INFECTION AND IMMUNITY, Jan. 1993, p. 23–31
0019-9567/93/010023-09$02.00/0

Vol. 61, No. 1

Association between Virulence of *Yersinia pestis* and Suppression of Gamma Interferon and Tumor Necrosis Factor Alpha

RYOHEI NAKAJIMA† AND ROBERT R. BRUBAKER*

Department of Microbiology, Michigan State University, East Lansing, Michigan 48824-1101

Received 10 August 1992/Accepted 20 October 1992

It is established that *Yersinia pestis*, the causative agent of bubonic plague, and enteropathogenic *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* share a ca. 70-kb low-calcium response or Lcr plasmid (Lcr$^+$). The latter is known to encode regulatory functions that restrict growth at 37°C in Ca^{2+}-deficient medium and virulence factors that are expressed only in vitro within this environment (e.g., certain Yops and V antigen). In this study, gamma interferon (IFN-γ) was never detected in mice infected with 10 minimum lethal doses (MLD) of Lcr$^+$ cells of *Y. pestis*, and significant levels of tumor necrosis factor alpha (TNF-α) arose only prior to death. Prompt and marked synthesis of these cytokines was observed upon infection with avirulent Lcr$^-$ mutants. Treatment of mice with exogenous IFN-γ plus TNF-α inhibited multiplication of Lcr$^+$ yersiniae in vivo, thereby providing protection against challenge with 10 MLD. Administration of both cytokines was required for absolute survival, suggesting a synergistic rather than cumulative interaction. This protective effect entailed cytokine priming as judged by subsequent detection of substantial levels of endogenous IFN-γ and TNF-α. Monospecific anti-V-antigen, known to provide passive immunity against 10 MLD of Lcr$^+$ *Y. pestis*, permitted significant synthesis of endogenous IFN-γ and TNF-α. These findings demonstrate that Lcr$^+$ yersiniae suppress synthesis of cytokines and suggest that this effect is mediated by one or more Lcr plasmid-encoded virulence factors.

In vitro vegetative growth of wild-type *Yersinia pestis*, the causative agent of bubonic plague, and enteropathogenic *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is dependent at 37°C, but not at 26°C, upon addition to synthetic culture media of that amount of Ca^{2+} normally present in mammalian vascular fluid (ca. 2.5 mM). Cultivation at 37°C in Ca^{2+}-deficient medium, an environment simulating mammalian intracellular fluid, results in expression of a unique low-calcium response characterized by a reduced adenylate energy charge, shutoff of stable RNA synthesis, and inhibition of cell division. However, this form of ordered stepdown, hereafter termed restriction, results in selective synthesis of numerous virulence factors (e.g., certain Yops and LcrV or V antigen), all of which are encoded on a shared ca. 70-kb low-calcium response or Lcr plasmid. The latter also contains regulatory genes that mediate restriction with induction of virulence factors (Lcr$^+$). A surface adhesin encoded by *yadA* serves as an additional Lcr$^+$-specific invasin of enteropathogenic yersiniae; this activity is not expressed in *Y. pestis*. Lcr$^-$ mutants cured of the Lcr plasmid or isolates lacking certain essential structural genes or regulatory functions encoded on this element are avirulent (12, 20, 24, 32).

After induction in vitro at 37°C by withdrawal of Ca^{2+}, Yops of enteropathogenic yersiniae typically undergo release into culture supernatant fluid (27, 40, 41), although significant levels also accumulated at the outer membrane (7, 52, 64). In contrast, most but not all Yops produced in vitro by wild-type cells of *Y. pestis* undergo immediate posttranslational degradation (39, 58) catalyzed by an outer membrane plasminogen activator encoded on a species-specific 10-kb pesticin or Pst plasmid (Pst$^+$) (4, 60, 61). The few Yops of Lcr$^+$ Pst$^+$ cells of *Y. pestis* not attacked by this hydrolase are released in intact form into culture supernatants (36, 62), whereas all Yops undergo net accumulation in cultures of induced Lcr$^+$ Pst$^-$ yersiniae (39, 58). Two degradable Yops shown to be virulence factors are E (56, 63) and H (8, 55, 63), which are known to disrupt actin (56) and dephosphorylate protein tyrosine (6, 23), respectively. Additional Yops required for expression of virulence were degradable K and L (65) and released M (35). The former were necessary for sustained growth in organs, suggesting a role in immunosuppression (65), and the latter exhibited similarity to platelet surface protein GPIbα (36).

Mutants lacking V antigen, which is encoded in the *lcrGVH-yopBD* operon of the Lcr plasmid (5, 51, 53, 54), lack the temperature-dependent nutritional requirement for Ca^{2+} typical of wild-type organisms and are avirulent (5, 53). Presumed monospecific rabbit polyclonal anti-V-antigen (72, 73) and mouse monoclonal 15F anti-V-antigen (59) prevented lethality, and the former promoted formation of protective granulomas (73), suggesting that this autoproteolytic peptide (13) functions as an immunosuppressor. However, the nature of the events affecting the formation of protective granulomas by yersiniae has not been resolved. Recent studies have demonstrated central roles for the cytokines gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) in inflammatory reactions (1, 3, 26, 74) associated with development of cellular immunity directed against a variety of facultative (14, 21, 26, 31, 34, 38, 42, 44–50) and obligate (75, 76) intracellular procaryote parasites.

