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Suppression of Cytokines in Mice by Protein A-V Antigen Fusion Peptide and Restoration of Synthesis by Active Immunization

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It is established that an ~70-kb Lcr plasmid enables *Yersinia pestis*, the causative agent of bubonic plague, to multiply in focal necrotic lesions within visceral organs of mice by preventing net synthesis of the cytokines tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ), thereby minimizing inflammation (Lcr^+). Rabbit antiserum raised against cloned staphylococcal protein A-V antigen fusion peptide (PAV) is known to passively immunize mice against 10 minimum lethal doses of intravenously injected Lcr^+ cells of *Y. pestis*. In this study, injected PAV suppressed TNF-α and IFN-γ in mice challenged with avirulent V antigen-deficient *Y. pestis* (*lcrV* or Lcr^-) and promoted survival in vivo of these isolates as well as salmonellae and *Listeria monocytogenes* (with which the outcome was lethal). Active immunization of mice with PAV protected against 1,000 minimum lethal doses of intravenously injected Lcr^+ cells of *Y. pestis* and *Yersinia pseudotuberculosis* but not *Yersinia enterocolitica*. The progressive necrosis provoked by Lcr^+ cells of *Y. pestis* in visceral organs of nonimmunized mice was replaced after active immunization with PAV by massive infiltration of neutrophils and mononuclear cells (which generated protective granulomas indistinguishable from those formed against avirulent Lcr^- mutants in nonimmunized mice). Distinct multiple abscesses typical of Lcr^+ cells of *Y. pseudotuberculosis* were prevented by similar immunization. Significant synthesis of TNF-α and IFN-γ occurred in spleens of mice actively immunized with PAV after challenge with Lcr^+ cells of *Y. pestis*. These findings suggest that V antigen contributes to disease by suppressing the normal inflammatory response.

Yersinia pestis, the causative agent of bubonic plague, and enteropathogenic *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* cause disease in mice due, in part, to expression of major virulence factors encoded on a shared ~70-kb low-calcium response (25), or Lcr, plasmid (9, 43, 45, 46). These determinants include cytotoxic enzymes (e.g., YopE, YopH, and YpkA) (6, 24, 38–40, 46) delivered via YopB and YopD upon bacterium-host cell contact (20, 26, 41) and an excreted peptide termed V antigen (LcrV) (8–10, 12, 30, 33). Simulation of host cell cytoplasm with respect to temperature (37°C), Ca^{2+} (≤1.0 mM), and Mg^{2+} (~20 mM) (29) promotes full induction of Yops (6, 31, 32, 36, 42) as well as V antigen (11, 30, 50). However, addition of the extracellular cation Na^+ to this environment, especially as the L-glutamate salt (21), causes bacteriostasis (27) due to loss of adenylate energy charge (51) with attendant stepdown of stable RNA transcription (18) and shutoff of vegetative protein synthesis (31, 32) (Lcr^+). Mutational loss of the whole Lcr plasmid (Lcr^-), certain resident regulatory genes, or the structural genes encoding the virulence factors noted above causes outright avirulence (mouse 50% lethal dose, $>10^7$ bacteria) (9, 45, 46).

As first shown by Une et al. (49), necrotic focal centers within visceral organs containing released host cell cytoplasm constitute the major niche colonized by Lcr^+ cells of *Y. pestis* in the mouse. A single bacterium can initiate a lesion of this type which progressively increases in size and eventually coalesces with similar neighboring foci, resulting in ultimate loss of organ function. At a time corresponding to the onset of clinical morbidity, bacteria spill from such fully developed lesions into the vascular system (34, 44, 47–49), where they become available for consumption by the flea vector. In contrast, infectious centers formed in visceral organs by Lcr^- mutants of *Y. pestis* rapidly attract inflammatory cells that promote formation of protective granulomas (49). These findings have been verified (44) and extended to *Y. pseudotuberculosis*, which caused formation of abscesses rather than necrotic focal lesions (49); analogous results for *Y. enterocolitica* were reported independently by Autenrieth et al. (2). The Lcr plasmid thus both promotes cytotoxicity and inhibits inflammatory-cell chemotaxis, preventing formation of protective granulomas (9).

