

Gamma Interferon-Mediated Cytotoxicity Related to Murine *Chlamydia trachomatis* Infection

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Received 22 February 1988/Accepted 13 May 1988

After infection with the mouse pneumonitis agent (MoPn; murine *Chlamydia trachomatis*), heterozygous (*nu/+*) but not nude athymic (*nu/nu*) mice produced enhanced amounts of gamma interferon (IFN- γ) in vitro in response to MoPn antigen that exhibited cytotoxic activity when added to host cells already infected with chlamydiae. Antibody-complement lysis showed the cytotoxic activity to be dependent, at least in part, on L3T4⁺ T cells for production. The cytotoxic responses were directed primarily against *Chlamydia*-infected target cells, but a second type of toxicity was demonstrable against uninfected target cells after treatment of the generating cell population with anti-Lyt-2 antibody plus complement at certain time points after infection. This additional nonspecific cytotoxic activity was presumably due to a second factor (factor X) acting in concert with IFN- γ . Lyt-2⁺ cells, however, also were shown to play a role in IFN- γ production and cytotoxicity directed against infected targets at later time points after infection. Neutralization of IFN- γ in the samples containing cytotoxic activity abrogated the cytotoxicity against both infected and uninfected targets, but cloned murine IFN- γ exhibited toxicity in a dose-dependent manner only against infected target cells. The data provides evidence that cytotoxicity against infected targets is due to antigen-specific induction of IFN- γ , but other cytokine activity, most demonstrable after removal of Lyt-2.2⁺ cells and cytotoxic to uninfected targets, also is present.

The immunology and immunopathology of host defense against infection with *Chlamydia trachomatis* are not well understood. Byrne and Krueger (4) have demonstrated that supernatant fluid prepared by incubation of *Chlamydia psittaci*-immunized murine spleen cells with mitogen (concanavalin A) contained factor-mediated cytotoxic activity against mouse fibroblasts infected with *C. psittaci*. The cell type responsible for production of the cytotoxic factor or factors was not clear, nor was the identity of the factor(s). Cytotoxicity during murine infection with *C. psittaci* also has been described by Lammert (6). In that study, the cytotoxicity was mediated by spleen cells, not supernatant. Early in infection, the cytotoxicity was relatively nonspecific, affecting both infected and uninfected target cells (fibroblasts or macrophages). Later, it became relatively specific for *Chlamydia*-infected targets. The cell type(s) responsible for the cytotoxicity was not defined. The relationship between the factor-mediated and cell-mediated cytotoxicity of these two studies is not clear. In both studies, incubation times were longer than that used in the traditional 4-h assay used to look for cytotoxic T-cell activity. Pavia and Schachter (8) employed the traditional assay (4-h chromium release) and syngeneic target cells to look for T-cell-mediated cytotoxicity during murine infection with *C. trachomatis*. They were unable to detect cytotoxic activity; thus, the role of cytotoxicity in *Chlamydia* infection and the factor(s) and cell type(s) responsible for it remain unclear. We decided to further explore the question of cytotoxicity during *Chlamydia* infection by using our model of chlamydial pneumonia in the nude athymic mouse (*nu/nu*) and its heterozygous littermate (13–15). The infecting agent was the mouse pneumonitis agent

(MoPn; murine *Chlamydia trachomatis*). In this model, resistance to infection is T-cell dependent in that the *nu/nu* mouse is significantly more susceptible to MoPn than the *nu/+* mouse is, a defect correctable by adoptively giving the *nu/nu* mouse T cells. In this model, specific antibody production also is T-cell dependent (13–15).

Recently, using this model, we have shown that spleen cells from MoPn infected *nu/+* but not *nu/nu* mice produced gamma interferon (IFN- γ) in vitro in response to MoPn antigen (3). Chlamydial replication also was inhibited by a factor produced only by immunized *nu/+* mice, and there was a good correlation between inhibition of replication and IFN production (3). We used this same model and looked for factor-mediated cytotoxicity in the MoPn-mouse system. An important distinction between this and previous work is that inhibition of replication was observed when host cells were treated with IFN- γ for at least 24 h prior to infection. In contrast, cytotoxicity was observed in the present study only when IFN- γ was added after infection of the target host cells. We have, for the first time, demonstrated factor-mediated cytotoxicity generated in response to infection with *C. trachomatis*, have shown that the effect is, at least in part, T-cell dependent, and have provided evidence that IFN- γ is involved in the cytotoxic phenomenon.