Little is known about the role of cytokines in mediating immunity to yersiniae other than that the enteropathogenic species-specific *yadA* product promotes inhibition of an anti-invasive effect of interferon (9). The purpose of this report is to provide evidence demonstrating that Lcr$^+$ but not Lcr$^-$ cells of *Y. pestis* strongly suppress generation of endogenous TNF-α and, especially, IFN-γ in vivo. Nevertheless, significant synthesis of both cytokines occurred

* Corresponding author.
† Present address: Exploratory Research Laboratories I, Daiichi Pharmaceutical Co., Ltd., 1-16-13, Endogawa, Tokyo 134, Japan.

after infection, provided that the mice either received anti-V-antigen or underwent cytokine priming by injection of exogenous TNF-α plus IFN-γ. Either one of these therapeutic regimens provided absolute protection against 10 minimum lethal doses (MLD) of Lcr^+ yersiniae.

MATERIALS AND METHODS

Bacteria. A conditionally virulent Lcr^+ Pst^+ but nonpigmented (28) isolate of *Y. pestis* KIM (10), lethal by the intravenous route but not by peripheral routes of injection (29, 71), and an isogenic avirulent mutant lacking the Lcr plasmid (19) were used in most experiments. The protective capabilities of new lots of rabbit polyclonal and mouse monoclonal anti-V-antigen were also assayed against wild-type cells of *Y. pseudotuberculosis* PB1 (15) and *Y. enterocolitica* WA (16). An Lcr^+ Pst^- mutant of *Y. pestis* KIM capable of net synthesis of all known Yops (39, 58) was used for absorption of polyclonal anti-V-antigen.

Cultivation. Bacteria were stored at −20°C in buffered glycerol as previously described (4), directly inoculated onto slopes of tryptose blood agar base (Difco Laboratories, Detroit, Mich.), and incubated at 26°C for 1 day (enteropathogenic yersiniae) or 2 days (*Y. pestis*). The organisms were then removed in 0.033 M potassium phosphate buffer, pH 7.0 (phosphate buffer), and appropriately diluted in the same buffer for use as inocula. Bacteria required in large numbers for use in preparation of V antigen for immunization or for absorption of rabbit polyclonal anti-V-antigen were cultivated in oxalated fermenter medium as previously described (13). Lcr^+ Pst^- cells of *Y. pestis* used for preparation of outer membranes or yersiniae used in immunoblots were grown at 37°C in chemically defined medium containing 20 mM Mg^{2+} and no added Ca^{2+} as defined elsewhere (78).

Antisera. V antigen was purified from a sterile cytoplasmic extract of *Y. pestis* by an established method (13) and then used for immunization of rabbits as already reported (72). After absorption of the resulting hyperimmune antiserum with disrupted and lyophilized cells of Lcr^- yersiniae, followed by removal of insoluble debris by centrifugation (72), putative remaining contaminating antibodies (e.g., anti-Yops) were eliminated by absorption with outer membranes (10 mg of protein per ml of serum) prepared from Lcr^+ Pst^- cells of *Y. pestis* as previously described (64). This mixture was stirred gently at 37°C for 30 min and then overnight at 4°C; all particulate matter was then removed by centrifugation (150,000 × *g* for 3 h), and the remaining soluble immunoglobulin was purified by precipitation with 50% saturated $(NH_4)_2SO_4$, chromatography on DEAE-cellulose (Whatman, Inc., Clifton, N.J.), and then chromatography on Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) (72). The resulting reagent was homogeneous gammaglobulin (13, 72) lacking detectable anti-Yop or anti-porin activity as judged by immunoblotting against Lcr^+ enteropathogenic yersiniae or *Escherichia coli* K-12 (data not shown). This preparation was dialyzed overnight against cold phosphate buffer prior to use in promoting passive immunity with concomitant synthesis of endogenous cytokines.

The process used to prepare monoclonal 15F anti-V-antigen has been described previously (59). Briefly, the method involved injection of BALB/c mice once a week for 5 weeks with purified V antigen (13) (10 μg of protein in complete Freund's adjuvant). Spleen lymphocytes were then isolated, fused with strain X-63-Ag8-653 mouse myeloma cells (22), and subcultured, and then selected clones were either replicated in RPMI 1640 medium supplemented with 10% fetal calf serum or injected into the peritoneal cavities of BALB/c mice previously treated with pristane. Antibody secreted from hybridomas was identified by an enzyme-linked immunosorbent assay utilizing goat anti-mouse immunoglobulin G and M conjugated with horseradish peroxidase (18). The monoclonal antibody, 15F, used in the present experiments was precipitated with 50% saturated $(NH_4)_2SO_4$ to eliminate foreign material from the hybridoma culture medium and then dialyzed overnight against cold phosphate buffer prior to use in experiments.

Animals. Outbred female Swiss-Webster mice (Harlan-Sprague Dawley, Inc., Indianapolis, Ind.), 6 weeks of age, were used to determine the capability of the refined preparations of anti-V-antigen described above to provide passive immunity to experimental plague and yersiniosis. Inbred female C57BL/6 mice (Harlan-Sprague-Dawley), 6 to 9 weeks of age, were used in experiments concerned with cytokine production and suppression. Mice were kept in a room maintained at a constant temperature of 18.5°C and a relative humidity of 35%; commercial food and drinking water were provided ad libitum.