A possible basis for this anti-inflammatory effect was provided by demonstrating that mice infected with Lcr^+ yersiniae failed to express the cytokines gamma interferon (IFN-γ) or tumor necrosis factor alpha (TNF-α) during the initial period required for the occurrence of massive bacterial growth within necrotic lesions of visceral organs (34). Furthermore, injection of TNF-α plus IFN-γ was found to provide concentration-dependent protection against infection with 10 minimal lethal doses (MLD) of Lcr^+ cells of *Y. pestis* (34). Autenrieth et al. (1) independently reported that removal of endogenous IFN-γ enhanced the virulence of Lcr^+ cells of *Y. enterocolitica*. Injection of mice with rabbit polyclonal anti-V antigen provided similar protection against 10 MLD of *Y. pestis* and permitted normal synthesis of TNF-α and IFN-γ, suggesting that suppression of these cytokines was promoted by V antigen per se (34), although Beuscher et al. (5) recently attributed cytokine suppression to YopB. To further define this relationship, we constructed a fusion between the structural gene of staphylococcal protein A (PA) present on the vector plasmid pRIT5 and that of V antigen (*lcrV*) obtained from the Lcr plasmid of *Y. pseudotuberculosis* (33). The resulting PA-V antigen fusion

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peptide (PAV), encoded on pPAV13 carried by protease-deficient *Escherichia coli* BL21, contained 305 N-terminal amino acids from PA plus 259 C-terminal amino acids from V antigen and could be purified to homogeneity in one step by immunoglobulin G affinity chromatography. Rabbit antibodies raised against one or more epitopes present within an internal portion of the V antigen domain of PAV (amino acids 168 to 275) accounted for protection against experimental plague.

We showed in the present study that injected PAV but not PA markedly suppressed TNF-α and IFN-γ normally induced upon infection of control mice with avirulent *lcrV* or Lcr$^-$ mutants of *Y. pestis* and promoted in vivo survival of these isolates as well as salmonellae and *Listeria monocytogenes*. Furthermore, active immunization of mice with PAV but not PA provided protection against intravenous challenge with 1,000 MLD of *Y. pestis* while permitting normal production of TNF-α and IFN-γ. This restoration of the ability to express cytokines was correlated with a rapid and effective inflammatory response that culminated in formation of protective granulomas.

(Portions of this research were presented at the Sixth International Symposium on *Yersinia*, Rome, Italy, 26 to 28 September 1994.)

MATERIALS AND METHODS

Bacteria. Conditionally virulent Lcr$^+$ but nonpigmented *Y. pestis* KIM (7), which is virulent in mice when administered by intravenous injection (50% lethal dose, ~10 bacteria [47]) but avirulent when administered by peripheral routes (50% lethal dose, $>10^7$ bacteria [28]), and its isogenic avirulent Lcr$^-$ mutant (intravenous 50% lethal dose, $>10^7$ bacteria [7]), which is known to be cured of the Lcr plasmid (19), were used for challenge, determination of histopathological changes, and analysis of cytokine expression. Also utilized for these purposes were Lcr$^+$ and Lcr$^-$ isolates of *Y. pseudotuberculosis* PB1 (15) and *Y. enterocolitica* WA (16), as well as *Salmonella typhimurium* W112 and *L. monocytogenes* EGD (obtained from the Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan). A nonpolar *lcrV* mutant of *Y. pestis* KIM (37) was received through the courtesy of Susan C. Straley and utilized in experiments concerned with suppression of endogenous cytokines by PAV.

Cultivation. Bacteria were stored at −20°C in buffered glycerol as previously described (3), directly inoculated onto slopes of tryptose blood agar base (Difco Laboratories, Detroit, Mich.) (for yersiniae and salmonellae) or 5% rabbit tryptose blood agar (for *L. monocytogenes*), and incubated at 26°C for 1 day (for enteropathogenic yersiniae) or 2 days (for *Y. pestis* and *L. monocytogenes*). The organisms were then suspended in sterile 0.033 M potassium phosphate buffer, pH 7.0 (phosphate buffer), and appropriately diluted in the same buffer for use as inocula for challenge.

Mice. Female Swiss-Webster mice (Charles River Laboratories, Wilmington, Mass.) 5 to 6 weeks of age were used throughout this study. The animals received tap water and commercial food ad libitum in a room controlled at 18.5°C and 35% humidity.

