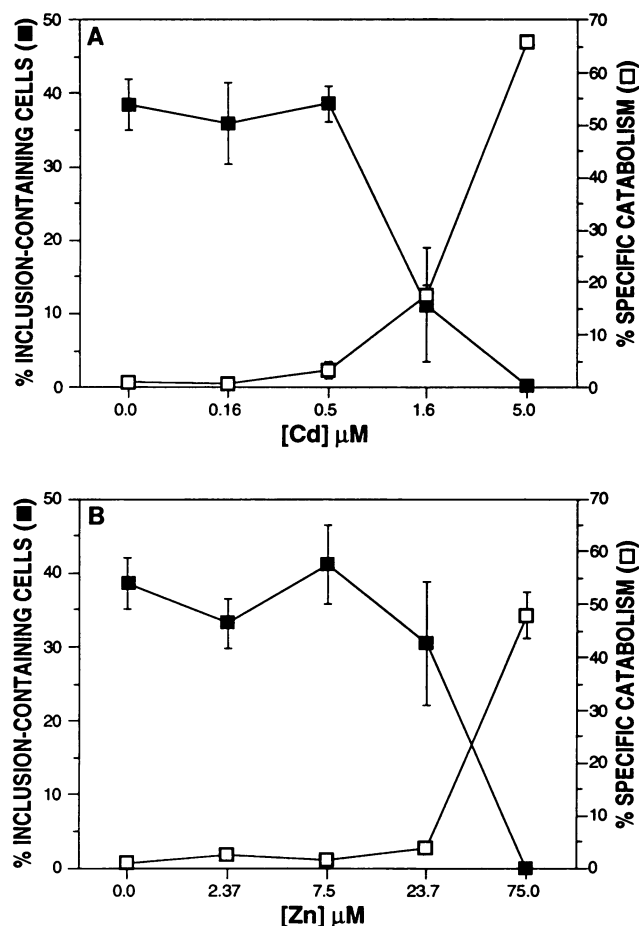


FIG. 4. Effect of increasing concentrations of Cd²⁺ (A) and Zn²⁺ (B) on the induction of INDO activity (□) and the inhibition of chlamydial growth (■) in cells transfected with the sense construct of INDO cDNA (1.4 kb) in the pMEP4 vector. Chlamydial growth and INDO activity are expressed as described in the legend to Fig. 2. Error bars represent SD.

concentration) to chelate Cd²⁺, which was inhibitory to *T. gondii* replication, and the cultures were infected with *T. gondii*. The growth of the parasite was measured by the incorporation of [³H]uracil 2 days later. The results showed >90% inhibition of *T. gondii* growth in cells transfected with the sense construct after treatment with Cd²⁺. No inhibition of *T. gondii* growth was observed in cells transfected with the antisense construct after treatment with Cd²⁺ (data not shown).

While our studies were in progress, Habara-Ohkubo et al. (15) reported that expression of mouse INDO cDNA in murine cells blocked the growth of *T. gondii*. Our experiments showed a similar result with human INDO in human cells. In view of this published report (15), we did not carry out any further experiments to study the effect of INDO expression on *T. gondii* growth.

INDO expression and cell growth. IFN- γ has been shown to have an antiproliferative effect on tumor cells in culture (9, 25, 30, 35, 36), which has been correlated with the induction of INDO enzyme. We examined whether the expression of INDO activity alone in transfected cells would have a cytostatic and/or cytotoxic effect in the absence of IFN- γ . Cells transfected with

