Characterization of Gamma Interferon-Mediated Cytotoxicity to Chlamydia-Infected Fibroblasts

GERALD I. BYRNE,1* CHARLES S. SCHOBERT,1 DWIGHT M. WILLIAMS,2 AND DAVID A. KRUEGER1

Department of Medical Microbiology, University of Wisconsin Medical School, Madison, Wisconsin 53706,1 and Audie L. Murphy Memorial Veterans Administration Hospital, University of Texas Health Science Center, San Antonio, Texas 782842

Received 7 September 1988/Accepted 12 December 1988

Addition of murine recombinant gamma interferon (IFN-γ) to mouse fibroblast cultures infected with Chlamydia psittaci was found to induce a cytotoxic response that was dependent on the concentration of IFN-γ added and the multiplicity of infection given. No cytotoxicity was observed for uninfected cells treated with IFN-γ, nor did infection alone elicit cytotoxicity. Cytotoxicity was detected only if IFN-γ was present for at least the first 18 h of a 30-h incubation period. Cytotoxic activity was not observed when infected cells were treated with 50 µg of chloramphenicol per ml, a drug which inhibits differentiation of infectious elementary bodies to noninfectious relictuate bodies. Cytotoxic activity was restored if addition of chloramphenicol was delayed until 18 h postinfection. Addition of 100 U of penicillin per ml to infected host cells reduced but did not abolish cytotoxic activity. Treatment of host cells with as little as 0.2 µg of cycloheximide per ml inhibited cytotoxicity without interfering with chlamydial growth. When addition of cycloheximide was delayed until 12 h after infection and IFN-γ treatment, cytotoxicity was restored. These data indicate that IFN-γ functions as a cytotoxic cytokine against chlamydia-infected fibroblasts. Cytotoxicity was found to be dependent on chlamydial multiplicity of infection, differentiation of chlamydiae to the metabolically active form, and host cell protein synthesis.

Elements of the immune response to chlamydiae that may play a role in protection or in recovery from infection have not been clearly defined. Evidence has accumulated to support a role for gamma interferon (IFN-γ) as a host cell activator in the restriction of intracellular replication of both Chlamydia psittaci (3, 12) and C. trachomatis (8). In those studies, host cells were treated for at least 1 day prior to infection, and in some experimental systems, the tryptophan catabolic enzyme indoleamine 2,3-dioxygenase was found to be an important IFN-γ-induced protein that mediated oxygen-independent inhibition of intracellular chlamydial replication (5).

Induction of antimicrobial activity has been recognized as an important IFN-γ-mediated event in several host-pathogen models (6, 11). It does not appear that there is a single mechanism of IFN-γ-mediated antimicrobial activity induced in host cells against intracellular pathogens. Some intracellular microbes appear to be susceptible to oxygen-dependent activity that becomes elevated in cells treated with IFN-γ. Other microbes, however, appear to be inhibited by mechanisms unrelated to the generation of toxic intermediates of oxygen metabolism. In either case, the net result of the effects of IFN-γ on potential host cells is the same. The host cell becomes activated in the presence of IFN-γ, and subsequent intracellular pathogen growth is curtailed within the treated host cell. In this report, we further characterize an additional activity associated with IFN-γ-mediated events related to intracellular chlamydial replication. In the system reported here, IFN-γ, when added to host cells after infection with chlamydiae, evoked a cytolytic response that was dependent on the multiplicity of infection (MOI), the concentration of IFN-γ, the time of incubation, and the de novo production of proteins by the infected host cells. Thus, IFN-γ has now been characterized as a cytolytic cytokine that apparently exhibits specificity for host cells only after they have become infected. Some characteristics of this newly described activity are reported here.

MATERIALS AND METHODS

Growth of chlamydiae and maintenance of cell lines. The 6BC strain of C. psittaci was grown in L-cell monolayers as described previously (4). Cells were collected 48 h after infection and lysed by sonication, and the chlamydiae were partially purified by differential centrifugation. Small samples of harvested chlamydiae in phosphate-buffered saline supplemented with 2% heat-inactivated fetal bovine serum and 0.25 M sucrose were stored at −70°C. A representative sample was thawed, and the number of viable elementary bodies (EBs) was estimated by the 50% infective dose (ID50) method as described by Hatch (9). Frozen samples were thawed just before being used, and all experiments were done with a single, standardized stock of chlamydiae.

