Gamma Interferon as a Crucial Host Defense against
Rickettsia conorii In Vivo

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Received 17 November 1986/Accepted 2 February 1987

Gamma interferon (IFN-γ) plays an important role as a host defense in rickettsial infection. Swiss Webster mice, which are resistant to Rickettsia conorii (Malish 7 strain) infection, were treated with a monoclonal antibody against mouse IFN-γ. When the antibody-treated mice were inoculated with 12.5% tissue culture infective doses of R. conorii, the mortality was 47% and the morbidity was 100%. None of the control mice, which received the same dose of R. conorii, died or became ill. The enumeration of rickettsiae in organs by direct immunofluorescence in paraffin sections demonstrated higher quantities of rickettsiae in the spleen and liver of IFN-γ-depleted mice as compared with those of the infected controls. The kinetic analysis of IFN-γ levels in sera showed depletion in the treated mice. These results indicate that IFN-γ plays an important role as a host defense in the early stage of rickettsial infection. Survival of some mice despite continued treatment with antibody to IFN-γ suggests that other immune mechanisms may also be important.

The mechanisms of host defense against rickettsial infection have been studied extensively. It has been demonstrated that the development of cell-mediated immunity is a very important antirickettsial response (8, 9, 11, 16, 23). Lymphokines from the supernatant of cultured lymphocytes stimulated with rickettsial antigens or concanavalin A activated macrophages and inhibited the multiplication of rickettsiae in macrophages, macrophagelike cells, fibroblasts, and endothelial cells (12, 13, 18).

The activity of lymphokines on cells to induce antimicrobial effects shares many characteristics with gamma interferon (IFN-γ) (20, 21, 25). Expression of activity requires protein synthesis by host cells, and the antimicrobial activity is destroyed by exposure to pH 2, heat (80°C), and trypsin. Either IFN-γ-like lymphokines or cloned mouse IFN-γ can inhibit multiplication of rickettsiae in infected cells (14, 15, 19) and induce cytolysis of rickettsia-infected phagocytic cells (21, 25). This suggests that the interaction between the infected cell and the effector mechanism is a part of antirickettsial immunity. Elimination of lymphokine-mediated antimicrobial activity by monoclonal antibody specific for murine IFN-γ in vivo assays suggested that IFN-γ is an important factor in the antirickettsial activity of lymphokines (5).

This study presents evidence that IFN-γ plays a key role in host resistance to rickettsial infection in vivo. Swiss Webster mice, which are resistant to a high challenge dose of Rickettsia conorii, died from overwhelming rickettsial infection when depleted of IFN-γ by a monoclonal antibody and infected with a low dose of R. conorii.

MATERIALS AND METHODS

Mice. Male Swiss Webster mice, 6 to 8 weeks of age, were purchased from Jackson Laboratory, Bar Harbor, Maine.

Rat anti-murine IFN-γ monoclonal antibody. The rat hybridoma R4-6A2 producing monoclonal anti-murine IFN-γ antibody was prepared, and the antibody was characterized by Spitalny and Havell (17). Antibody used in the present study was obtained by growing hybridoma cells in BALB/c athymic mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) and collecting the ascitic fluid from the mice. The ascitic fluid was stored at −70°C and filter sterilized before use. The anti-IFN-γ titers of the ascitic fluid was 4.2 × 10⁴ neutralizing units per 0.1 ml, as determined by neutralization of antiviral activity of a standardized lymphokine preparation as previously described (17).

Mouse monoclonal antibody against Vero cytoskeleton. A mouse monoclonal antibody against a Vero cell cytoskeletal antigen was prepared in this laboratory as described previously (2). The titers by direct immunofluorescence assay was 1:10,240.

Rickettsia. R. conorii (Malish 7 strain) was obtained from the American Type Culture Collection, Rockville, Md., and was plaque purified and shown to be mycoplasma-free. The rickettsial inoculum was cultivated in yolk sacs of 5-day-old specific-pathogen-free embryonated chicken eggs (SPAFAS, Inc., Norwich, Conn.); 10% infectious yolk sac suspension contained 10⁶-10⁷ 50% tissue culture infective doses (TCID₅₀) per ml for primary chicken embryo cells. Rickettsiae were stored at −70°C before dilution in sucrose phosphate glutamate buffer, pH 7.0 (0.218 M sucrose, 0.0038 M KH₂PO₄, 0.0086 M Na₂HPO₄, 0.0049 M glutamate) for inoculation of 12 TCID₅₀ per mouse.