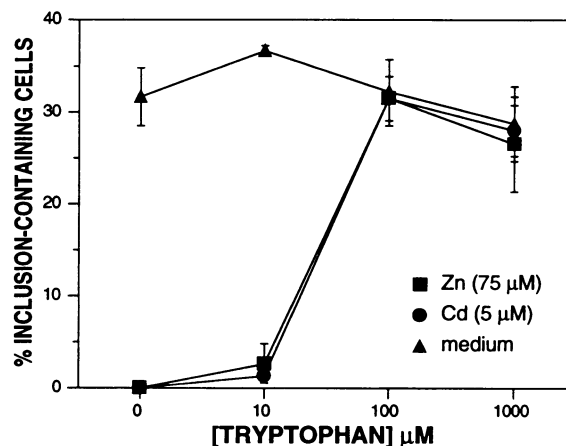


FIG. 5. Inhibition of chlamydial growth by Cd²⁺ and Zn²⁺ in cells transfected with the INDO cDNA sense construct and its reversal by the addition of excess tryptophan. Error bars represent SD.

the sense or antisense INDO construct were seeded in 24-well plates, and duplicate wells were treated with either IFN- γ (250 U/ml) or CdCl₂ (5 μM) or left untreated (control). The effect on cell growth was determined by measuring the incorporation of [³H]thymidine after 3 or 5 days. The results showed that thymidine incorporation was strongly inhibited in cells transfected with the INDO sense construct after treatment with Cd²⁺ (Fig. 6A) but not in cells transfected with the antisense construct (Fig. 6B). Both cell populations showed comparable inhibition after treatment with IFN- γ (Fig. 6). Continued treatment with Cd²⁺ resulted in profound cytotoxicity in cells transfected with the sense INDO construct (Fig. 7, right), whereas the cells transfected with the antisense construct continued to grow normally (Fig. 7, left), indicating that the cytotoxicity observed was not due to the presence of Cd²⁺. Both cell populations showed similar cytotoxicity in response to IFN- γ (Fig. 7). These results indicate that the growth-

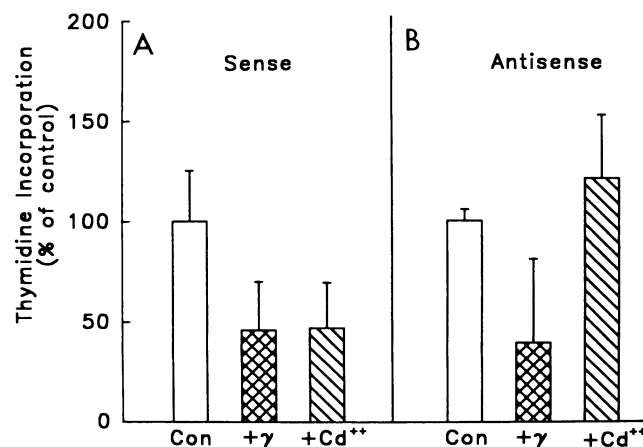


FIG. 6. Incorporation of [³H]thymidine into cells transfected with the sense construct (A) or antisense construct (B) of INDO cDNA after treatment with IFN- γ (250 U/ml) or CdCl₂ (5 μM) for 3 days compared with the corresponding controls (Con). The average counts in control samples were normalized to 100%, and the relative values in different samples were calculated. Error bars represent SD.

