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Intracellular Tryptophan Pool Sizes May Account for Differences in Gamma Interferon-Mediated Inhibition and Persistence of Chlamydial Growth in Polarized and Nonpolarized Cells

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Gamma interferon (IFN-γ) is an important factor in the modulating inhibition of intracellular chlamydial growth and persistence. In human epithelial cells and macrophages, this inhibition is the result of depletion of the essential amino acid tryptophan via the IFN-γ-induced enzyme indoleamine 2,3-dioxygenase. Under these conditions, chlamydiae must successfully compete with the host cell for limited resources in order to maintain viability. We provide evidence to support the hypothesis that the host cell polarization state influences the host-pathogen interplay and outcome of IFN-γ-mediated inhibition. In polarized cells, intracellular soluble tryptophan pools were larger than those in nonpolarized cells despite only small differences in the initial uptake rate of this amino acid compared to that in nonpolarized cells. Furthermore, in *Chlamydia trachomatis*-infected cells, the amounts of tryptophan consumed by the organisms were similar for cells grown in either state. We propose that intracellular tryptophan pool sizes can account for differences in IFN-γ-mediated chlamydial persistence and growth inhibition in polarized and nonpolarized cells. Collectively, these results argue that polarized cell models, which more accurately reflect the conditions in vivo, may be more relevant than conventionally cultured cells in the study of intimate intracellular host-parasite interactions.

Chlamydiae are among the most common human bacterial pathogens. *Chlamydia trachomatis* is the most prevalent sexually transmitted disease in industrialized countries and also the world's leading cause of preventable infectious blindness. Chlamydial upper genital tract infections in women can have very serious repercussions, including the onset of pelvic inflammatory disease. This syndrome often results in severe and irreversible sequelae, such as infertility or ectopic pregnancy, most likely the consequence of local immune reactivity and fallopian tube scarring (32).

One hallmark of chlamydial infections is that they are often asymptomatic and may persist for long periods of time if left untreated. Considerable efforts have been made to define the immune response to these infections due to their chronic nature and resultant immunopathology. One mechanism of control is through the induction of the immune response-regulated cytokine interferon gamma (IFN-γ). In humans, IFN-γ is known to be elicited during natural chlamydial infections and has been detected in the cervices of *Chlamydia*-infected women (1). Employing a mouse model of genital tract disease, several recent studies have demonstrated the importance of Th1 cells in the resolution of infection (8, 17, 21). This protection is afforded, in part, through the production of IFN-γ (15, 17, 21). Cytotoxic T cells may also contribute to protection as a source of this cytokine (27).

IFN-γ has been shown to inhibit the growth of *Chlamydia psittaci* (6, 7, 23), *C. trachomatis* (25), and *C. pneumoniae* (28) in cell culture. The mechanism of action in cultured human cell lines has been elucidated and involves the induction of a host cell enzyme, indoleamine 2,3-dioxygenase (IDO), which catalyzes oxidative decyclization of the essential amino acid tryptophan to *N*-formylkynurenine (7, 30). IFN-γ-stimulated IDO production is common to many cell types, including fibroblasts, primary epithelial cells, peripheral blood mononuclear cells, and human macrophages (14, 22, 29). The inhibitory effect of IFN-γ is reversed by the addition of superphysiologic concentrations of tryptophan to the medium (19, 22, 24), indicating that access to tryptophan is required for chlamydial growth.

Previous studies demonstrated that under some conditions IFN-γ-mediated IDO induction and subsequent tryptophan limitation lead to an altered developmental form of *Chlamydia* known as persistence (2). Chlamydial persistence is characterized by aberrant morphology and a failure to recover infectious organisms after IFN-γ treatment (3–5). In addition, persistent organisms express reduced amounts of the chlamydial major outer membrane protein, a potential protective antigen (36), and increased levels of the chlamydial 57-kDa heat shock protein, which may contribute to disease pathogenesis (20). It is apparent that intracellular levels of tryptophan modulate chlamydial growth; reduced levels of this amino acid result in chlamydial persistence while the absence of tryptophan leads to growth inhibition.