Passive immunity. The outbred mice were challenged intravenously with 10^2 Lcr^+ Pst^+ cells of *Y. pestis*, 10^2 cells of Lcr^+ *Y. pseudotuberculosis*, and 10^3 cells of Lcr^+ *Y. enterocolitica*; these values closely approximate 10 MLD (70–72) and were also found to be equivalent to 10 MLD for the inbred mice. Passive immunization was performed as previously described (72); i.e., mice challenged on day 0 received intravenously either rabbit polyclonal anti-V-antigen (100 μg of protein in 0.1 ml of phosphate buffer) or mouse monoclonal 15F anti-V-antigen (200 μg of protein in 0.2 ml of phosphate buffer) on postinfection days 1, 3, and 5. A single injection of the polyclonal antiserum on postinfection day 1 typically provided absolute passive protection against Lcr^+ *Y. pestis* but not the enteropathogenic yersiniae (71; data not shown).

Cytokines. The inbred mice were injected intravenously with either 10^2 Lcr^+ or 10^6 Lcr^- cells of *Y. pestis* in experiments involving cytokines. Mouse recombinant IFN-γ (Genentech, Inc., San Francisco, Calif.) and mouse recombinant TNF-α (Genzyme Corp., Boston, Mass.) were always used in this study. The specific activity of IFN-γ, determined by antiviral activity (2), was 5×10^6 U/mg, and that of TNF-α, assayed by cytotoxicity for L929 cells in the presence of actinomycin D (1), was 5×10^7 U/mg. Each reagent was diluted appropriately in phosphate buffer immediately before use in injections. Mice were treated intraperitoneally with IFN-γ and then TNF-α at 6 and 2 h, respectively, before challenge with yersiniae. The doses used were 10^5 U of IFN-γ and 10^4 U of TNF-α per mouse, unless specified otherwise.

Cytokine assays. A minor modification of an established process (48) was used to prepare samples for assay of cytokines. Mice in groups of three were sacrificed at intervals by terminal bleeding under anesthesia prior to removal of livers and spleens. Pooled blood was allowed to clot overnight at 4°C, and resulting samples of serum, separated by centrifugation at 10,000 × *g* for 30 min, were stored at −70°C prior to assay for cytokines. Spleens and livers were individually homogenized at a concentration of 100 mg/ml of Hank's balanced salt solution (GIBCO BRL, Gaithersburg, Md.) containing 1% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (Sigma Chemical Co., St. Louis, Mo.). After extraction of cytokines for 1 h at 4°C, the

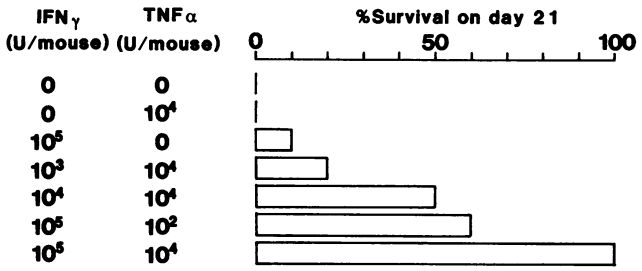

FIG. 1. Protection of C57BL/6 mice by combinations of intraperitoneally injected mouse recombinant IFN-γ and TNF-α against intravenous challenge against 10 MLD of Lcr$^+$ cells of *Y. pestis* KIM. IFN-γ and TNF-α were injected 6 and 2 h, respectively, before infection.

samples were centrifuged (10,000 × *g* for 30 min) and supernatants were preserved at −70°C until assay.

Commercial solid-phase enzyme immunoassays utilizing the multiple-antibody sandwich principal were used to determine cytokines in biological samples. In these experiments, TNF-α and IFN-γ were determined with the mouse Factor-TEST TNF kit (Genzyme) and mouse InterTEST-γ kit (Genzyme), respectively. Concentrations of cytokines were measured by reading optical density at 490 nm and then calculated in reference to values obtained in standard curves generated at each assay. To negate possible adverse effects of serum or homogenates on development of the assay, the materials used for standard curves were diluted in normal biological fluids identical in concentration to those present in the test samples. Assays of pooled sera were repeated three times, and those of individual organ homogenates were performed in duplicate.

Growth in vivo. Groups of three mice infected with Lcr$^+$ Pst$^+$ cells of *Y. pestis* were sacrificed at intervals, and spleens were removed aseptically, weighed, suspended at a concentration of 100 mg/ml of phosphate buffer, homogenized, and diluted appropriately in the same buffer. Samples of 0.1 ml were then spread on the surface of tryptose blood agar base contained in petri plates, and after incubation for 2 days at 26°C, CFU were counted. The lower limit of detection by this process was 10^2 cells per g of spleen.

Miscellaneous. Purity of immunoglobulin was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions as illustrated earlier (13, 72), and specificity was assayed by immunoblotting via methods previously described in detail (68). Protein was determined by the method of Lowry et al. (37) and located in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels by silver staining (43).