Determination of IFN-γ and TNF-α levels. Cytokines were assayed by use of an established process (35) utilized previously (34). Briefly, organs of sacrificed infected animals were either pooled or homogenized individually in Hanks balanced salt solution (GIBCO BRL, Gaithersburg, Md.) containing 1% (wt/vol) 3-[(3-cholamidopropyl)-diamethylammonio]-propanesulfonate (Sigma Chemical Co., St. Louis, Mo.) (100 μg/ml). After extraction of cytokines for 1 h at 4°C, the homogenates were centrifuged (10,000 × *g* for 30 min) and supernatants were preserved at −70°C until assay. Levels of TNF-α and IFN-γ were determined with the mouse Factor-TEST kit (Genzyme Corp., Boston, Mass.) and mouse InterTEST-gamma kit (Genzyme), respectively. The limits of detection of IFN-γ and TNF-α were <4.0 and <1.5 ng/g of organ or ml of blood, respectively.

Suppression of cytokine production by PAV. The plasmids pPAV13 and pPRIT5, encoding PAV and PA, respectively, were previously described, as were the expression of these peptides in *E. coli* BL21 and their purification to homogeneity by affinity chromatography, a process that ensured removal of significant lipopolysaccharide (33). Normal mice were challenged intravenously with 10^6 *lcrV* or Lcr$^-$ cells of *Y. pestis* and then treated for 6 days, starting on postinfection day 0, by intravenous injection of 0.1 ml of phosphate buffer alone or the same volume of phosphate buffer containing 100 μg of PA or PAV. Spleens and livers from four mice were pooled at intervals and processed for the determination of cytokine levels as described above.

Active immunization. Homogeneous PA or PAV was diluted in phosphate buffer to 2 mg/ml and emulsified separately with an equal volume of the adjuvant TiterMax (Hunter's TiterMax R-1; CytRx Corp., Norcross, Ga.). Mice received a primary immunization on day 0 consisting of 25 μl of emulsion (containing 25 μg of PA or PAV) by both subcutaneous and intraperitoneal routes of injection (50 μg per mouse), and then identical booster immunizations were given on days 21 and 35. Adjuvant emulsified with an equal volume of phosphate buffer alone was also used as a negative control.

Serum antibody titration. Blood samples obtained during the course of immunization were stored overnight at 4°C prior to centrifugation at 10,000 × *g* for 30 min; after decanting, the resulting sera were preserved at −70°C before assay for anti-V antigen. An enzyme-linked immunosorbant assay (ELISA) utilizing the multiple-antibody-sandwich principle was employed for titration of serum antibodies specific to V antigen. Wells of ELISA plates (Corning, Inc., Corning, N.Y.) were coated with rabbit immunoglobulin G (10 μg/ml) prepared from anti-PAV absorbed as described previously with a disrupted suspension of *E. coli* BL21(DE3)/pBluescript SK$^+$ (33). The wells were washed extensively with borate-buffered saline (0.17 M H_3BO_4 and 0.12 M NaCl adjusted to pH 8.5 with NaOH) and then treated with blocking solution (0.25% bovine serum albumin) (Calbiochem, La Jolla, Calif.) to prevent nonspecific binding of immunoglobulins. Highly purified cloned V antigen from *E. coli* BL21(pKVE14) (33) was then added to each well at a concentration of 100 ng/ml. After incubation for 2 h at 26°C, the plates were washed with borate-buffered saline, and then experimental mouse serum diluted 1:1,000 with blocking solution was added to each well. The plates were again incubated for 2 h at 26°C, and the presence of antibodies recognizing V antigen was detected with anti-mouse immunoglobulin G labeled with alkaline phosphatase (Sigma) by reaction with 3 mM *p*-nitrophenyl phosphate (Sigma). The specific colormetric intensity was read at 405 nm and yielded a maximum value of ~0.6 on week 6.