C. trachomatis is an obligate intracellular bacterium which normally grows in columnar epithelial cells of the conjunctiva or genital tract. These cells are polarized in the body, with distinct apical and basolateral domains separated by tight junctions (26). A number of recent studies indicate that bacteria interact with cells grown in this manner differently than with cells grown in traditional culture (16, 31). These in vitro polarized culture systems resemble the environment encountered by pathogens in vivo more closely than do traditional cultures and thus may be a more appropriate way to assess host-pathogen interactions. We recently demonstrated that IFN-γ-mediated chlamydial persistence occurs in a polarized human epithelial culture system (18). The characteristics of persistent chlamydiae in polarized cells are the same as those described

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previously for nonpolarized cells, with one intriguing difference: substantially larger quantities of IFN-γ are required to induce persistence or growth inhibition in the polarized cells.

The purpose of this study is to define the basis behind the differences in IFN-γ-responsiveness between polarized and nonpolarized cells in the context of chlamydial persistence and growth inhibition. We hypothesize that chlamydial growth is more difficult to inhibit in polarized cells because they have larger intracellular tryptophan pools, perhaps as a result of more efficient amino acid transport when cells are grown in this orientation. To test this, intracellular levels of tryptophan in both polarized and nonpolarized cells were examined at various times after IFN-γ treatment. In addition, the rate of tryptophan transport into cells grown in the two orientations was assessed. The kinetics of tryptophan transport were not significantly different between cells grown in either orientation, although more time was required to reach steady-state levels in polarized cells. Moreover, polarized cells consistently had larger intracellular pools of tryptophan and these pools were not depleted as readily in the presence of IFN-γ as those in nonpolarized cells.

MATERIALS AND METHODS

Cell culture and propagation of chlamydiae. ME-180 cells, a human cervical epithelial cell line, were maintained at 37°C in 5% CO_2 in Eagle's minimal essential medium (EMEM; BioWhittaker, Walkersville, Md.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, Utah), 2 mM glutamine, 0.1 mM nonessential amino acids, and 10 μg of gentamicin per ml and 50 μg of vancomycin per ml (Sigma Chemical Co., St. Louis, Mo.).

C. trachomatis serovar A/HAR-13 was grown in HeLa 229 cells in EMEM supplemented as described above with the addition of 2 μg of cycloheximide per ml. Chlamydial elementary bodies were harvested from infected cells and purified over discontinuous Renografin gradients (Squibb Diagnostics, New Brunswick, N.J.) as described previously (9).

Preparation of infected cultures. Cells for nonpolarized cultures were plated into 96-well trays at a density of 5×10^4 cells per well. Polarized cultures were obtained by plating cells at the same density onto 6.5-mm-diameter polycarbonate Transwell filter inserts (Costar, Cambridge, Mass.) that previously had been coated with 0.15 mg of collagen (types VI and X from human placenta) (Sigma) per ml as described by Wyrick et al. (33).

Polarized and nonpolarized cells were infected on the apical surface with 100 μl of SPG (10 mM sodium phosphate [pH 7.2], 0.25 M sucrose, 5 mM L-glutamic acid) containing *C. trachomatis* at a multiplicity of infection of 1. Cultures were incubated at 37°C for 1 h on a platform rocker and then held stationary at 37°C for 1 h. Following adsorption, the inoculum was removed and replaced with medium containing radiolabeled tryptophan as described below.