RESULTS

Cytokine-mediated resistance. Groups of 10 mice were treated intraperitoneally with phosphate buffer or IFN-γ at doses ranging from 10^3 to 10^5 U per mouse, and 4 h later, the animals similarly received phosphate buffer or TNF-α at doses of 10^2 to 10^4 U per mouse. After 2 h, the mice were challenged intravenously with 10 MLD of Lcr$^+$ cells of *Y. pestis* and observed for 3 weeks. As shown in Fig. 1, IFN-γ in combination with TNF-α promoted protection against lethality in a dose-dependent fashion. This effect is emphasized by the complete survival of mice that received the highest doses of cytokines (10^5 U of IFN-γ followed by 10^4 U of TNF-α). IFN-γ or TNF-α administered alone at these doses failed to provide significant protection, indicating that the two cytokines worked synergistically rather than additively to augment protective mechanisms of host defense.

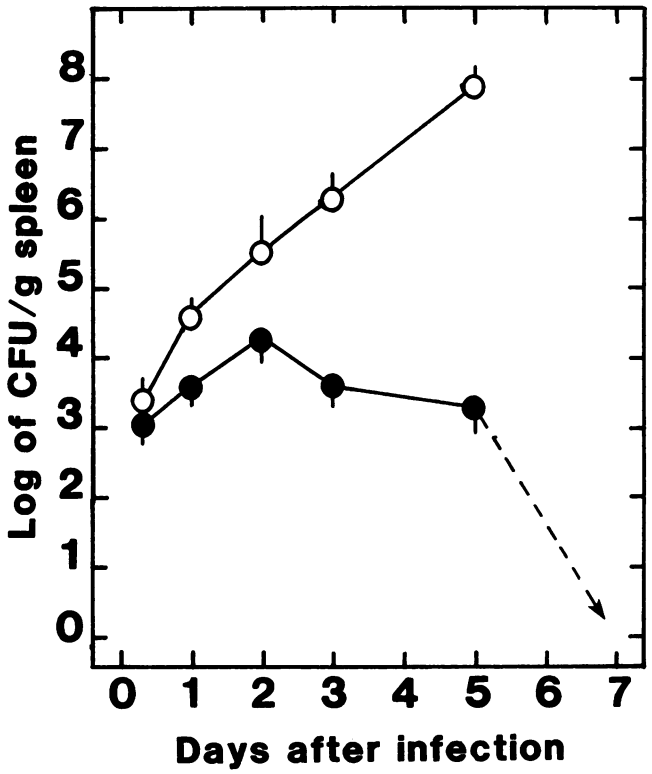

FIG. 2. Population of Lcr$^+$ cells of *Y. pestis* KIM in spleens of control (○) and cytokine-treated (●) mice after intravenous challenge with 10 MLD. Treatment consisted of intraperitoneal injection of 10^5 U of mouse recombinant IFN-γ and 10^4 U of mouse recombinant TNF-α at 6 and 2 h, respectively, before infection. Each symbol and vertical bar represents the respective mean and standard deviation of three mice, and the broken line indicates a decrease to undetectable levels; differences between the two groups at each interval were always significant ($P < 0.05$), except at 12 h postinfection.

Cytokine-inhibited growth in vivo. An experiment was done to define the effect of exogenous cytokines on growth in vivo. A population of about 10^3 Lcr$^+$ cells per g of spleen was observed in control mice 12 h after intravenous injection of 10 MLD (Fig. 2). The bacteria grew thereafter at a near-logarithmic rate, exceeding a concentration of 10^7 cells per g of spleen on postinfection day 5, at which time all mice were moribund. In striking contrast, treatment with the fully protective dose of cytokines (10^5 U of IFN-γ followed by 10^4 U of TNF-α) prevented initial rapid growth and then prompted clearance of the bacteria from infected spleens by postinfection day 7.

Fate of exogenous cytokines. Normal mice were given the optimal cytokine treatment regimen found to provide complete protection against challenge with *Y. pestis*. The distribution and disappearance of each cytokine in vivo was then determined. As shown in Table 1, exogenous IFN-γ, initially injected at a concentration of 10^5 U, underwent distribution in blood and organs prior to disappearance by 4 h without promotion of detectable endogenous TNF-α. At this time, the mice received 10^4 U of exogenous TNF-α, which again

TABLE 1. Distribution and disappearance of intraperitoneally injected IFN-γ and TNF-α in normal mice[a]

Time (min)	IFN-γ (ng/ml or g)			TNF-α (ng/ml or g)		
	Serum	Spleen	Liver	Serum	Spleen	Liver
0	<4.0[b]	<4.0	<4.0	<1.5[c]	<1.5	<1.5
5	6,450.0 ± 220.0	1,1200.0 ± 108.0	253.0 ± 21.0	<1.5	<1.5	
15	450.0 ± 21.0	970.0 ± 13.2	249.0 ± 12.0	<1.5	<1.5	<1.5
30	97.5 ± 2.8	199.0 ± 3.6	57.4 ± 3.8	<1.5	<1.5	<1.5
60	12.0 ± 1.8	23.9 ± 1.8	6.5 ± 0.3	<1.5	<1.5	<1.5
120	<4.0	5.3 ± 1.1	4.8 ± 0.3	<1.5	<1.5	<1.5
240	<4.0	<4.0	<4.0	<1.5	<1.5	<1.5
245	<4.0	<4.0	<4.0	5.6 ± 1.2	6.3 ± 1.1	2.0 ± 0.3
255	<4.0	<4.0	<4.0	3.4 ± 0.2	4.4 ± 0.9	1.8 ± 0.1
270	<4.0	<4.0	<4.0	2.1 ± 0.1	2.2 ± 0.2	1.6 ± 0.1
300	<4.0	<4.0	<4.0	<1.5	<1.5	<1.5
360	<4.0	<4.0	<4.0	<1.5	<1.5	<1.5