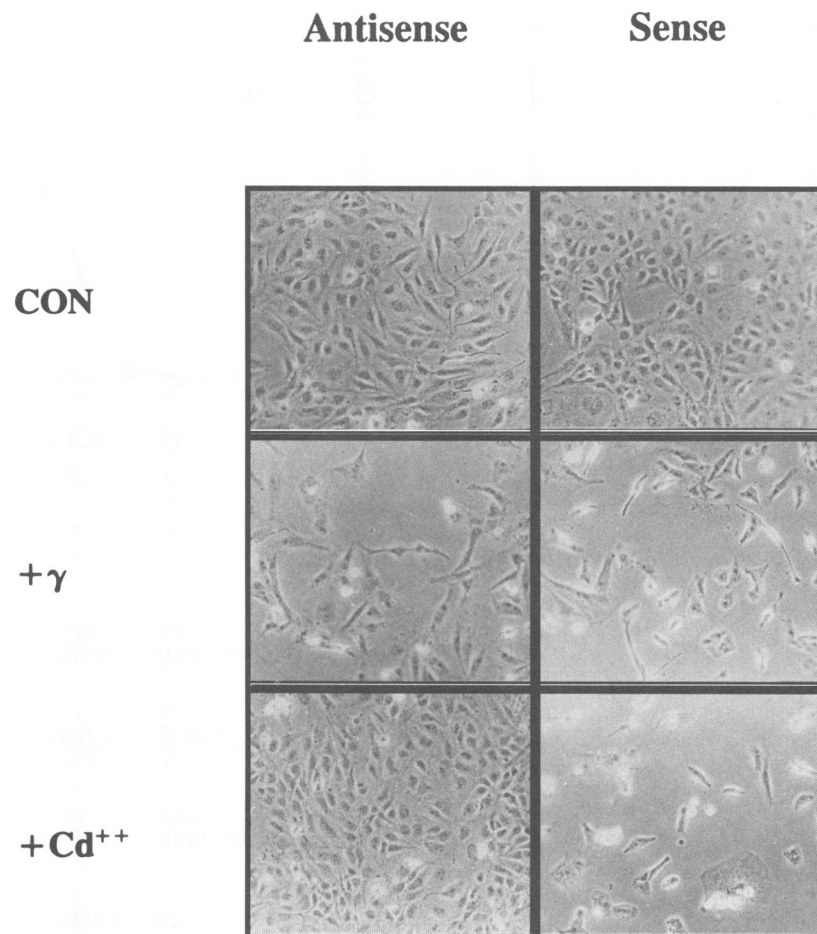


FIG. 7. Effect of IFN- γ and CdCl₂ on the growth and survival of cells transfected with the INDO sense construct (right panels) or antisense construct (left panels). See Materials and Methods for details. Cells were treated for 6 days before pictures were taken. CON, control.

inhibitory and cytotoxic effects of IFN- γ could be reproduced by the expression of INDO enzyme alone (Fig. 6 and 7).

DISCUSSION

The antimicrobial activities of IFN- γ have been correlated with the activation of various different mechanisms. In order to assess the relative importance of these effector mechanisms, it is important to determine the role of each pathway independent of other IFN-induced mechanisms. We have tested whether expression of INDO enzyme in cells transfected with INDO cDNA in an expression vector would block the growth of two pathogens, *C. psittaci* and *T. gondii*, in the absence of IFN- γ . The results presented here demonstrate that the growth of the two microbes was strongly inhibited by the expression of INDO activity. In experiments with *C. psittaci*, the inhibition of the microbe was closely correlated with the induction of INDO activity, as reflected by tryptophan degradation, and could be reversed by the addition of excess tryptophan to the medium (Fig. 4 and 5). These results provide strong support to the view that induction of INDO enzyme is an important factor in the antimicrobial activity of IFN- γ . Earlier studies showed that purified recombinant murine IFN- γ did not induce measurable levels of INDO activity in murine fibroblasts and failed to inhibit the growth of *T. gondii* (32). Expression of INDO

activity in mouse cells by transfection conferred inhibition of *T. gondii* growth (15), indicating an important role for INDO expression in the inhibition of *T. gondii*. Our results with INDO-transfected human fibroblasts support this conclusion and are in agreement with the recent report that mutant human cells deficient in IFN- γ -induced expression of the INDO gene showed reduced capacity to suppress *Chlamydia* and *Toxoplasma* growth (35a).

The results presented here also showed that expression of INDO activity alone from transfected cDNA resulted in the inhibition of cell growth and led to cytotoxicity, as shown earlier with IFN- γ (9, 25, 30, 35, 36). Thus, the antiproliferative and cytotoxic effects of IFN- γ may be due in part to the induction of INDO and the resulting starvation for tryptophan, an essential amino acid.