Tryptophan transport assays. Polarized and nonpolarized cells were incubated in EMEM containing recombinant human IFN-γ (10 U/ng; Genzyme Diagnostics, Cambridge, Mass.) at various concentrations for 48 h. Cells were rinsed with Hanks balanced salt solution (HBSS) and incubated with HBSS containing 10 μCi of L-[^{3}H]tryptophan (specific activity, 27 to 33 Ci/mmol; Amersham, Arlington Heights, Ill.) per ml and 50 μM unlabeled L-tryptophan carrier (Sigma) at 37°C. For long-term assays (>1 h), cells were incubated with tryptophan-deficient MEM (MEM Select-Amine; Gibco BRL, Grand Island, N.Y.) containing 10% dialyzed heat-inactivated FBS (11), IFN-γ, and tryptophan as described above. At specified intervals, supernatants were collected, and cells were rinsed three times with HBSS and extracted with ice-cold 10% trichloroacetic acid (TCA). Each filter was excised from the plastic support with a scalpel and placed in a screw-cap vial before TCA extraction. Tryptophan and catabolites were detected in the supernatants and acid soluble fractions by reversed-phase high-performance liquid chromatography (HPLC) as described previously (35). Samples were injected into a μBondapak C_{18} column (Waters, Milford, Mass.), eluted in 1 mM KH_2PO_4 (pH 4) in 10% methanol, and detected by continuous monitoring of the effluent with a radioactive flow detector (Flo-one; Radiomatic Instruments and Chemical Co., Tampa, Fla.). TCA precipitates were pelleted by centrifugation before TCA supernatants were loaded into autosampler vials.

RESULTS

Chlamydial infection affects intracellular tryptophan pool sizes in polarized and nonpolarized cells equally. Preliminary studies suggested that chlamydial infection did not affect intracellular tryptophan levels of the host cells. To test this, cells were incubated in medium containing L-[^{3}H]tryptophan immediately following infection with *C. trachomatis*, and intracellular levels of tryptophan were determined up to 48 h postinfection. Infected, polarized and nonpolarized ME-180 cells contained less intracellular tryptophan than did uninfected controls, presumably due to chlamydial consumption of this amino acid (Fig. 1). The difference in tryptophan levels between infected and uninfected cells, regardless of host cell polarization state, remained constant from 24 to 48 h postinfection. Furthermore, the relative difference in tryptophan levels in infected and uninfected cells was nearly identical for cells grown in either orientation; infected cells contained between 60 and 75% of the amount of intracellular soluble tryptophan found in uninfected cells. These results demonstrated that chlamydiae utilize a constant amount of tryptophan for growth in each cell type.

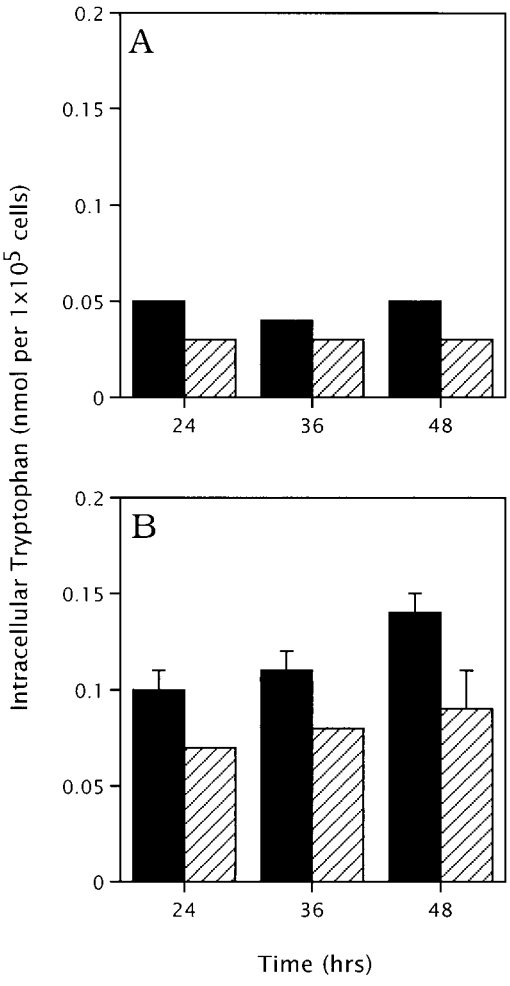

FIG. 1. Intracellular tryptophan concentrations after long-term labeling of *C. trachomatis*-infected cells. Nonpolarized (A) and polarized (B), uninfected (■) and infected (▨) cells were incubated with tryptophan-deficient EMEM containing 10 μCi of L-[^{3}H]tryptophan and 50 μM unlabeled L-tryptophan carrier at the time of infection. Samples were collected at 24, 36, and 48 h postinfection and extracted with TCA. Soluble intracellular tryptophan concentrations were determined by HPLC with these extracts. Values represent the means ± the standard deviations (error bars). For some samples, the standard deviations are too low (<1% of the means) to be visible on the figure.