Experimental design. Mice were divided into four groups. (i) Group 1 consisted of a total of 40 mice that received a total dose of 4.2 × 10⁴ units of monoclonal anti-murine IFN-γ antibody per mouse by intraperitoneal injection of 8.4 × 10⁴ units of antibody per dose and were infected with 12 TCID₅₀ of R. conorii. (ii) Group 2 consisted of 30 mice that received monoclonal antibody against Vero cell cytoskeleton and 12 TCID₅₀ of R. conorii. (iii) Group 3 consisted of 15 mice that received only monoclonal anti-murine IFN-γ antibody (4.2 × 10⁴ units per mouse, total dose). (iv) Group 4 consisted of 15 mice that received 12 TCID₅₀ of R. conorii only.

All the mice that received either monoclonal antibody were treated on days 0, 1, 3, 5, and 7 after infection. Rat
monoclonal anti-murine IFN-γ antibody was diluted in phosphate-buffered saline, pH 7.2 (0.137 M NaCl, 0.027 M KCl, 0.088 M Na₂HPO₄, 0.0015 M KH₂PO₄). Mouse monoclonal antibody against the Vero cell antigen was diluted similarly in phosphate-buffered saline. All the inocula of rickettsiae and antibodies were administered intraperitoneally. Mice were sacrificed on days 5, 7, 8, and 10 after infection, and their spleens and livers were collected, fixed in 4% buffered formaldehyde, and embedded in paraffin. Sections were cut at 4-μm thickness for direct immunofluorescence demonstration of rickettsiae. Two mice each from groups 1 and 2 were bled by cardiac puncture on each of days 2, 4, 6, 8, 10, 12, and 14 postinfection. Sera were collected and stored at −70°C for IFN assay.

**Enumeration of rickettsiae in organs.** Rickettsiae were enumerated in formaldehyde-fixed, paraffin-embedded tissue by an immunofluorescence technique (22). Sections were affixed to slides with Weld Wood white glue (Roberts Consolidated Industries). The deparaffinized, trypsin-digested slides were reacted with a specific fluorescent isothiocyanate-conjugated globulin fraction of rabbit antiserum reactive with spotted fever group rickettsiae obtained from the Centers for Disease Control, Atlanta, Ga., as described previously (22). The slides were examined by UV microscopy with barrier and exciter filters for fluorescent isothiocyanate. Rickettsiae in 10 fields were counted at ×1,000 magnification, and the average number of rickettsiae per field was calculated.

**Assay for serum IFN-γ.** A cytopathic effect inhibition assay which involved protection of L-929 cells against infection with vesicular stomatitis virus, modified from that of Havell and Vileček (3, 14), was used to measure IFN levels. Twofold dilutions of sera were prepared in Eagle minimum essential medium supplemented with 10% fetal bovine serum and added to monolayers of L-929 cells in 96-well plates. After incubation at 37°C for 18 to 24 h, the monolayers were washed, and vesicular stomatitis virus in Eagle minimum essential medium with 2% fetal bovine serum was added. After incubation at 37°C for 48 h, monolayers were examined for cytopathic effect. The dilution at which 50% protection was observed is the endpoint.

**Assay of serum anti-IFN-γ antibody.** The presence and quantity of antibody to IFN-γ in mouse serum were assayed by the neutralization of antiviral activity in a lymphokine preparation containing IFN-γ, as described previously (17). A lymphokine preparation containing 10 U of IFN-γ per ml and sera collected from anti-IFN-γ-treated mice were added to monolayers of L-929 cells in 96-well plates. Then, the monolayers were infected with vesicular stomatitis virus. The dilution of serum that neutralized the antiviral activity of 10 U of IFN is the titer of antibody to IFN-γ.

**RESULTS**

All the mice in group 1, which received antibody to murine IFN-γ and *R. conorii*, were ill on day 3 after infection. They were thin and inactive with ruffled fur. These animals began to die from rickettsial infection on day 7 postinfection. The morbidity was 100%, and mortality was 47%. No mice from the three control groups became ill or died. The IFN levels are presented in Table 1. The antiviral activity of the sera of mice treated with antibody to murine IFN-γ was lower than that of the infected control mice. IFN-γ was detected in a few samples and at low titer in the anti-IFN-γ-treated mice. In contrast, IFN-γ was first noted in the sera of all untreated, infected controls on day 6 after infection and persisted for 1 week. IFN-α/β as defined by pH 2 stability was found in the sera of mice treated with anti-murine IFN-γ during the early stage of infection, and beyond 6 days after infection, it was no longer detectable. No IFN-α/β was detected in the sera of untreated mice. Antibody to IFN-γ in the sera of treated mice was detectable during the first 8 days of infection, the first 7 days of which animals received antibody to IFN-γ. The average anti-IFN-γ titer in sera of treated mice for this period was 1:16. After day 8, the titer of residual circulating anti-IFN-γ antibody decreased and was undetectable by days 12 to 14.