[a] Mice in groups of three individually received 10^5 U of IFN-γ at 0 min and 10^4 U of TNF-α at 240 min; levels of cytokines in serum and organs were determined as described in the text.
[b] Lower detection limit of IFN-γ.
[c] Lower detection limit of TNF-α.

disappeared rapidly without causing appearance of detectable endogenous IFN-γ. These findings demonstrate that injected IFN-γ and TNF-α underwent distribution in tissues but were rapidly removed thereafter and that injection of one of these cytokines did not cause production of the other.

Cytokines during infection. Production of endogenous IFN-γ and TNF-α was compared in infected mice either left untreated or primed with the optimal regimen of exogenous cytokines defined above. As expected from results obtained with normal mice (Table 1), neither IFN-γ (Fig. 3) nor TNF-α (Fig. 4) was present at the time of challenge with 10 MLD of Lcr^+ cells of *Y. pestis*. Infected control mice not primed with exogenous cytokines failed to produce detectable levels of IFN-γ throughout the course of infection, although considerable TNF-α was expressed as the animals became moribund. In contrast, a significant concentration of IFN-γ was detected in spleens and livers of cytokine-primed mice at 6 h after challenge; levels of this cytokine peaked at postinfection day 1 and then decreased, becoming undetectable by postinfection day 7 (Fig. 3). Levels of TNF-α in cytokine-primed mice increased steadily, starting immediately after challenge, peaked on postinfection day 2, and

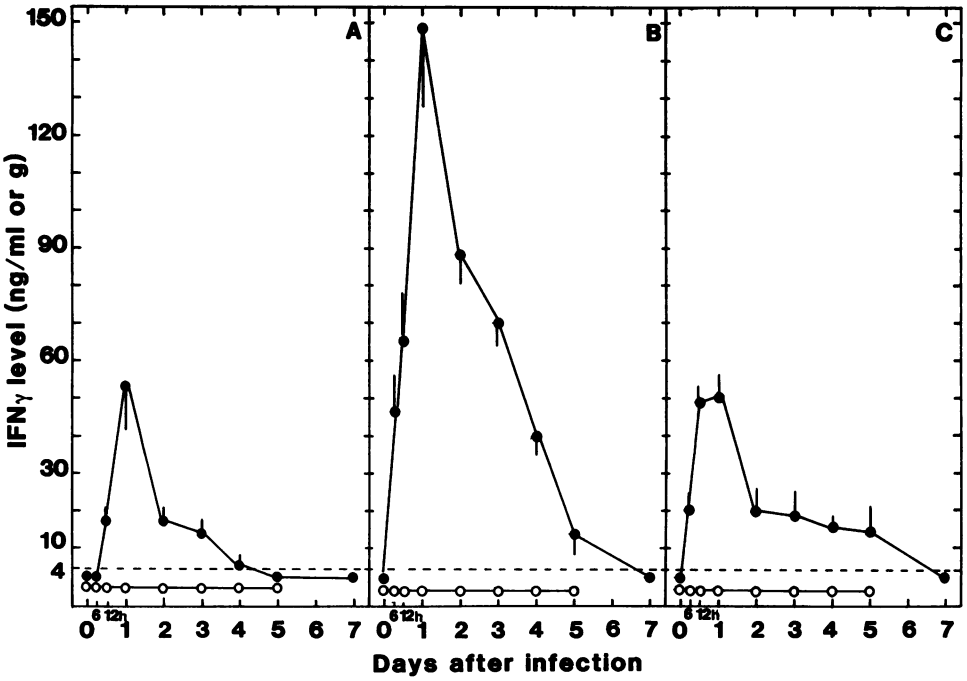

FIG. 3. Effect of exogenous cytokine priming (intraperitoneal injection of 10^5 U of mouse recombinant IFN-γ and 10^4 U of mouse recombinant TNF-α at 6 and 2 h, respectively, before intravenous challenge with 10 MLD of Lcr^+ cells of *Y. pestis* KIM) on production of exogenous IFN-γ in sera (A), spleens (B), and livers (C) of unprimed control (○) and cytokine-primed (●) mice. Each symbol and vertical bar represents the mean and standard deviation of three mice, respectively, and the dashed line indicates the lower limit of detection (4.0 ng/ml or g).