Kinetics of tryptophan uptake in polarized and nonpolarized cells. Since chlamydiae utilized the same proportion of tryptophan regardless of host cell polarization status, we ex-

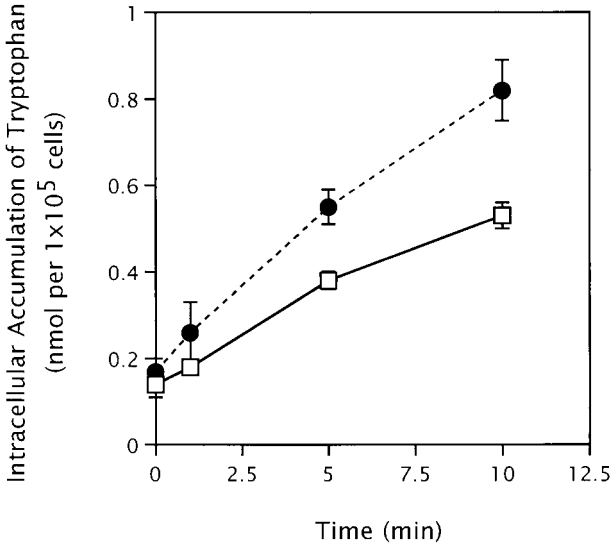

FIG. 2. Accumulation of L-tryptophan in intracellular soluble pools in polarized (●) and nonpolarized (□) ME-180 cells over time. Cells were incubated with L-[^{3}H]tryptophan and carrier in HBSS. At the indicated times, samples were collected and assayed as described for Fig. 1. Uptake was linear up to 10 min ($R^2 = 0.985$ for nonpolarized and 0.992 for polarized cells). Values represent the means ± standard deviations (error bars). For some samples, the standard deviations are too low (<1% of the means) to be visible on the figure.

amined the ability of the host cells to supply this amino acid. One possible mechanism was that polarized cells were able to import tryptophan at a faster rate than do conventionally cultured cells. To test this, polarized and nonpolarized ME-180 cells were incubated with L-[^{3}H]tryptophan plus carrier, and at various times, intracellular tryptophan levels were assessed in the TCA-soluble fractions. Tryptophan uptake was shown to be linear with respect to time up to 10 min in cells grown in either polarization state (Fig. 2). Polarized cells transported tryptophan at a slightly faster rate (0.065 nmol tryptophan/10^5 cells/min) than did nonpolarized cells (0.040 nmol tryptophan/10^5 cells/min), although these differences were not significant (Student's t test of slopes, $P > 0.05$).

When the accumulation of tryptophan was examined beyond the linear portion of the curve, maximum levels were reached sooner in conventionally grown cells than in polarized cells (Fig. 3A). Nonpolarized cells were able to completely replace their intracellular tryptophan pools with radiolabeled amino acid and carrier within 10 to 30 min, compared to at least 60 min or more for polarized cells. In addition, more than twice as much tryptophan was taken into polarized cells than in nonpolarized cells in the same amount of time. This finding was the first indication that polarized cells have larger intracellular stores of tryptophan.

Next we examined the effect of IFN-γ treatment on tryptophan uptake by cells grown in a polarized or nonpolarized fashion. When cells were pretreated with 0.1 ng of IFN-γ/ml for 48 h before labeling, tryptophan uptake into cells of either orientation exhibited kinetics similar to those of untreated cells (Fig. 3C). At this dose of IFN-γ, tryptophan decyclization, assessed by the presence of the catabolite kynurenine, was apparent in polarized cells as early as 1 min postlabeling (Fig. 3D). Intracellular kynurenine levels slowly increased in polarized cells over the course of the assay. In contrast, tryptophan catabolites were not detected inside nonpolarized cells for at least 30 min, although these products did accumulate to a small extent late in the assay. No intracellular kynurenine was detected in the absence of IFN-γ treatment (Fig. 3B).