Challenge and survival. Mice were challenged intravenously 14 days after the last immunization with 100 Lcr$^+$ cells of *Y. pestis*, 100 Lcr$^+$ cells of *Y. pseudotuberculosis*, or 1,000 Lcr$^+$ cells of *Y. enterocolitica*; these values are approximately 10 MLD (48, 49). This dose was increased in later determinations designed to define the limits of protection or patterns of growth in vivo. Challenged mice were observed daily for 3 weeks to ascertain the mean survival time. Groups of four infected immunized or nonimmunized control mice were sacrificed at periodic intervals by terminal bleeding under anesthesia prior to aseptic removal of the spleen, liver, and lung. Blood was mixed immediately with heparin (5 U/ml) to prevent clotting, and organs were weighed and homogenized separately with phosphate buffer at a concentration of 100 mg/ml. These preparations were appropriately diluted in the same buffer, and samples of 0.1 ml were spread in triplicate on the surface of tryptose blood agar base in petri dishes. Resulting colonies were counted after incubation at 26°C for 1 day (for enteropathogenic yersiniae) or 2 days (for *Y. pestis*). The lower limit of detection by this process was 10 bacteria per ml of blood or 100 bacteria per g of organ.

Release of cytokine suppression by active immunization with PAV. Mice receiving adjuvant alone or those actively immunized with PA or PAV were challenged with 10 MLD of Lcr$^+$ cells of *Y. pestis*. Spleens from four mice of each group were individually processed at intervals and analyzed for IFN-γ and TNF-α as described above.

PAV-mediated survival of bacteria in vivo. Normal mice were challenged with 1×10^6 *lcrV* or Lcr$^-$ cells of *Y. pestis*, 1×10^3 cells of *L. monocytogenes*, or 5×10^4 cells of *S. typhimurium* and then treated by intravenous injection on postinfection days 0 through 6 with 0.1 ml of phosphate buffer alone or the same volume of phosphate buffer containing 100 μg of PA or PAV. At intervals, the numbers of bacteria in individual spleens of four mice were determined as described above; numbers of listeriae were estimated on blood agar.

Passive immunization. The procedures used for raising anti-PA and anti-PAV in rabbits and the utilization of purified immunoglobulin G prepared from the latter antisera for passive immunization of mice have been described previously (33, 34, 48).

Histopathology. Immunized and nonimmunized control mice were sacrificed at periodic intervals after infection, and spleens, livers, and lungs were removed and fixed in 10% formalin–phosphate-buffered saline for 7 days at room temperature for preparation of thin sections. The tissues were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

RESULTS

Suppression of cytokines by PAV. Normal mice received 10^6 avirulent Lcr$^-$ cells of *Y. pestis* (lacking the Lcr plasmid) by intravenous injection to induce synthesis of IFN-γ and TNF-α as defined previously (34) and were then treated with increasing doses of PA or PAV. As shown in Fig. 1, administration of PA did not significantly reduce the concentration of IFN-γ or TNF-α generated 24 h after challenge, whereas PAV promoted dose-dependent suppression of both cytokines. Identical results were obtained by similar injection of 10^6 *lcrV* mutant cells (unable to synthesize V antigen) to induce cytokine synthesis (not illustrated). Following challenge with 10^6 *lcrV* yersiniae, the extent of suppression was monitored for 10 days

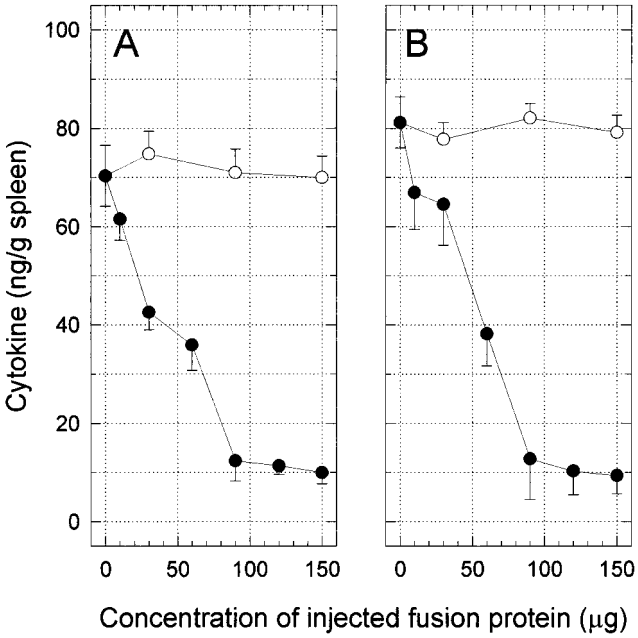

FIG. 1. Levels of TNF-α (A) and IFN-γ (B) in spleens of normal mice induced 24 h previously by intravenous injection of 10^6 Lcr$^-$ cells of *Y. pestis* KIM followed 30 min later by intravenous injection of the indicated concentrations of truncated PA (○) or PAV (●). Values represent means and standard deviations for four mice.