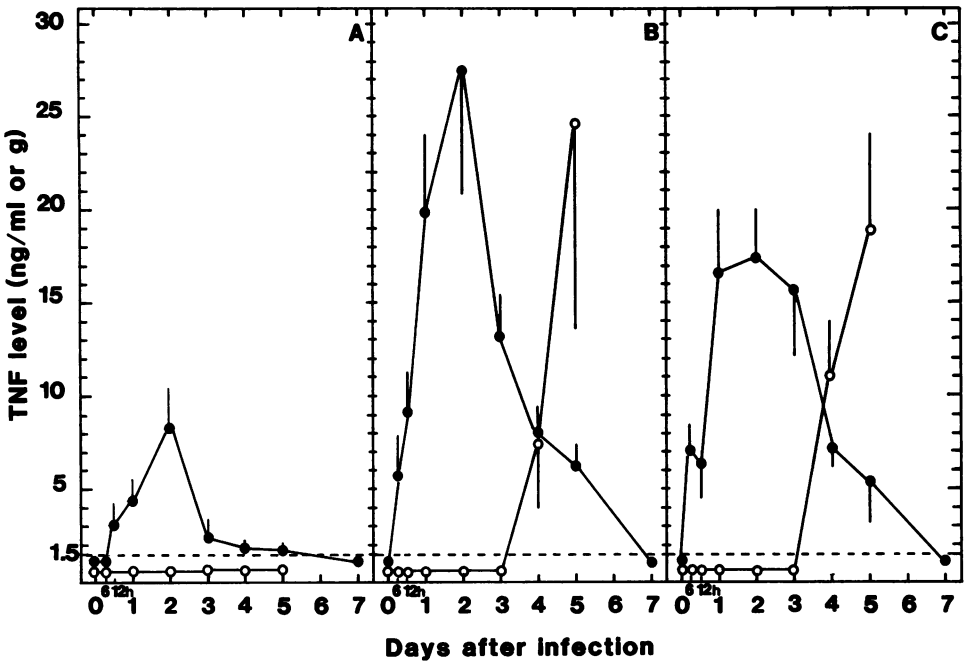

FIG. 4. Same as Fig. 3, except that production of exogenous TNF-α is illustrated. The dashed line indicates the lower limit of detection (1.5 ng/ml or g).

then declined gradually to become undetectable by postinfection day 7 (Fig. 4).

Passive immunity with anti-V-antigen. A refined lot of rabbit monospecific polyclonal anti-V-antigen was prepared as described in Materials and Methods and compared (Fig. 5) with known protective mouse monoclonal 15F anti-V-antigen (78) for specificity and ability to provide passive immunity in mice. This preparation was monospecific as judged by immunoblotting with restricted Lcr$^+$ cells of *Y. pestis* and enteropathogenic yersiniae (data not shown). It was more effective than the protective monoclonal antibody in providing passive immunity against *Y. pestis* and enteropathogenic yersiniae (Table 2).

Cytokines after passive immunization. The pattern of cytokine production in mice passively immunized with anti-V-antigen and challenged with Lcr$^+$ yersiniae resembled that which occurred in normal mice challenged with Lcr$^-$ organisms. In the latter case, production of endogenous IFN-γ was immediate, and typical biphasic induction (74) occurred in the spleen, with appearance of the first peak 12 h after challenge and the second on postinfection days 3 to 4 (Fig. 6). Passive immunization with rabbit monospecific polyclonal anti-V-antigen after challenge with Lcr$^+$ cells also prompted significant and sustained production of IFN-γ. TNF-α in spleens of mice infected with Lcr$^-$ organisms was detectable after 6 h and peaked at 12 h; a gradual decline occurred thereafter until the cytokine became undetectable on postinfection day 5 (Fig. 7). Treatment of mice with the polyclonal antiserum after challenge with Lcr$^+$ yersiniae permitted marked synthesis of endogenous TNF-α beginning 1 day after immunization and peaking on postinfection day 3. Similar results were obtained in the liver (data not shown) and in both the spleen and the liver upon use of the protective mouse monoclonal 15F V-antigen antibody (data not shown).

DISCUSSION

It is established that extracellular procaryote parasites typically cause purulent infections, are controlled primarily by humoral immune mechanisms, and are rapidly killed after uptake by professional phagocytes. In contrast, generalized facultative intracellular parasites commonly cause granulomatous disease, are frequently vulnerable to processes of cell-mediated immunity, and are capable of growth within professional phagocytes (11, 25). The latter group contains yersiniae, as judged by their previously noted potential to promote granulomatous lesions (65, 73) and ability to survive within professional phagocytes (17, 30, 66, 69, 70). However, both humoral and cellular processes of immunity can operate against wild-type cells of *Y. pestis*. This relationship is illustrated by the proven effectiveness of antibodies directed against certain virulence factors, including V antigen (33, 59, 72, 73), in protecting against experimental plague. Similarly, a T-lymphocyte-mediated process prompted by exposure to Lcr$^-$ yersiniae provided equally effective immunity (77).