After treatment with a larger dose of IFN-γ (1 ng/ml), the initial rate of tryptophan uptake into nonpolarized cells was similar to that in cells treated with 0.1 ng of IFN-γ/ml, although tryptophan did not plateau at levels as high as those in untreated cells (Fig. 3E). In contrast, no intracellular tryptophan could be detected in polarized cells treated with 1 ng of IFN-γ/ml. At this dose of IFN-γ, intracellular kynurenine was detected in cells of both orientations very shortly after labeling and accumulated over the course of the assay (Fig. 3F). The accumulation of intracellular kynurenine in polarized cells treated with 1 ng of IFN-γ/ml mimicked the kinetics of tryptophan uptake in untreated cells. These results indicated that in polarized cells, tryptophan was taken up at the same rate whether IDO activity was absent (Fig. 3A) or maximally induced (Fig. 3F), although tryptophan was catabolized immediately under the latter conditions.

Polarized cells have larger intracellular tryptophan pools than do nonpolarized cells. An alternative hypothesis to explain differences in the ability of host cells to provide tryptophan to the chlamydiae is that polarized cells have larger intracellular pools of this amino acid than do nonpolarized cells. To examine this, polarized and nonpolarized cells were incubated with IFN-γ and L-[^{3}H]tryptophan simultaneously to assess steady-state levels of intracellular tryptophan at times related to active chlamydial growth. In the absence of IFN-γ treatment, polarized cells consistently had more than three times the amount of intracellular tryptophan in nonpolarized cells up to 48 h after labeling (Fig. 4A). When cells were treated with low doses of IFN-γ (0.1 ng/ml, a persistence-inducing dose for nonpolarized cells), polarized cells had considerably more intracellular tryptophan during the first 24 h of incubation (Fig. 4B). At late times, intracellular tryptophan was not detected in nonpolarized cells. However, polarized cells still contained tryptophan concentrations similar to those in untreated, nonpolarized cells. At higher IFN-γ concentrations (1 ng/ml, chlamydial growth-inhibiting dose in nonpolarized and persistence-inducing dose in polarized cells [18]), polarized cells still had much higher intracellular tryptophan levels than did nonpolarized cells until 24 h posttreatment (Fig. 4C). No intracellular tryptophan was detected in cells of either orientation 24 h after treatment with this dose of IFN-γ. These results indicate that a certain minimum level of tryptophan must be maintained within the host cell, at least early in infection, to enable normal or even persistent chlamydial development.

DISCUSSION

These findings reinforce the importance of choosing an appropriate culture system to study chlamydial growth in vitro. Many distinctions between cells grown in a polarized or nonpolarized manner have been documented. These differences also have been noted to affect the way in which chlamydiae interact with their host cells; Wyrick et al. have demonstrated an accelerated developmental cycle in polarized cells (34), and previously we have shown a difference in IFN-γ-mediated growth restriction based on host cell polarization status (18).

Chlamydial infection did not affect the tryptophan transport rates into polarized or nonpolarized cells, suggesting that the size of intracellular soluble tryptophan pools may be an important factor in chlamydial growth modulation. This finding agrees with that of previous studies examining nutritional requirements for chlamydial growth which indicated that intracellular amino acid pools of the host cell were sufficient for *C.*