L cells were maintained as monolayers in M199 medium supplemented with 10 µg of gentamicin sulfate per ml, 50 µg of vancomycin per ml, and 10% (vol/vol) heat-inactivated fetal bovine serum (growth medium). Cells were routinely trypsinized and subcultured at less-than-confluent densities twice weekly in 75-cm² cell culture flasks.

Cytotoxicity assay. A thymidine release assay was used to quantitate the amount of cytolysis exhibited by cells infected and then treated with various concentrations of IFN-γ. L-cell monolayers, in 75-cm² tissue culture flasks, were radiolabeled by incubation for 20 to 24 h in growth medium in the presence of 2 µCi of [3H]thymidine per ml (Dupont, NEN Research Products, Boston, Mass.; specific activity, 6.7 Ci/mM). Radiolabeled cells were washed free of [3H]thymidine-containing medium and infected with appropriate doses of the 6BC strain of C. psittaci. An inoculum size of 1 ID50 was used for most experiments, but the
The inoculum was varied from 0.03 to 4.0 ID₅₀ for some experiments. A mock-infected tissue culture flask of radiolabeled cells was always included as a control for nonspecific cytolyis. The chlamydial inoculum was adsorbed for 2 h on an environmentally controlled platform shaker at 37°C. Infected or mock-infected cells then were removed from the plastic cell culture flask substrate by trypsinization and transferred to 96-well microtiter plates at a density of 2 × 10⁵ cells per microtiter well. Some wells were treated with appropriate dilutions of murine recombinant IFN-γ (Genentech, South San Francisco, Calif.). Others were reserved for determining maximum radiolabel release by adding a final concentration of 0.5% sodium dodecyl sulfate, and the remaining wells were reserved for determining spontaneously released radioactivity for both infected and uninfected L cells. The final volume of medium in each well was adjusted to 200 µl, and all parameters were set up and tested in quadruplicate. Plates were incubated for 30 h at 37°C in an atmosphere composed of 5% CO₂ in air, and then 100 µl of medium was removed and the amount of radioactivity was determined in a liquid scintillation spectrometer. Results were recorded as percent specific cytotoxicity according to the following formula: percent specific cytotoxicity = [(cpm sample - cpm background) / (cpm maximum - cpm background)] × 100, where cpm is counts per minute. Background counts per minute were always below 10% of the maximum number of counts per minute released from sodium dodecyl sulfate-treated samples for both infected and uninfected L-cell samples. All data have been reported as mean values ± standard deviations.

Effect of metabolic inhibitors on cytotoxicity. For some experiments, the effects of procaryotic- or eucaryotic-specific metabolic inhibitors on IFN-γ-mediated cytotoxicity were measured. To determine the effects of chloramphenicol (which prevents differentiation of EBs to reticulate bodies [RBs]) or penicillin (which interferes with chlamydial division but not metabolic activity), cells were infected and plated in 96-well microtiter plates as usual. Chloramphenicol was added to one set of wells at a final concentration of 50 µg/ml, and penicillin was added to another set of wells at a final concentration of 100 U/ml. Cells were incubated and processed for percent specific cytotoxicity determinations as described above. The effect of time of addition of chloramphenicol was also measured. For these experiments, radiolabeled cells were infected and plated as described. Chloramphenicol (50 µg/ml) was then added to sets of wells at specified times after plating (2, 6, 12, 18, and 24 h), and samples were processed for percent specific cytotoxicity determinations as described.

To determine whether new host protein synthesis was required for the induction of IFN-γ-mediated cytotoxicity, infected and IFN-γ-treated cells were incubated for the full 30-h period with concentrations of cycloheximide ranging from 0.02 to 0.5 µg/ml. Control wells included samples not treated with IFN-γ but incubated with cycloheximide and cells not infected but treated with either IFN-γ, cycloheximide, or both. Time course experiments also were done to determine what point during the IFN-γ treatment period proteins related to induction of cytotoxicity were produced. For these experiments, cells were radiolabeled, infected, plated, and treated with IFN-γ as described above. Cycloheximide, at a final concentration of 0.05 µg/ml, was added to sets of wells after 2, 6, 12, 18, and 24 h of incubation. Cells were processed for percent specific cytotoxicity determinations after 30 h of incubation.