IFN- γ , produced by activated T cells and natural killer cells, is a pleiotropic cytokine with multiple biological activities. The biological effects of IFN- γ are apparently mediated through the induction of multiple cellular genes. The fact that the expression of INDO alone could reproduce some of the biological effects of IFN- γ suggests that it may be an important factor in the biological activities of IFN- γ . It is not implied, however, that INDO induction is the sole mechanism for the antimicrobial activity of IFN- γ . Other mechanisms activated by IFN- γ may be equally important in host defense against

microbial pathogens in different cell types and different host species. The induction of INDO by IFN- γ may also play a role in the pathogenesis of certain diseases. For example, increased levels of IFN- γ have been found in the serum of AIDS patients who are seropositive for human immunodeficiency virus type 1 antibodies (13), which has been correlated with reduced levels of tryptophan and serotonin (a product derived from tryptophan) in these patients, apparently due to the induction of INDO by circulating IFN- γ (12, 20). Furthermore, greatly increased levels of quinolinic acid, a neurotoxic metabolite of tryptophan, have been found in the serum and cerebrospinal fluid of AIDS patients, especially those with neurological dysfunction, i.e., AIDS dementia complex (17, 18), suggesting a correlation. Increased INDO activity and toxic levels of quinolinic acid were also found in the spinal cord and the cerebrospinal fluid of macaques infected with poliovirus, suggesting a possible role in neurological disease (19). Therefore, the induction of INDO activity by IFN- γ may be a significant factor in the physiological resistance to pathogens as well as the pathogenesis of various diseases involving IFN- γ .

ACKNOWLEDGMENTS

This work was supported in part by U.S. Public Health Service grants AI 33762 (to S.L.G.), AI 32483 (to W.D.), AI 25187 (to E.R.P.), and AI 32741 and a Research Challenge grant from the Ohio Board of Regents (to J.M.C.).

We thank Genentech, Inc., for the generous gift of purified recombinant human IFN- γ and Susan Borotz for skilled technical assistance.