In all experiments performed, some animals in the group treated with anti-murine IFN-γ and infected with *R. conorii* survived the infection. Recovery from the rickettsial infection was evident on day 9 after infection, and even if anti-murine IFN-γ was given continuously after day 9 of infection, animals alive on day 9 survived the infection (data not shown).

The enumeration of rickettsiae in organs by direct immunofluorescence on paraffin-embedded sections demonstrated higher quantities of rickettsiae in the spleens and livers of mice which received the monoclonal antibody to murine IFN-γ and *R. conorii* than in those of the control groups (Table 2). From day 5 after infection, IFN-γ-depleted mice had numerous rickettsiae in the spleen and liver; only a few rickettsiae could be found in the organs of the control mice. On day 10 after infection, the quantity of rickettsiae in the organs of the treated mice decreased dramatically and approached that of control mice. Among the organs examined, the spleen was infected the most severely.
DISCUSSION

This study demonstrates directly for the first time that IFN-γ is an important host defense factor in resistance to rickettsial infection in vivo. Monoclonal antibody against murine IFN-γ converts an animal model of complete resistance to R. conorii into a susceptible model with significant lethality by depletion of IFN-γ from the animal. Previous studies have indicated that IFN-γ is responsible for both antirickettsial and cytolytic actions in vitro (19, 21, 25). The antirickettsial activity requires protein synthesis by host cells, and the activity can be destroyed by trypsin, heating to 80°C, and exposure to low pH. The cytolytic activity is specific for rickettsia-infected cells and does not act on uninfected cells (25). This situation suggests the possibility that the surface of the infected cells had been altered in some way by the intracellular rickettsial infection (25). Either rabbit antisera to purified murine IFN-γ or rat monoclonal antibody to murine IFN-γ neutralizes the antirickettsial activity of lymphokine preparations and cloned mouse IFN-γ (5, 20) in vitro. The removal of the ability of lymphokines to stimulate antirickettsial activity of macrophages by monoclonal antibody to IFN-γ conjugated to Sepharose and the ability of recombinant IFN-γ to induce macrophage antirickettsial activity suggest that IFN-γ is the major inducer of macrophage antirickettsial activity among the lymphokines. IFN-γ exerts regulatory activity at many points in the system, including enhanced expression of Ia and H-2 antigens on lymphocytes, macrophages, and endothelial cells, enhanced cytotoxic T cell activity, and enhanced NK cell killing activity (1, 10, 26), all of which may be important in antirickettsial immunity.

The protection against rickettsial infection afforded by IFN was observed nearly 20 years ago. It has been reported that an IFN-like inhibitor was found in mouse serum that protected mice from lethal rickettsial infection (6, 7, 24). In these studies, it was reported that the protective IFN was of the α/β type. In this study, we found IFN-α/β in the treated mouse blood at an early stage of infection, but it was not protective as compared with that of the control mice. Turco and Winkler reported that a preparation of virus-induced IFNs (α/β) had no detectable antirickettsial activity (20). It is not clear why the finding of pH 2-stable IFN in the sera of anti-murine IFN-γ-treated mice was relatively inconsistent; however, the levels were always low and near the level of detection in this assay. This and the fact that the mice used in this study were outbred perhaps are the reasons that pH 2-stable IFN was not always detectable in the sera of treated, infected animals.

The quantities of rickettsiae in organs of experimental and control groups are quite different. The lethal infection of IFN-γ-depleted mice is systemic and results from specific rickettsial infection. The reduction in levels of IFN-γ in the blood of treated mice suggests that the monoclonal antibody entered the circulation and neutralized IFN-γ.

The recovery of anti-murine IFN-γ-treated mice from infection is not associated with an increase in the level of IFN-γ in the blood of the animals. The IFN-γ levels remained undetectable in treated mice even after treatment with anti-murine IFN-γ was discontinued. Moreover, mice which received antibody continually even after day 9 still survived. That there are other components of the immune system that are activated during the infection is suggested since recovery of mice did not result from an increase in IFN-γ levels. Palmer et al. reported that the peak of IFN-γ production occurred earlier than did the peak of protection from animal death (14). This also suggests that immunity to rickettsiae is due not only to IFN-γ but also to some other immune mechanism(s) or nonspecific host defense mechanism, as described for R. tsutsugamushi (4).

ACKNOWLEDGMENTS

We thank Lorraine Zeiler for preparation of the manuscript. This work was supported in part by Public Health Service grant AI-21242 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

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