in mice receiving daily injections of 100 μg of PA or PAV on postinduction days 0 through 6 (Fig. 2). PAV but not PA promoted marked suppression of IFN-γ and TNF-α during this period, although the levels of both cytokines eventually rose after treatment was terminated; repetition of this experiment with Lcr$^-$ mutants yielded identical results (not illustrated). These observations indicate that the V antigen but not the PA domain of PAV can suppress the ability of mice to generate IFN-γ and TNF-α in response to infection by Lcr$^-$ or *lcrV* yersiniae.

PAV-mediated survival of bacteria in vivo. To ascertain if the suppression defined above favored survival in vivo, we determined the number of viable *lcrV* yersiniae present in spleens of infected normal mice during treatment with phosphate buffer, PA, or PAV. As shown in Fig. 3A, viability fell precipitously except in the case of PAV, which promoted significant survival. Identical results were obtained when this experiment was repeated with Lcr$^-$ cells of *Y. pestis* (not illustrated). To determine if this effect was limited to yersiniae, mice were infected with *L. monocytogenes* or *S. typhimurium* and viability in the spleen was similarly monitored during treatment with phosphate buffer, PA, or PAV. Growth of *L. monocytogenes* was modest and self-limiting in mice receiving PA (Fig. 3B); an identical pattern was observed during treatment with phosphate buffer alone (not illustrated). In contrast, unrestrained proliferation of listeriae occurred during treatment with PAV, resulting in death between postinfection days 6 and 9. Growth of *S. typhimurium* in mice treated with PAV (Fig. 3C) was enhanced to a lesser extent over that obtained with PA, which yielded a pattern identical to that observed for phosphate buffer alone (not illustrated).

Production of anti-V antigen in mice. During immunization of mice with PAV, serum antibodies directed against highly purified recombinant V antigen (33) became evident by week 4 and achieved a maximum titer (optical density of ~0.6) by week 6. An identical regimen with PA did not raise detectable anti-V antigen.

Active immunization with PAV. Mice were challenged intravenously with Lcr$^+$ (wild-type) yersiniae after completing the schedule of active immunization described above. PAV but not PA provided absolute protection against 10 MLD of *Y. pestis* and *Y. pseudotuberculosis* but was ineffective against *Y. entero-*