MATERIALS AND METHODS

Mice. The mice used in these studies were pathogen-free "super-clean" *nu/+* and *nu/nu* mice on a BALB/c background, as we have used in our previous studies. They were free of *Mycoplasma* spp., *Chlamydia* spp., viruses, and pathogenic bacteria by culture and serology (13–15). They were maintained under barrier conditions and veterinary supervision as described in our previous studies (3, 13–15).

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MoPn. The mouse biovar of *C. trachomatis* was obtained as previously described and maintained in embryonated hen eggs. Dilutions of the organism for intranasal infection of mice were made in McCoy modified 5A medium (Difco Laboratories, Detroit, Mich.). Mice were infected with 10^4 inclusion-forming units of MoPn in a volume of 0.05 ml as previously described (3, 13–15).

MoPn antigen. The antigen used for cytokine production in vitro was elementary bodies of MoPn grown in HeLa-229 cells and purified by Renografin (E. R. Squibb & Sons, Princeton, N.J.) gradient separation as previously described (13). It contained no egg yolk material. The protein content was 2.16 mg/ml. Inactivation of the antigen was accomplished by UV light. The inactivated antigen was diluted in phosphate-buffered saline and stored in small aliquots at -70°C .

Preparation of whole spleen cells and generation of cytokines. This was accomplished as in our previous study of IFN generation (3). We obtained spleens in an aseptic manner from infected or uninfected *nu/+* or *nu/nu* mice. For some experiments, cells were depleted of L3T4^+ cells or Lyt-2^+ cells by incubation with the appropriate antibody for 1 h on ice, followed by washing. Antibody-treated cells then were incubated for 1 h at 37°C with a 1:10 dilution of low-toxicity rabbit complement (Pel-Freez, Rogers, Alaska). Antibodies used in depletion studies were the monoclonal antibody GK 1.5 (gift of Katherine Wall and John Kung, University of Texas Health Science Center, San Antonio) and anti-Lyt-2.2 (Cederlane, London, Ontario, Canada). Antibodies were used at a 1:10 dilution. Cell suspensions were incubated with $5\ \mu\text{g}$ of UV-inactivated MoPn antigen per ml in a final volume of 20 ml with 5.0×10^6 cells per ml. Cells were incubated for 48 h at 37°C in 5% CO_2 in air. Cells then were centrifuged at $500 \times g$ for 5 min, and the supernatant fluids were collected and filtered through a $0.45\text{-}\mu\text{m}$ -pore-size Nalgene filter. Supernatant fluids were stored at -70°C until being assayed for the presence of IFN or cytokine activity. Cytokine was neutralized for IFN- γ activity by incubation with monoclonal anti-mouse IFN- γ (hybridoma R4-6A2 developed by George Spitalny and Edward Havell and obtained from Lee Biomolecular, San Diego, Calif.). The amount of monoclonal antibody used was twice that calculated to be needed on the basis of the IFN titer of the sample.

Measurement of IFN. IFN activity was assayed with a microtiter procedure against a cytocidal infection with vesicular stomatitis virus (2). Samples, when indicated, were evaluated with and without IFN neutralizing antibody in parallel and were coassayed against the National Institutes of Health reference standards.

Cytotoxicity assay. Cytotoxicity was determined by a modification of the [^3H]thymidine-release assay used for this purpose in a previous publication by Byrne and Krueger (4). L-cell monolayers in 75-cm 2 flasks were incubated with $2\ \mu\text{Ci}$ of [^3H]thymidine (Amersham-Searle, Arlington Heights, Ill.) per ml for 16 to 20 h. After incubation, cells were washed extensively with Hanks balanced salt solution and then were infected with *C. psittaci* at 5 50% infective doses. The inoculum size was routinely verified by concomitant titration in L cells. Sham-infected cells were incubated as controls. After adsorption and uptake of the inoculum, infected L cells were trypsinized, counted, and distributed as 100- μl portions into wells of 96-well, flat-bottom microtiter plates at a density of 2×10^4 cells per well (2×10^5 cells per ml). Cells were also plated in Lab-tek multiwell slides for subsequent confirmation of the percent infection. Each