The ability of a host to form protective granulomas is the hallmark of delayed hypersensitivity (67). Results of recent studies have demonstrated that the granulomatous response characteristic of this state of cellular immunity is mediated by cytokines, especially IFN-γ and TNF-α (31, 42). Findings provided in this report illustrate that production of these cytokines in mice infected with Lcr$^+$ but not Lcr$^-$ yersiniae was inhibited unless the animals received sufficient anti-V-antigen to provide passive immunity. This evident form of immunosuppression may thus account for the ability of Lcr$^+$ cells of *Y. pestis* to form necrotic lesions in organs without prompting infiltration of inflammatory cells (65, 73). Since Lcr$^+$ organisms do promote protective granulomas similar to those caused by Lcr$^-$ yersiniae after passive immunization

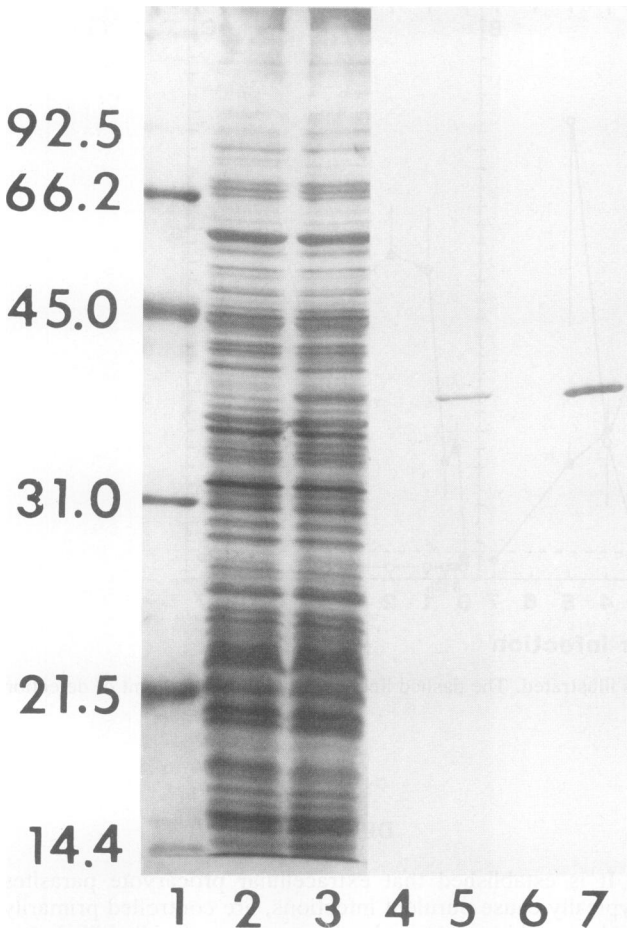

FIG. 5. Specificity of antisera used to provide passive immunity and restore ability to express cytokines in mice challenged with Lcr^+ cells of *Y. pestis* KIM. Lanes: 1, silver-stained molecular weight standards (10^3); 2, silver-stained Ca^{2+}-starved Lcr^- cells of *Y. pestis* grown at 37°C; 3, silver-stained Ca^{2+}-starved Lcr^+ cells of *Y. pestis* grown at 37°C; 4, Ca^{2+}-starved Lcr^- cells of *Y. pestis* grown at 37°C immunoblotted with mouse monoclonal 15F anti-V-antigen; 5, Ca^{2+}-starved Lcr^+ cells of *Y. pestis* grown at 37°C immunoblotted with mouse monoclonal 15F anti-V-antigen; 6, Ca^{2+}-starved Lcr^- cells of *Y. pestis* grown at 37°C immunoblotted with rabbit polyclonal anti-V-antigen; 7, Ca^{2+}-starved Lcr^+ cells of *Y. pestis* grown at 37°C immunoblotted with rabbit polyclonal anti-V-antigen. V antigen is detectable as a major silver-stained peptide with a molecular weight of 38,000 (lane 3).

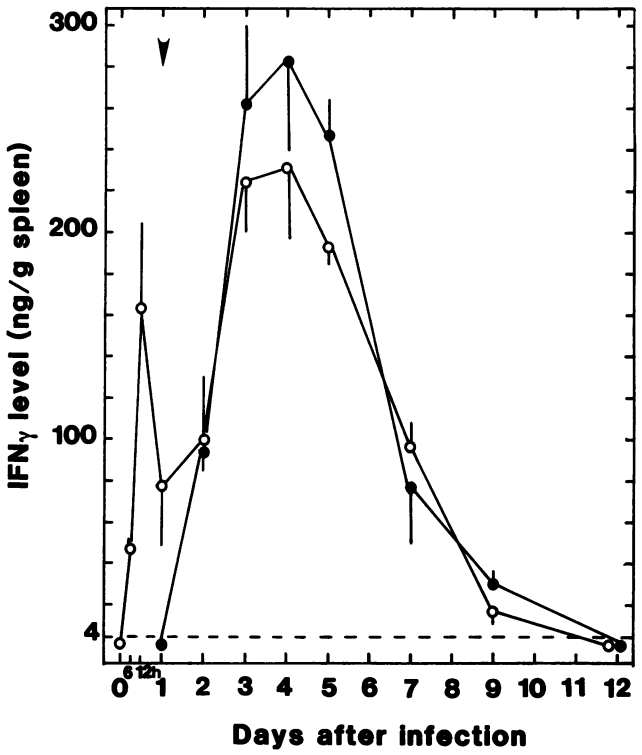

FIG. 6. Expression of endogenous IFN-γ in spleens of mice after intravenous challenge with 10^6 Lcr^- cells of *Y. pestis* KIM (○) or 10 MLD of isogenic Lcr^+ cells (●) treated on postinfection day 1 with rabbit monospecific polyclonal anti-V-antigen (100 μg per mouse). IFN-γ was assayed as described in the text. Symbols and vertical bars represent the means and standard deviations, respectively, of groups of three mice; the dashed line shows the lower detection limit (4.0 ng/g of spleen).

with anti-V-antigen (73), it is tempting to assume that V antigen per se suppresses production of cytokines.