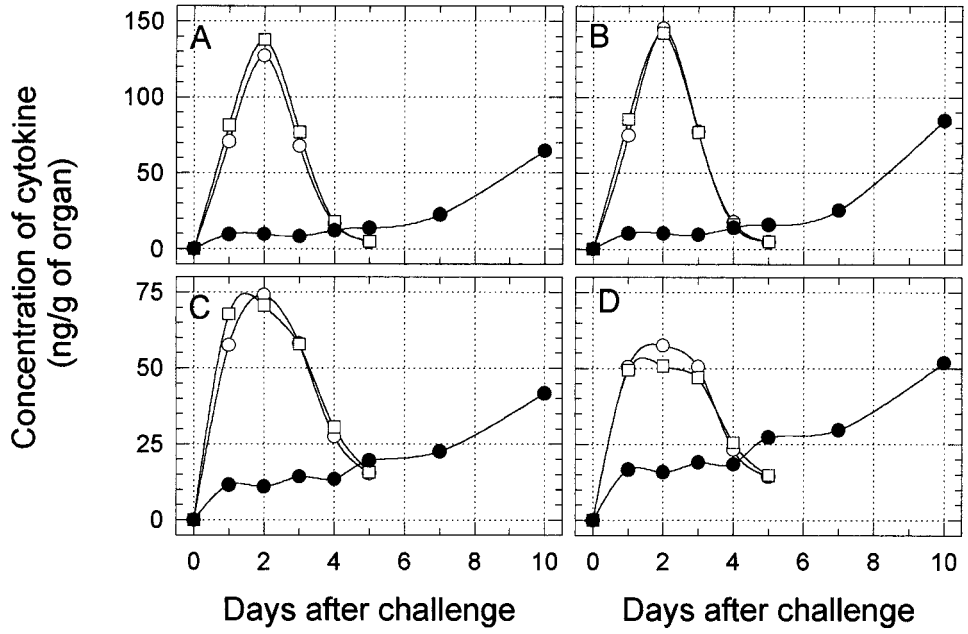

FIG. 2. Levels of IFN-γ in spleens (A) and livers (B) or of TNF-α in spleens (C) and livers (D) of normal mice following induction of cytokine synthesis on day 0 by intravenous injection of 10^6 *lcrV* cells of *Y. pestis* KIM. Mice received daily intravenous injections of 0.1 ml of 0.033 M potassium phosphate buffer, pH 7.0 (□), or 100 μg of either truncated PA (○) or PAV (●) in 0.1 ml of the same buffer on postinduction days 0 through 5. Values represent averages determined for four mice.

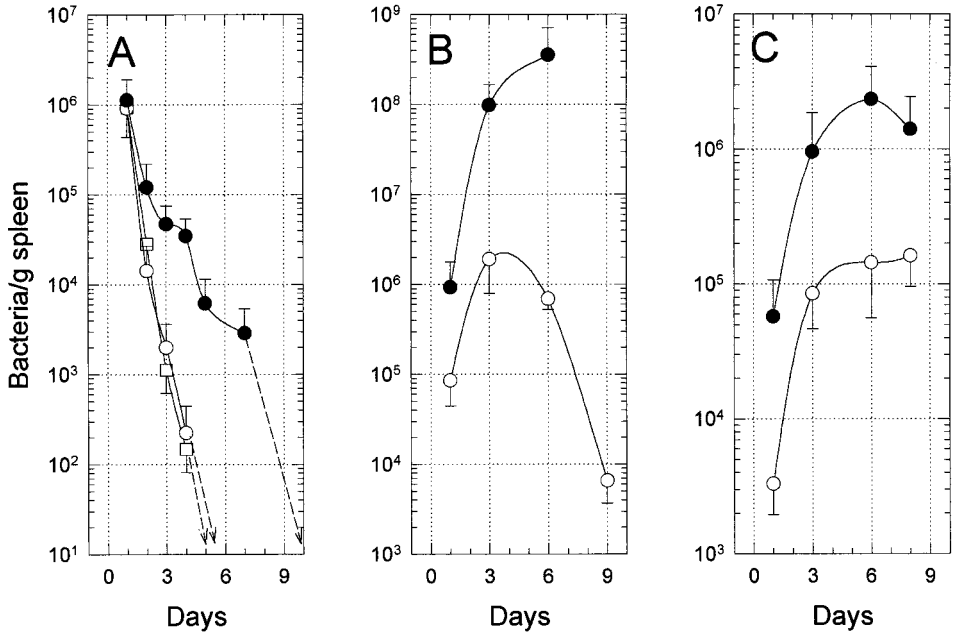

FIG. 3. Viabilities of 1×10^6 intravenously injected *lcrV* cells of *Y. pestis* KIM (A), 1×10^3 intravenously injected cells of wild-type *L. monocytogenes* EGD (B), or 5×10^4 intravenously injected cells of wild-type *S. typhimurium* W112 (C) in spleens of normal mice that received daily intravenous injections of 0.1 ml of 0.033 M potassium phosphate buffer, pH 7.0 (□), or 100 μg of either truncated PA (○) or PAV (●) in 0.1 ml of the same buffer on postinduction days 0 through 5. Values represent the means and standard deviations for four mice (all mice receiving *L. monocytogenes* plus PAV died between postinfection days 6 and 9). Note the differences in the scales of the *y* axes.

colitica (Table 1). Active immunity against *Y. pestis* (Table 1) and *Y. pseudotuberculosis* (not illustrated) extended to 1,000 MLD (10^4 bacteria) but not to 10,000 MLD.

Growth in normal and immunized mice. The multiplication of Lcr^+ cells of *Y. pestis* in blood and organs of mice immunized with PA was determined. After intravenous injection of 1,000 MLD, prompt logarithmic growth commenced in the liver (Fig. 4B), lung (Fig. 4C), and spleen (Fig. 4D), with significant spillover into blood on postinfection day 3 (Fig. 4A) and death by postinfection day 6. Identical results were observed for nonimmunized control mice (not illustrated) (34, 47–49). Similar challenge of mice actively immunized with PAV resulted in the removal, without a further net increase in number, of *Y. pestis* from the vascular system by the liver (Fig. 4B) and spleen (Fig. 4D); colonization of the lung or reappearance of bacteria in the vascular system was not detected.