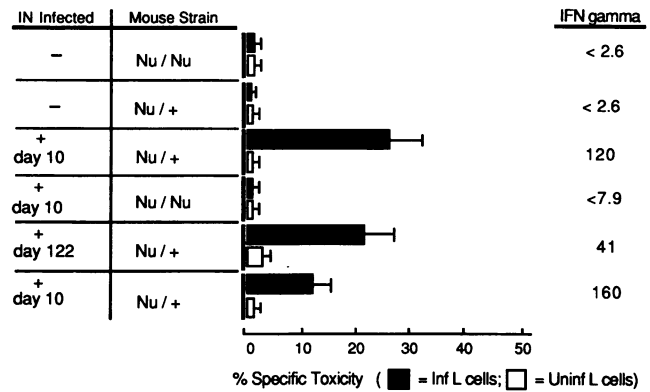


FIG. 1. Cytotoxic activity for chlamydia-infected (Inf) or uninfected (Uninf) L cells generated by spleen cells taken from intranasally MoPn-infected *nu/nu* or *nu/+* BALB/c mice 10 or 122 days after infection or activity generated by spleen cells from uninfected mice. Spleen cells were incubated in vitro with chlamydial antigen, and supernatant fluids were tested for cytotoxic activity. Each bar represents the mean of quadruplicate determinations, and standard deviations are given. Acid-labile interferon (IFN gamma) titers (in units per milliliter) are included for comparison.

microtiter plate well was brought to a final volume of 200 μl by adding various dilutions of cytokine. Microtiter plates were incubated for 36 h at 37°C in a CO_2 incubator and then processed to measure the amount of [^3H]thymidine released into the culture medium. Plates were centrifuged for 5 min at $500 \times g$, and 100 μl was drawn off and added to 3 ml of water-soluble scintillant. The maximal counts per minute (cpm) released was determined by adding 0.1% sodium dodecyl sulfate to quadruplicate samples. The cpm released spontaneously also was measured. Spontaneous release was consistently below 10% of the maximum cpm released by postdetergent treatment for both infected and uninfected cell populations. All samples were set up in quadruplicate, and the percent specific [^3H]thymidine released was determined by the following relation: percent specific ^3H release = [(experimental cpm - spontaneous cpm)/(maximum cpm - spontaneous cpm)] $\times 100$.

Statistics. The Student *t* test with correction for unequal variances was used to calculate statistics. Results were termed significant when $P < 0.05$.

RESULTS

Figure 1 shows the results of the cytotoxicity assay by using cytokine generated by MoPn antigen from 10- or 122-day infected or uninfected *nu/+* spleen cells and *nu/nu* day 10 infected spleen cells. This activity was correlated with IFN- γ levels in the same sample. Uninfected *nu/+* and *nu/nu* mice exhibited neither cytotoxicity nor IFN- γ production. Significant production of cytokine was only seen in two separate samples from *nu/+* mice infected for 10 or 122 days. IFN- γ also was produced only by the same 3 groups, although relative amounts of IFN (120, 41, and 160 U) did not correlate exactly with specific cytotoxicity against infected targets ($28.4 \pm 3.5\%$, $23 \pm 4.4\%$, and $12.8 \pm 2.9\%$, respectively).

Figure 2 shows data from individual (anti-Lyt3T4+C', C' only) or pooled (+, -) mouse spleens from *nu/+* mice infected for 10 to 96 days. Some nonspecific cytotoxicity was observed when spleen cells had been incubated with complement alone, for reasons that were not clear. However,