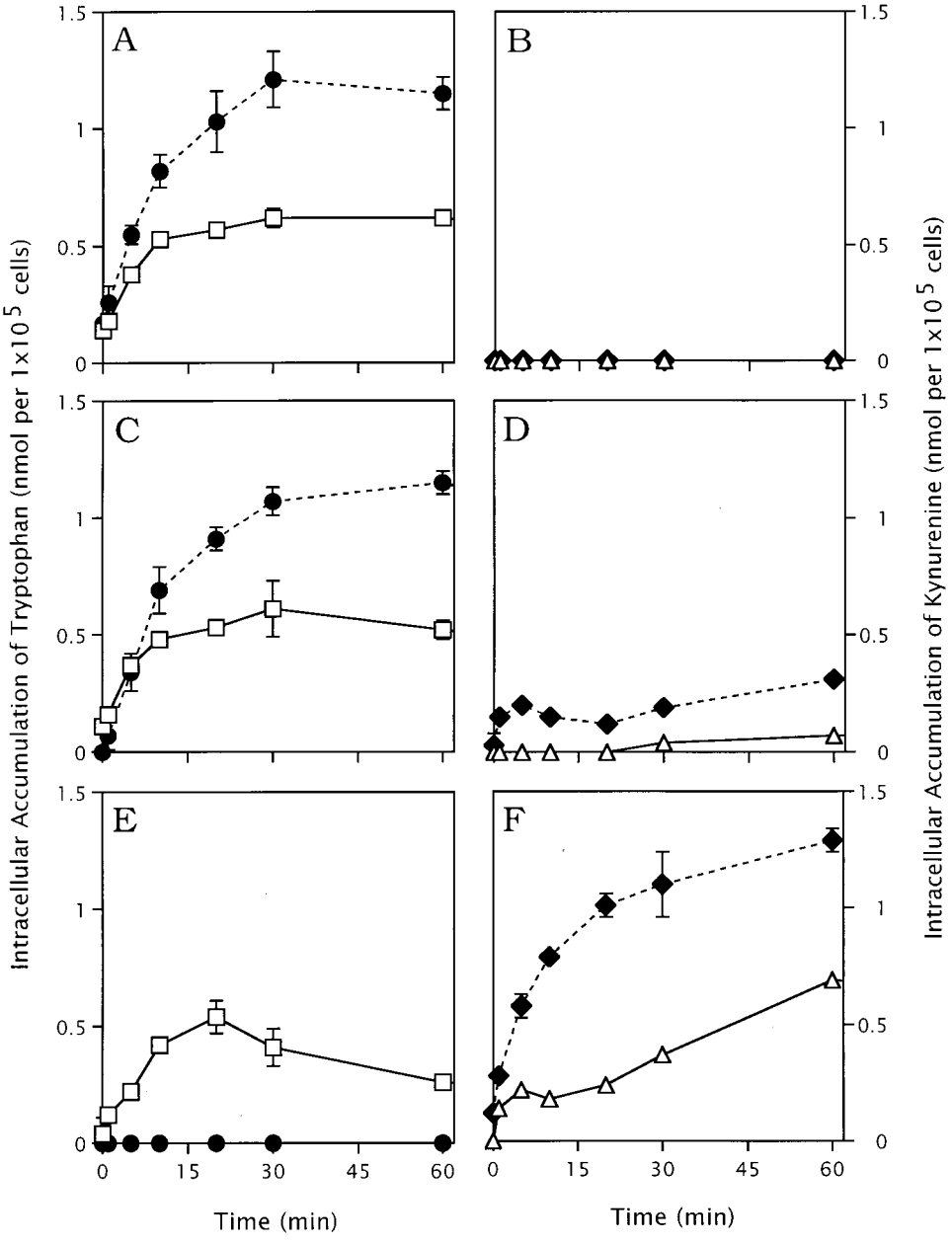

FIG. 3. Tryptophan transport into IFN-γ-treated, polarized (filled symbols) and nonpolarized (open symbols) ME-180 cells over time. Cells were untreated (A and B) or pretreated with 0.1 ng (C and D) or 1 ng (E and F) of IFN-γ/ml for 48 h before labeling to induce IDO activity. At the indicated times, intracellular tryptophan (A, C, and E) and kynurenine (B, D and F) concentrations were determined by HPLC with TCA-soluble cell extracts. Values represent the means ± standard deviations (error bars). For some samples, the standard deviations are too low (<1% of the means) to be visible on the figure.

psittaci growth since changes in extracellular amino acid concentrations could not be detected (11).