TABLE 1. Abilities of adjuvant alone, PA, and PAV to provide to mice active immunity against intravenously injected Lcr^+ cells of *Y. pestis* KIM, *Y. pseudotuberculosis* PB1, and *Y. enterocolitica* WA

Expt	Species	Injected bacteria		Vaccine[a]	No. of mice surviving on postinfection day:															No. dead/ total no.
		No.	MLD		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	21	
1	*Y. pestis*	10^2	10^1	None	10	10	10	7	3	2	2	0	0	0	0	0	0	0	0	0	10/10
		10^2	10^1	PA	10	10	10	6	3	3	2	2	0	0	0	0	0	0	0	0	10/10
		10^2	10^1	PAV	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	0/10[b]
	Y. pseudotuberculosis	10^2	10^1	None	10	10	10	9	7	7	4	0	0	0	0	0	0	0	0	0	10/10
		10^2	10^1	PA	10	10	10	10	7	7	5	0	0	0	0	0	0	0	0	0	10/10
		10^2	10^1	PAV	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	0/10[b]
	Y. enterocolitica	10^3	10^1	None	8	8	8	8	8	5	5	2	2	0	0	0	0	0	0	0	8/8
		10^3	10^1	PA	10	10	10	10	10	10	5	2	2	0	0	0	0	0	0	0	10/10
		10^3	10^1	PAV	8	8	8	8	8	8	5	5	5	4	4	4	3	3	3	2	6/8[c]
2	*Y. pestis*	10^2	10^1	None	5	5	5	4	1	1	0	0	0	0	0	0	0	0	0	0	5/5
		10^2	10^1	PA	5	5	5	4	2	2	1	0	0	0	0	0	0	0	0	0	5/5
		10^2	10^1	PAV	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	0/5[b]
		10^3	10^2	PAV	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	0/5[b]
		10^4	10^3	PAV	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	0/5[b]
		10^5	10^4	PAV	5	5	5	5	4	2	1	0	0	0	0	0	0	0	0	0	5/5

[a] None indicates immunization with adjuvant alone; PA and PAV were provided as vaccines in the same adjuvant.
[b] $P < 0.005$ by Fisher's exact test.
[c] Not statistically significant.