REFERENCES

- Adams, L. B., J. B. Hibbs, Jr., R. R. Taintor, and J. L. Krahenbuhl. 1990. Microbiostatic effect of murine activated macrophages for *Toxoplasma gondii*. *J. Immunol.* **144**:2725–2729.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1990. Current protocols in molecular biology. John Wiley & Sons, New York.
- Belosevic, M., D. S. Finbloom, P. H. van der Meider, M. V. Slayter, and C. A. Nacy. 1989. Administration of monoclonal anti-IFN- γ antibodies *in vivo* abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. *J. Immunol.* **143**:266–274.
- Buchmeier, N. A., and R. D. Schreiber. 1985. Requirement of endogenous interferon-gamma production for resolution of *Listeria monocytogenes* infection. *Proc. Natl. Acad. Sci. USA* **82**:7404–7408.
- Byrne, G. I., and C. L. Faubion. 1982. Lymphokine-mediated microbiostatic mechanisms restrict *Chlamydia psittaci* growth in macrophages. *J. Immunol.* **128**:469–474.
- Byrne, G. I., L. K. Lehmann, and G. J. Landry. 1986. Induction of tryptophan catabolism is the mechanism for gamma interferon-mediated inhibition of intracellular *Chlamydia psittaci* replication in T24 cells. *Infect. Immun.* **53**:347–351.
- Caplen, H. S., and S. L. Gupta. 1988. Differential regulation of a cellular gene by human interferon- γ and interferon- α . *J. Biol. Chem.* **263**:332–339.
- a. Carlin, J. M., E. C. Borden, and G. I. Byrne. 1989. Interferon-induced indoleamine 2,3-dioxygenase activity inhibits *Chlamydia psittaci* replication in human macrophages. *J. Interferon Res.* **9**:329–337.
- b. Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159.
- Dai, W., and S. L. Gupta. 1990. Molecular cloning, sequencing and expression of human interferon- γ -inducible indoleamine 2,3-dioxygenase cDNA. *Biochem. Biophys. Res. Commun.* **168**:1–8.
- de la Maza, L. M., and E. M. Peterson. 1988. Dependence of the *in vitro* antiproliferative activity of recombinant human γ -interferons on the concentration of tryptophan in culture media. *Cancer Res.* **48**:346–350.
- Farrar, M. A., and R. A. Schreiber. 1993. The molecular and cell biology of interferon- γ and its receptor. *Annu. Rev. Immunol.* **11**:571–611.
- Feng, G.-S., and M. W. Taylor. 1989. Interferon- γ -resistant mutants are defective in the induction of indoleamine 2,3-dioxygenase. *Proc. Natl. Acad. Sci. USA* **86**:7144–7148.
- Fuchs, D., A. Forsman, L. Hagberg, M. Larsson, G. Norkrans, G. Reibnegger, E. R. Werner, and H. Wachter. 1990. Immune activation and decreased tryptophan in patients with HIV-1 infection. *J. Interferon Res.* **10**:599–603.
- Fuchs, D., A. Hausen, G. Reibnegger, E. R. Werner, G. Werner-Felmayer, M. P. Dierich, and H. Wachter. 1989. Interferon-gamma concentrations are increased in sera from individuals with human immune deficiency virus type 1 infection. *J. Acquired Immune Defic. Syndr.* **2**:158–162.
- Green, S. J., R. M. Crawford, J. T. Hockmeyer, M. S. Meltzer, and C. A. Nacy. 1990. *Leishmania major* amastigotes initiate the L-arginine dependent killing mechanism in IFN- γ stimulated macrophages by induction of tumor necrosis factor. *J. Immunol.* **145**:4290–4297.
- Habara-Ohkubo, A., T. Shirahata, O. Takikawa, and R. Yoshida. 1993. Establishment of antitoxoplasma state by stable expression of mouse indoleamine 2,3-dioxygenase. *Infect. Immun.* **61**:1810–1813.
- Hatch, T. P. 1975. Competition between *Chlamydia psittaci* and L cells for host isoleucine pools: a limiting factor in chlamydia replication. *Infect. Immun.* **12**:211–220.
- Heyes, M. P., B. J. Brew, A. Martin, R. W. Price, A. M. Salazar, J. J. Sidtis, J. A. Yergey, M. M. Mouradian, A. E. Sadler, J. Keilp, D. Rubinow, and S. P. Markey. 1991. Increased cerebrospinal fluid concentrations of the excitotoxin quinolinic acid in human immune deficiency virus infection and AIDS dementia complex. *Ann. Neurol.* **29**:202–209.
- Heyes, M. P., D. Rubinow, C. Lane, and S. P. Markey. 1989. Cerebrospinal fluid quinolinic acid concentrations are increased in acquired immune deficiency syndrome. *Ann. Neurol.* **26**:275–277.
- Heyes, M. P., K. Saito, D. Jacobowitz, S. P. Markey, O. Takikawa, and J. H. Vickers. 1992. Poliovirus induces indoleamine 2,3-dioxygenase and quinolinic acid synthesis in macaque brain. *FASEB J.* **6**:2977–2989.
- Launay, J. M., L. Copel, J. Callebort, N. Corvaia, E. Lepage, F. Bricaire, F. Saal, and J. Peries. 1988. Decreased whole blood 5-hydroxytryptamine (serotonin) in AIDS patients. *J. Acquired Immune Defic. Syndr.* **1**:324–325.
- Mayer, J., M. L. Woods, Z. Vavrin, and J. B. Hibbs. 1993. Gamma interferon-induced nitric oxide production reduces *Chlamydia trachomatis* infectivity in McCoy cells. *Infect. Immun.* **61**:491–497.
- Murray, H. W. 1988. Interferon-gamma, the activated macrophage, and host defense against microbial challenge. *Ann. Intern. Med.* **108**:595–608.
- Murray, H. W. 1992. The interferons, macrophage activation, and host defense against non-viral pathogens. *J. Interferon Res.* **12**:319–322.
- Nathan, C., and J. B. Hibbs, Jr. 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.* **3**:65–70.
- Ozaki, Y., M. P. Edelstein, and D. S. Duch. 1988. Induction of indoleamine 2,3-dioxygenase: a mechanism of the antitumor activity of interferon- γ . *Proc. Natl. Acad. Sci. USA* **85**:1242–1246.
- Pfefferkorn, E. R. 1984. Interferon- γ blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan. *Proc. Natl. Acad. Sci. USA* **81**:908–912.
- Pfefferkorn, E. R., M. Eckel, and S. Rebhun. 1986. Interferon- γ suppresses the growth of *Toxoplasma gondii* in human fibroblasts through starvation for tryptophan. *Mol. Biochem. Parasitol.* **20**:215–224.
- Pfefferkorn, E. R., and P. M. Guyre. 1984. Inhibition of growth of *Toxoplasma gondii* in cultured fibroblasts by human recombinant gamma interferon. *Infect. Immun.* **44**:211–216.
- Pfefferkorn, E. R., and L. C. Pfefferkorn. 1976. *Toxoplasma gondii*: isolation and preliminary characterization of temperature-sensitive mutants. *Exp. Parasitol.* **39**:365–376.
- Rubin, B. Y., and S. L. Gupta. 1980. Differential efficacies of