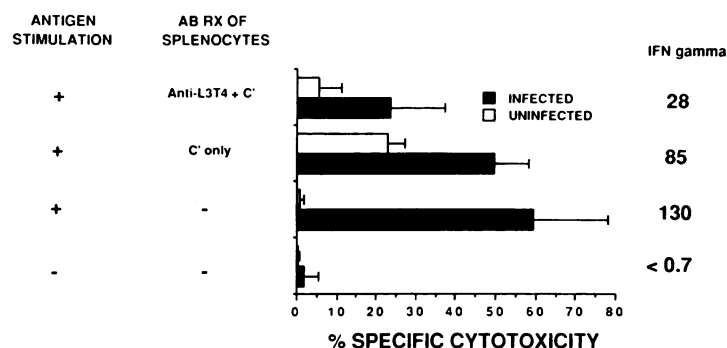


FIG. 2. Effect of anti-L3T4 antibody plus complement (C') on IFN- γ levels and cytotoxic activity on infected or uninfected targets from splenocytes of *nu*/+ -infected BALB/c mice. Each bar represents the mean of quadruplicate samples. Standard deviations are given. Cells were first treated with complement alone, complement plus anti-L3T4, or medium and then were centrifuged and incubated with antigen as described in Materials and Methods. Acid-labile interferon (IFN gamma) titers (in units per milliliter) are included for comparison.

both IFN- γ and cytotoxicity against infected and uninfected targets treated with complement alone were significantly reduced by removal of L3T4⁺ spleen cells by antibody-complement lysis. Incubation of splenocytes with complement alone did not abrogate cytotoxicity nor IFN- γ levels. A high level of both IFN- γ and cytotoxicity directed against infected targets was observed for antigen-stimulated spleen cells. No toxicity was observed when unstimulated spleen cell supernatants were used.

A different result was observed when spleen cells from infected mice were removed and incubated with anti-Lyt-2.2 plus complement at specified times after infection (Table 1). At day 10, treatment with anti-Lyt-2.2 plus complement did not reduce IFN- γ levels nor cytotoxic activity against infected targets when compared with untreated whole spleen cell preparations. With day 70 samples, anti-Lyt-2.2 treatment reduced both IFN- γ production and cytotoxicity against infected targets when compared with either untreated samples or day 10 results. Surprisingly, cytotoxicity against uninfected targets was significantly increased after anti-Lyt-2.2 plus complement at both time points.

Thus, on the basis of these experiments, it can be concluded that a cytotoxic factor(s) was produced by *nu*/+ whole spleen cells and increased significantly after MoPn infection. This was not observed for *nu/nu* mice, suggesting a T-cell dependence for both cytotoxic activity and IFN- γ production. Further, antibody-complement treatment to remove L3T4⁺ cells reduced both specific cytotoxicity and IFN- γ activity at both early and late time periods during infection. The effect of anti-Lyt-2⁺ and complement, however, was more variable.

Because of the partial correlation of IFN- γ production and cytotoxicity and clear L3T4⁺ T-cell dependence of both, the effect on cytotoxicity of neutralization of a IFN- γ activity by specific monoclonal antibody was determined. Anti-IFN- γ antibody significantly decreased both IFN- γ activity (to less than 3 U/ml in all cases) and cytotoxicity. These data are presented in Fig. 3. Thus, there was a relationship between cytotoxicity and IFN- γ activity in our samples. This experiment used cytokine generated from day 70 *nu*/+ whole spleen cells treated with anti-Lyt-2 antibody. Cytotoxicity was seen against both infected and uninfected target cells, and anti-IFN- γ neutralized cytotoxicity against both kinds of cells.

To determine whether all or part of the cytotoxicity was dependent on the presence of IFN- γ alone, studies to determine the toxicity of cloned murine IFN- γ (Genentech, South San Francisco, Calif.) were performed (Fig. 4). Addition of cloned IFN- γ to infected or uninfected L cells led to cytotoxic activity only in infected L cells. The cytotoxic activity against uninfected L cells seen in Table 1 and Fig. 3 after anti-Lyt-2.2 treatment was therefore not due solely to the presence of IFN- γ . The anti-Lyt-2.2 monoclonal antibody was not itself toxic to L cells (data not shown).