Chlamydial infection led to a minor reduction in intracellular tryptophan levels, which remained constant through 48 h postinfection. The differences in the total amounts of tryptophan used in infected, polarized and nonpolarized cells also remained constant throughout the experiment, suggesting that chlamydiae utilize a discrete amount of this essential amino acid regardless of the host pool size under nutrient-replete conditions. It would be interesting to see if an increased chlamydial burden on the host cell affects these intracellular levels proportionally and if different serovars respond differently as suggested by Coles and Pearce (11).

To determine if differences in persistent chlamydial growth could be explained by an increased rate of tryptophan transport into polarized cells, uptake of this amino acid was examined. Transport rates into both types of host cells were not significantly different, suggesting that another mechanism must account for these differences in response to IFN-γ. The amount of tryptophan taken up into both polarized and nonpolarized host cells over time was linear for up to 10 min. Similar kinetics have been reported for the uptake of methio-

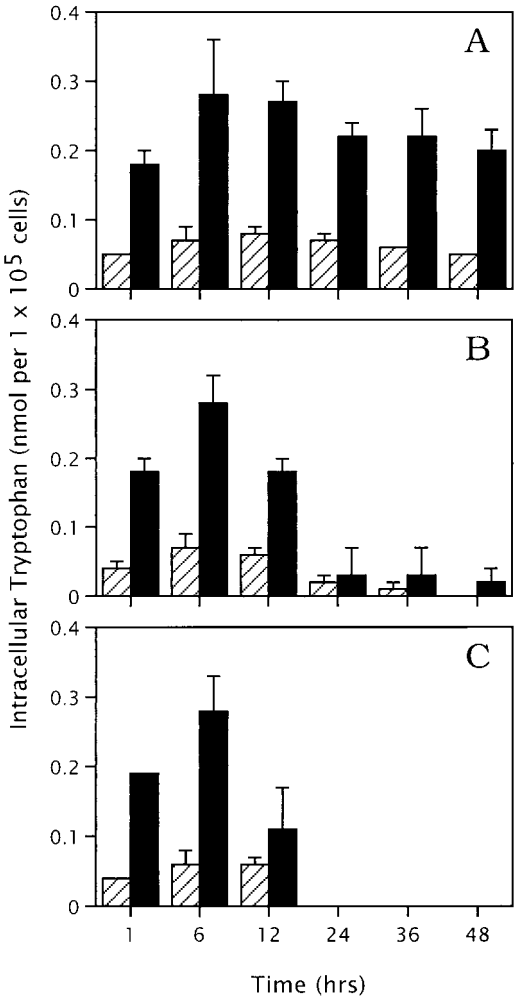

FIG. 4. Intracellular tryptophan concentrations after long-term labeling of IFN-γ-treated cells. Polarized (■) and nonpolarized (▨) cells were incubated with tryptophan-deficient EMEM containing L-[^{3}H]tryptophan carrier and no IFN-γ (A), 0.1 ng of IFN-γ/ml (B), or 1 ng of IFN-γ/ml (C) and assayed as described for Fig. 1. Values represent the means ± standard deviations (error bars). For some samples, the standard deviations are too low (<1% of the means) to be visible on the figure.

nine and lysine into polarized intestinal epithelial cells (10, 13), suggesting that amino acid transport in our system may be similar to that in other established systems. However, those studies focused on transcellular transport in intestinal epithelial culture systems as they relate to acquisition of amino acids from the diet rather than host-pathogen interactions.

In the absence of IFN-γ, longer incubation times were required to replace the native host cell pools with L-[^{3}H]tryptophan and carrier in polarized cells, and at least twice as much tryptophan was imported into these cells compared to that in conventional cultures. This result provided some evidence that polarized cells retained larger soluble tryptophan pools. When cells were treated with low (0.1 ng/ml) or high (1 ng/ml) doses of IFN-γ, IDO activity was induced as evidenced by the accumulation of catabolites over time. This enzyme induction did not appear to change the rate at which label was taken into the cells, implying that tryptophan transport may be the limiting step in this interaction.