- human type I and type II interferons as antiviral and antiproliferative agents. *Proc. Natl. Acad. Sci. USA* **77**:5928–5932.
31. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 32. Schwartzman, J. D., S. L. Gonias, and E. R. Pfefferkorn. 1990. Murine gamma interferon fails to inhibit *Toxoplasma gondii* growth in murine fibroblasts. *Infect. Immun.* **58**:833–834.
 33. Shemer, Y., R. Kol, and I. Sarov. 1987. Tryptophan reversal of recombinant human gamma interferon inhibition of *Chlamydia trachomatis* growth. *Curr. Microbiol.* **16**:9–13.
 34. Suzuki, Y., M. A. Orellana, R. D. Schreiber, and J. S. Remington. 1988. Interferon- γ : the major mediator of resistance against *Toxoplasma gondii*. *Science* **240**:516–518.
 35. Takikawa, O., T. Kuroiwa, F. Yamazaki, and R. Kido. 1988. Mechanism of interferon- γ action: characterization of indoleamine 2,3-dioxygenase in cultured human cells induced by interferon- γ and evaluation of the enzyme-mediated tryptophan degradation in its anticellular activity. *J. Biol. Chem.* **263**:2041–2048.
 - 35a. Thomas, S. M., L. F. Garritty, C. R. Brandt, C. S. Schobert, G.-S. Feng, M. W. Taylor, J. M. Carlin, and G. I. Byrne. 1993. IFN- γ -mediated antimicrobial response: indoleamine 2,3-dioxygenase-deficient mutant host cells no longer inhibit intracellular *Chlamydia* spp. or *Toxoplasma* growth. *J. Immunol.* **150**:5529–5534.
 36. Tying, S., G. R. Klimpel, W. R. Fleischmann, Jr., and S. Baron. 1982. Direct cytolysis by partially-purified preparations of immune interferon. *Int. J. Cancer* **30**:59–64.
 37. Vilcek, J., P. W. Gray, E. Rinderknecht, and C. S. Sevastopoulos. 1985. Interferon- γ : a lymphokine for all seasons, p. 1–32. *In* E. Pick (ed.), *Lymphokines*, vol. 11. Academic Press, Orlando, Fla.
 38. Weil, J., C. J. Epstein, L. B. Epstein, J. J. Sedmak, J. L. Sabran, and S. E. Grossberg. 1983. A unique set of polypeptides is induced by gamma interferon in addition to those induced with alpha and beta interferons. *Nature (London)* **301**:437–439.
 39. Young, S., and S. Lau. 1979. Rapid separation of tryptophan, kynurenine, and indoles using reversed phase high performance liquid chromatography. *J. Chromatogr.* **175**:343–347.
 40. Zhong, G., E. M. Peterson, C. W. Czarniecki, R. D. Schreiber, and L. M. de la Maza. 1989. Role of endogenous gamma interferon in host defense against *Chlamydia trachomatis* infection. *Infect. Immun.* **57**:152–157.