**Experimental results**

Cytotoxic activity of IFN-γ for chlamydia-infected fibroblasts. When murine fibroblasts (L cells) were infected with 1 ID₅₀ of C. psittaci 6BC and then treated with various concentrations of murine recombinant IFN-γ beginning 2 h after infection and incubated for a total of 30 h, a cytolytic response was observed that was dependent on the amount of IFN-γ present in the growth medium (Fig. 1). Concentrations of IFN-γ were varied from 0.5 to 10 U/ml. These concentrations represented amounts of IFN-γ ranging from 0.4 to 8 ng/ml on the basis of information provided by the manufacturer. Cytolysis of infected L cells was observed with as little as 1 U/ml and was markedly demonstrable at 5 and 10 U/ml. Cytolysis plateaued when higher amounts of IFN-γ were added, and the percent specific cytotoxicity achieved at the plateau was dependent on the inoculum added. Infected L cells incubated either in the absence of IFN-γ or with levels below 1 U/ml (0.8 ng/ml) did not exhibit a cytolytic response. Uninfected L cells did not exhibit demonstrable cytotoxicity in the presence of any concentration of IFN-γ tested.

When exposure of infected L cells to IFN-γ was delayed from 2 h after infection (28-h exposure; Fig. 2) to either 6, 18, or 24 h after infection (24-, 12-, and 6-h exposure times, respectively; Fig. 2), the IFN-γ-mediated cytolytic response was found to be dependent upon the time of exposure to IFN-γ. Only minimal cytotoxicity was measured when infected cells were exposed to IFN-γ for 24 h or less. Cytotoxic activity was clearly seen when infected cells were exposed to IFN-γ for 28 h, and the degree of cytotoxicity observed correlated roughly with the time of IFN-γ exposure.

The amount of cytotoxicity also varied with the inoculum size of strain 6BC administered to L cells (Fig. 3). Below 0.33 ID₅₀ (17% infected host cells), little IFN-γ-mediated cytotoxicity was seen. The proportion of cells exhibiting cytotoxic activity exhibited MOI-dependent kinetics at inocula greater than 0.33 ID₅₀. For example, at 0.67 ID₅₀,
approximately 25% of the cells were infected and 20% specific cytotoxicity was observed. At 1 ID₉₀, approximately 50% of the cells were infected and 35% specific cytotoxicity was observed. At 4 ID₉₀, approximately 70% of the cells were infected and 50% cytotoxicity was observed.

**Effect of metabolic inhibitors on IFN-γ-mediated cytotoxicity for 6BC-infected L cells.** Data presented in the previous section demonstrated that the IFN-γ-mediated cytolytic response was dependent both on the concentration of IFN-γ present in the medium and on the MOI. It was therefore of interest to determine whether the presence of chlamydial macromolecular synthesis in infected cells was sufficient to result in cytotoxicity in the presence of IFN-γ or whether chlamydial growth and metabolism were required. To determine this, infected cells were treated with either 50 μg of chloramphenicol per ml or 100 U of penicillin per ml. Chloramphenicol prevents differentiation of infectious EBs to metabolically active, noninfectious RBs if added at the time of infection and stops growth and metabolism of RBs when added at a time subsequent to the intracellular differentiation of EBs to RBs. In contrast, penicillin prevents binary fission of metabolizing RBs but does not significantly impair metabolic activity (10). When infected (3 ID₉₀s), IFN-γ-treated cells were incubated for the full 30-h duration in the presence of chloramphenicol, no cytotoxic activity was observed (Fig. 4), indicating that infection without differentiation of EBs to RBs was insufficient to establish conditions such that cytotoxicity could be manifest. For example, there could be a complete absence of chlamydial macromolecular synthesis in chloramphenicol-treated cells. Cytotoxic activity was observed, however, when infected, IFN-γ-treated cells were incubated in the presence of penicillin. The diminished amount of cytotoxicity observed when penicillin-treated cells were compared with cells treated only with IFN-γ probably reflected differences in the actual number of intracellular RBs contributing to the metabolic activity at the time cells were processed. When chlamydiae were allowed to metabolize for 18 h before being treated with chloramphenicol, cytotoxic activity was detectable, further substantiating that chlamydial differentiation, metabolism, and growth were required for IFN-γ-mediated cytotoxic activity. Maximal cytotoxic activity was observed for cells treated with chloramphenicol for only the last 8 h of the 30-h incubation period (data not shown).