It is more difficult to explain the apparent paradox seen between short-term and long-term labeling studies. Polarized cells appear to catabolize tryptophan more quickly and completely than do nonpolarized cells when examined shortly after labeling, but at late times they still have considerable intracellular tryptophan stores. Following 48 h of IFN-γ treatment in the short-term assay, it is likely that more than 1 h is required to overcome the effects of IDO-mediated tryptophan depletion and amass detectable levels of this amino acid.

Untreated, polarized cells consistently contain two to three times more intracellular tryptophan than did their nonpolarized counterparts for as long as 48 h after labeling. When conventionally grown cells are treated with a dose of IFN-γ sufficient to induce chlamydial persistence, the intracellular tryptophan levels diminish more rapidly and to a greater extent than in polarized cells. Perhaps continued access to moderate levels of tryptophan throughout the growth cycle (as seen in untreated, nonpolarized cells) or access to high levels of tryptophan early in the developmental cycle, despite limiting quantities later (in IFN-γ-treated, polarized cells), is sufficient for normal chlamydial development. It appears, then, that some threshold level of tryptophan must be maintained early in infection since large doses of IFN-γ can induce chlamydial persistence in polarized cells despite the presence of the amino acid for the first 12 h. Based on previous observations in our laboratory, detection of the persistence state does not occur until 36 h postinfection. Therefore, we postulate that the intracellular concentrations of tryptophan required for chlamydial growth are most critical during the middle of the developmental cycle (approximately 12 to 36 h postinfection).

Intracellular tryptophan pools were similar for both cell types under all IFN-γ treatment regimens until almost 12 h posttreatment. For many cell lines, IDO induction occurs 12 to 18 h after the addition of IFN-γ and plateaus by 36 to 48 h (29). In our system, substantial IDO activity was not detected until 24 h after IFN-γ treatment, with maximal enzyme activity at 36 h. This delay in detectable enzyme activity can fully account for the presence of relatively high levels of intracellular tryptophan in both polarized and nonpolarized cells up to 12 h after IFN-γ treatment.

Together these results indicate that polarized cells have larger intracellular pools of tryptophan than do nonpolarized cells. Polarized cells also acquire tryptophan more effectively and therefore can better replenish depleted intracellular pools, including those pools diminished as a result of IFN-γ-mediated induction of IDO under conditions sufficient to deplete this amino acid from nonpolarized cells. This ability to maintain higher intracellular tryptophan concentrations, even in the presence of IDO, may help explain why more IFN-γ is required to limit chlamydial growth or induce persistence when the host cells are grown in a polarized state. These results may also explain why persistence in polarized cells can be maintained over a much broader range of IFN-γ concentrations than in nonpolarized cells (18).

The differences observed in intracellular tryptophan stores based on the polarization state of the host cell raise additional questions. Previous studies have shown differences in the mechanisms of amino acid transport from the apical or basolateral surfaces of polarized epithelial cells (12, 13). Further study is needed to characterize the number and type of tryptophan transporters on the apical versus basolateral surfaces in this polarized culture system and how these compare to transporters in nonpolarized cells. Perhaps polarized cells can utilize more than one transporter to bring tryptophan into the cell, or the transporters may be more efficient when the cells are grown in the polarized orientation.

Additionally, other amino acids may influence intracellular

tryptophan availability. Coles and Pearce examined how amino acid interplay affects chlamydial growth and demonstrated an antagonistic effect between certain pairs of amino acids, one of which was tryptophan (11). This amino acid imbalance could be overcome not only by restoring the depleted amino acid but also by reducing the concentration of the antagonizing amino acid. Certainly, the competition between the host and parasite for limited available resources is complicated and impacts the ability of chlamydiae to grow productively or persistently. Whether other amino acids previously demonstrated to be required for growth of certain chlamydial strains compete for host cell amino acid transporters differently in polarized and nonpolarized cells is currently under investigation.

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