Although this may very well be the case, the available data are hardly sufficient to warrant the conclusion that V antigen serves as the sole immunosuppressor in question. The possibility exists, for example, that some contaminating antibody directed against an undetectable antigen accounts for protection. This possibility was minimized by demonstrating that administration of protective monoclonal antibody 15F, directed against V antigen, permitted enhanced synthesis of cytokines. Nevertheless, the epitope recognized by this antibody might be shared by some additional antigen actu-

TABLE 2. Ability of refined lots of monospecific rabbit polyclonal anti-V-antigen and mouse monoclonal 15F anti-V-antigen to provide passive immunity against intravenous infection with 10 MLD of Lcr^+ cells of yersiniae

Challenge organism[a]	No. of survivors/total no. infected (%)			
	Normal gamma globulin control[b]	Rabbit polyclonal anti-V-antigen[c]	Rabbit polyclonal anti-V-antigen[b]	Mouse monoclonal 15F anti-V-antigen[b]
Y. pestis KIM	0/8 (0)	8/8 (100)	8/8 (100)	6/8 (75)
Y. pseudotuberculosis PB1/+	0/10 (0)		7/8 (88)	3/8 (38)
Y. enterocolitica WA	0/10 (0)		6/8 (75)	0/8 (0)

[a] Doses were 10^2, 10^2, and 10^3 bacteria for *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, respectively; the determination was terminated after 21 days.
[b] Treatment on postinfection days 1, 3, and 5 as described in the text.
[c] Treatment on postinfection day 1 only as described in the text.

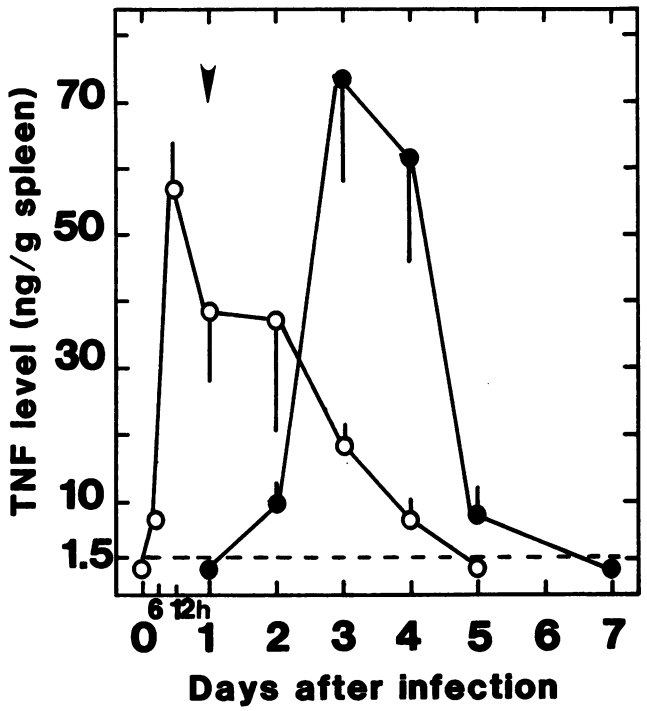

FIG. 7. Same as Fig. 6, except that production of endogenous TNF-α is illustrated. The dashed line shows the lower detection limit (1.5 ng/g of spleen).

ally responsible for immunosuppression. Presumably, a period of exposure to lipopolysaccharide and other surface components of Lcr$^-$ bacteria was necessary to induce the marked production of cytokines observed in these studies. In this case, any effective therapeutic regimen including antibiotic chemotherapy or passive immunization might indirectly result in release of the same inducers from killed Lcr$^+$ organisms with subsequent appearance of cytokines. Virulence factors in addition to V antigen may, of course, be required for the observed repression of cytokine synthesis. Likely prospects for this role obviously include those Yops known to function as determinants of disease (8, 35, 55–57, 63, 65).

The finding that Lcr$^+$ cells of *Y. pestis* can suppress expression of IFN-γ and TNF-α is an important new finding. This phenomenon probably accounts for the inability of the host to contain Lcr$^+$ yersiniae within protective granulomas as does occur in the case of Lcr$^-$ mutants (65, 73). This immunosuppressive effect may be distinct from the antiphagocytic roles attributed to the known biological activities of the essential Yops noted above. The possibility that V antigen serves as a component of the process that mediates this form of cytokine suppression is under active study. We hope that the mechanism will prove to be sufficiently straightforward so that the activity can be harnessed and utilized for controlled immunosuppression and investigation of the normal immune response.

ACKNOWLEDGMENTS

This study was supported by U.S. Public Health Service grant AI 19353 from the National Institute of Allergy and Infectious Diseases and by a generous gift from the Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan.

The excellent technical assistance of Janet M. Fowler is acknowledged, as is the valuable support and advice provided by Yasuaki Osada and Tsutomu Une.

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