It was also of interest to determine whether de novo host cell protein synthesis was required for IFN-γ-mediated cytotoxicity. Infected cells were treated with 5 U of IFN-γ per ml and then incubated in various concentrations of cycloheximide, a eucaryote-specific inhibitor of protein synthesis. It was found that as little as 0.02 μg of cycloheximide per ml added for the last 28 h of incubation was sufficient to
interfere with the cytolytic response (Fig. 5). It is notable that chlamydial growth continued unabated in cycloheximide-treated cells, providing further evidence that the observed IFN-γ-mediated cytotoxic activity was not merely a function of chlamydial growth but required active participation of the infected host cell. Cycloheximide treatment caused reversal of the cytotoxic response when the drug was added at either 2 or 6 h postinfection. If addition of cycloheximide was delayed until 12 h postinfection, then cytotoxicity was partially restored. When cells were exposed to the drug only for the last 12 h of incubation, maximal cytotoxicity was observed (data not shown). These data suggested that a protein made relatively soon after IFN-γ treatment was required for generation of the cytotoxic response.

DISCUSSION

A cytolytic function has been described elsewhere for IFN-γ (2), and in this report, the delineation of IFN-γ-mediated cytotoxicity has been extended and more completely characterized. It was found that IFN-γ-mediated cytotoxic activity was dependent upon the concentration of IFN-γ present in the incubation medium, the chlamydial MOI administered to L cells, intracellular differentiation and metabolism of chlamydiae, and host cell protein synthesis. An additional important parameter was found to be the specific sequence of host cell treatments that were required to establish conditions that led to cytolytic activity. It has been reported by several groups (3, 8, 12, 13) that treatment of potential chlamydial host cells with IFN-γ for 1 to 2 days prior to infection activates the host cell to restrict intracellular chlamydial growth without interfering with the uptake of infectious EBs into treated cells. In the human system, the induction of the tryptophan catabolic enzyme indoleamine 2,3-dioxygenase has been implicated as the IFN-γ-mediated mechanism crucial to inhibition of intracellular chlamydial development (5). The phenomenon of host cell activation and inhibition of intracellular chlamydial growth appears to be at least superficially similar in the murine system, but the specified IFN-γ-mediated mechanism has not been characterized, although it has been reported to be unrelated to induction of indoleamine 2,3-dioxygenase activity (7). The process of IFN-γ-mediated cytotoxicity is different from host cell activation in that it requires that host cells be infected before they are incubated in the presence of IFN-γ, and very low levels of IFN-γ are sufficient to mediate cytolytic activity. It is at present unclear what mechanisms induced by the combination of infection with chlamydiae and IFN-γ treatment mediate cytotoxicity, but the process appears to be a highly effective means of eliminating chlamydia-infected cells, at least in the cell culture system described here.

Although this activity may at first seem to be hastening the inevitable fate of an infected host cell, the potential for promoting premature lysis with the release of noninfectious chlamydiae would contribute to eradication of an ongoing infection and result in a heightened degree of antigen release that could serve to further stimulate the immune response.

It is apparent that induction of IFN-γ secretion plays a role in vivo during recovery from chlamydial infections (1) and in protective immunity against chlamydiae (14). It has not yet been established how activation of host cells to restrict intracellular chlamydial growth and induction of cytolytic activity for infected host cells contribute to protection against infection and resolution of acute disease. One could envisage a scenario in which, during the course of acute chlamydial disease, resolution results from the presence of IFN-γ at the site of infection would cause activation of uninfected host cells such that any viable chlamydiae that were released from infected cells would be inhibited from subsequent cycles of replication. This scenario is, of course, speculative at this time, but further work in this area should help substantiate the involvement of IFN-γ in immunity to chlamydiae.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants 19782 and 22380 from the National Institute of Allergy and Infectious Diseases, an award from the Edna McConnell Clark Foundation, and a grant from the General Medical Research Service of the Veterans Administration.

We thank Sherideen Stoll for her help in preparing the manuscript and Steve Riccio for his assistance in the laboratory.

LITERATURE CITED

5. Byrne, G. I., L. K. Lehamn, and G. J. Landry. 1986. Induction of tryptophan catabolism is the mechanism for gamma-interf-