DISCUSSION

The most significant finding of this work is that IFN- γ was found to exhibit cytolytic activity against chlamydia-infected fibroblasts. Thus, induction of IFN- γ now can be correlated with two distinct functions. Activation of uninfected host cells by IFN- γ results in inhibition of chlamydial develop-

TABLE 1. Interferon production and cytotoxic activity of spleen cell supernatant fluids from MoPn-infected *nu*/+ mice following antigen stimulation in vitro

Days after infection	Spleen cell treatment prior to antigen stimulation	Acid-labile IFN levels (U/ml) ^a	% Specific cytotoxicity for target cells	
			Infected ^b	Uninfected
10	Complement only	93 ± 14	33.9 ± 15.0	4.4 ± 10.4
	Anti-L3T4 + complement	26 ± 13	23.6 ± 6.8	1.6 ± 3.4
	Anti-Lyt-2.2 + complement	93 ± 14	49.5 ± 22.2	23.9 ± 27.9
70	Complement only	89 ± 7.5	43.2 ± 25.1	9.5 ± 5.5
	Anti-L3T4 + complement	10 ± 10	0.2 ± 7.9	3.9 ± 4.3
	Anti-Lyt-2.2 + complement	23 ± 16	14.5 ± 7.4	20.5 ± 23.1

^a International reference units determined as described in Materials and Methods.

^b L cells infected with the 6BC strain of *C. psittaci* as described in Materials and Methods.

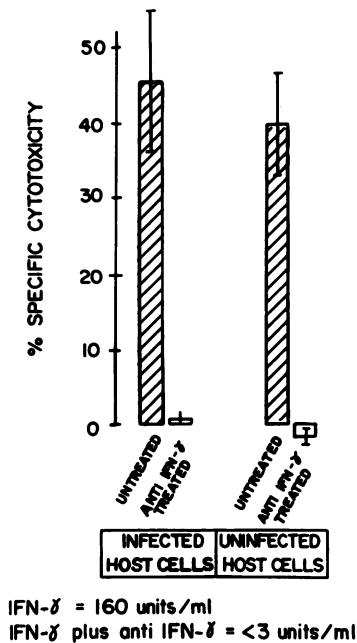


FIG. 3. Effect of treating supernatant fluids from anti-Lyt-2.2 antibody plus complement-treated spleen cells from intranasally infected *nu/+* BALB/c mice with anti-murine IFN- γ -immunoglobulin on the cytotoxic activity for chlamydia-infected and uninfected L cells. Each bar represents the mean of quadruplicate determinations, and standard deviations are indicated. The pre- and post-anti-IFN- γ -antibody titers for acid-labile interferon (IFN- γ) are indicated.

ment (3), whereas treatment of host cells already infected with chlamydiae manifests as a cytotoxic response. Several additional conclusions may be drawn from this work. (i) IFN- γ and factor X induction and/or increased production were demonstrated in *nu/+* but not *nu/nu* mice in response to MoPn infection. (ii) L3T4⁺ T cells clearly played a role in production of both IFN- γ and factor X. (iii) IFN- γ exhibited cytotoxicity activity for cells infected with chlamydiae in that specific neutralization of IFN- γ led to significantly reduced cytotoxicity. (iv) Cytotoxicity against uninfected fibroblasts also was noted after treatment of spleen cells taken at 10 or 70 days after infection with anti-Lyt-2.2 plus complement. The last phenomenon is consistent with the presence of a second cytokine induced in response to MoPn but suppressed by Lyt-2⁺ T cells. The observation that factor X was suppressed under normal conditions remains problematic. Perhaps a temporal association exists such that when Lyt-2⁺ cells become prominent this aspect of the potential immune response is no longer required.

The monoclonal antibody by itself was not toxic to L cells, nor did treatment of spleen cells with anti-Lyt-2.2 without complement induce cytokine production. The fact that anti-IFN- γ eliminated the cytokine against both infected and uninfected cells in anti-Lyt-2⁺-treated samples but that cloned murine IFN- γ had cytotoxicity only against infected L cells suggests the presence of a second cytotoxic factor which may synergize with IFN- γ or require it for activity. The identity of the factor responsible for cytotoxicity against uninfected L cells, however, remains undefined.

It is well known that IFN- γ may synergize in its cytotoxic effects with other cytokines (1, 7, 9, 11). IFN- γ can, for example, further augment the increased vulnerability of vesicular stomatitis virus-infected cells to lymphotoxin (1)

and increase the number of receptors and tumor necrosis factor- α (TNF- α) sensitivity of target cells (10). It also can synergize with lymphotoxin (putatively TNF- β) against uninfected targets (11). IFN- γ also can enhance the induction of lymphotoxin (12). The situation is further confused by the growing list of molecules which can act as direct mediators of cytotoxicity to include TNF- β (lymphotoxin), TNF- α (cachectin), interleukin-1, natural killer cell cytotoxic factor, leukoregulin, and others (5). Further, TNF- α and TNF- β are related molecules (5). In addition, although macrophages are the usual source of TNF- α and lymphocytes are the usual source for TNF- β , some T-cell clones make mRNA for both factors after concanavalin A stimulation (10).

Thus, it is quite possible that the additional factor causing cytotoxicity in our system (especially to uninfected targets after anti-Lyt-2 treatment) is TNF- β (produced by a T cell). It also may be TNF- α (produced by a macrophage). This factor could show an apparent T-cell dependence because of a need to synergize with IFN- γ (which is clearly L3T4⁺ T-cell dependent in our model). The characterization of cytotoxic factor in our model, in addition to IFN- γ , is the subject of ongoing work in our laboratories.

Data on cytotoxic responses generated after anti-Lyt-2.2 plus complement treatment also indicated that at early time points after infection, removal of Lyt-23.2⁺ cells did not result in diminution of IFN- γ production or cytotoxic activity. At later time points, Lyt-2.2⁺ cells appeared to contribute to both IFN- γ production and cytotoxicity. These results are consistent with a role for Lyt-2.2⁺ cells in cytokine induction as the infection proceeds from the acute to the chronic state or to resolution. It is not clear whether this observed temporal distinction in Lyt-2.2⁺ cells is a result of distinct subsets of Lyt-2.2⁺ cells or maturation of Lyt-2.2⁺ as acute disease is controlled.

The possible role of cytotoxic factors in host defense or immunopathology is also unknown at present. IFN- γ , when added 24 h prior to infections, inhibits the replication of MoPn in fibroblasts (G. I. Byrne, D. Williams, and J.

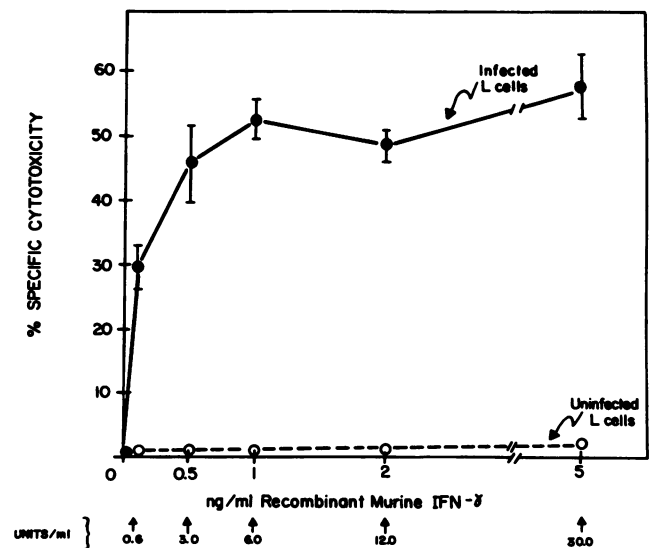


FIG. 4. Cytotoxic activity of recombinant murine IFN- γ on chlamydia-infected and uninfected L cells. Each point represents the mean of quadruplicate determinations, and standard deviations are given. IFN- γ concentrations are given as both nanograms per milliliter and international reference units per milliliter as provided by the supplier.

Schachter, unpublished data), but the effect is static and not lethal. It is possible, therefore, that cytotoxicity preferentially directed against host cells after infection represents a further host defense mechanism but one likely to cause significant immunopathology (to both infected and bystander cells). The cytotoxic assay itself was done by using a *C. psittaci* system because it is a well-characterized model thought to be representational of in vitro chlamydia-host cell interactions. Additional work needs to be done to determine whether chlamydial strains known to induce IFN- γ , such as MoPn, also are affected in the same way as described here. These studies are areas of further ongoing investigation.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-19782, AI-22380, and AI-21912 from the National Institutes of Health, by the General Medical Research Service of the Veterans Administration, and by an award by the Edna McConnell Clark Foundation.

We also thank Sherideen S. Stoll for her help in the preparation of the manuscript.

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