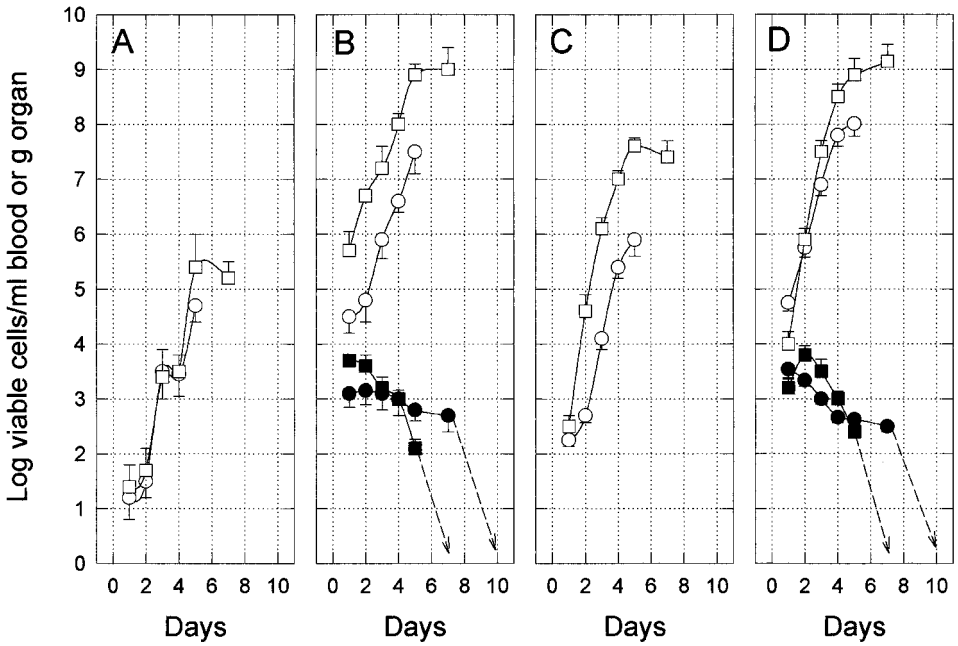

FIG. 4. Log viable bacteria per milliliter of blood (A) or per gram of liver (B), lung (C), or spleen (D) after challenge with 10^4 Lcr^+ cells of *Y. pestis* KIM of mice actively immunized with control PA (○) or PAV (●) or after challenge with 10^4 Lcr^+ cells of *Y. pseudotuberculosis* PB1 of mice actively immunized with control PA (□) or PAV (■). Dashed arrows show times when levels of surviving bacteria became undetectable. Individual values and vertical bars represent the means and standard deviations obtained for four mice. Yersiniae were never observed in blood or lungs of mice actively immunized with PAV; differences in the bacterial burden for comparable points are always significant by Student's *t* test.

These findings were essentially identical to those obtained for Lcr^+ cells of *Y. pseudotuberculosis* (Fig. 4) except that the latter bacteria achieved a higher density in blood and organs of normal mice before death and were eliminated more rapidly from mice actively immunized with PAV.

Histopathological findings. Intravenous injection of *Y. pestis* into nonimmunized control mice or those immunized with PA resulted in severe damage to the liver (Fig. 5A) and spleen (not illustrated) by postinfection day 3. Infiltration of inflammatory cells to these necrotic foci was never observed, underscoring the acute nature of the infection. In contrast, lesions formed in organs of mice immunized with PAV attracted massive numbers of neutrophils and mononuclear cells, resulting in their conversion to protective granulomas by postinfection day 3 (Fig. 5C). These granulomas closely resembled those formed in response to injection of Lcr^- cells into normal control mice (Fig. 5B) or of Lcr^+ cells into mice passively immunized with anti-PAV (Fig. 5D).

Injection of Lcr^+ cells of *Y. pseudotuberculosis* into mice immunized with PA caused formation of multiple abscesses in the liver, spleen, and lung by postinfection day 3; these abscesses were less severe than the necrotic foci generated by *Y. pestis*. For example, lesions formed in the liver showed marked infiltration of neutrophils surrounding central areas of bacterial colonization (Fig. 6A). In marked contrast, corresponding lesions in livers of mice immunized with PAV exhibited only slight infiltration of professional phagocytes and no detectable colonization (Fig. 6C). This modest change closely resembled that observed on postinfection day 3 in livers of mice passively immunized with anti-PAV (Fig. 6D) or in livers of normal mice challenged with Lcr^- cells of *Y. pseudotuberculosis* (Fig. 6B). These studies are consistent with the hypothesis that anti-V antigen abets infiltration of inflammatory cells to areas of bacterial colonization and that these cells subsequently effect formation of protective granulomas (34).

Cytokine production after active immunization. The levels of endogenous IFN-γ and TNF-α in spleens of outbred mice actively immunized with either PA or PAV were determined following intravenous challenge with 10 MLD of Lcr^+ cells of *Y. pestis* (Table 2). A significant amount of IFN-γ was not detected after immunization with PA, although TNF-α accumulated immediately prior to death. Abrupt synthesis of IFN-γ and TNF-α occurred on postinfection day 1 in mice immunized with PAV, and significant levels of both cytokines were maintained until postinfection day 4 (at which time formation of protective granulomas had been completed).

DISCUSSION

The enigma of V antigen is that its mutational loss results in both avirulence and removal of the temperature-dependent nutritional requirement for Ca^{2+} that is unique to Lcr^+ yersiniae (4, 37). Furthermore, nonpolar *lcrV* cells of *Y. pestis* were reported to produce a significantly lower level of Yops in Ca^{2+}-deficient medium than did those of the Lcr^+ parent (37), an observation that we have now amply confirmed (22). The discovery of this phenotype logically prompted the assignment of regulatory functions to V antigen and endorsed the hypothesis that avirulence of *lcrV* organisms represents a reduced ability to synthesize essential Yops rather than loss of V antigen per se (4, 37). This interpretation, however, is inconsistent with two circumstances. First, despite intensive study in many laboratories on regulation of the low-calcium response, no evidence has yet been reported to show that V antigen functions directly as a transcriptional regulator of Yops or of vegetative protein. Second, a considerable literature has accumulated which indicates that antibodies directed

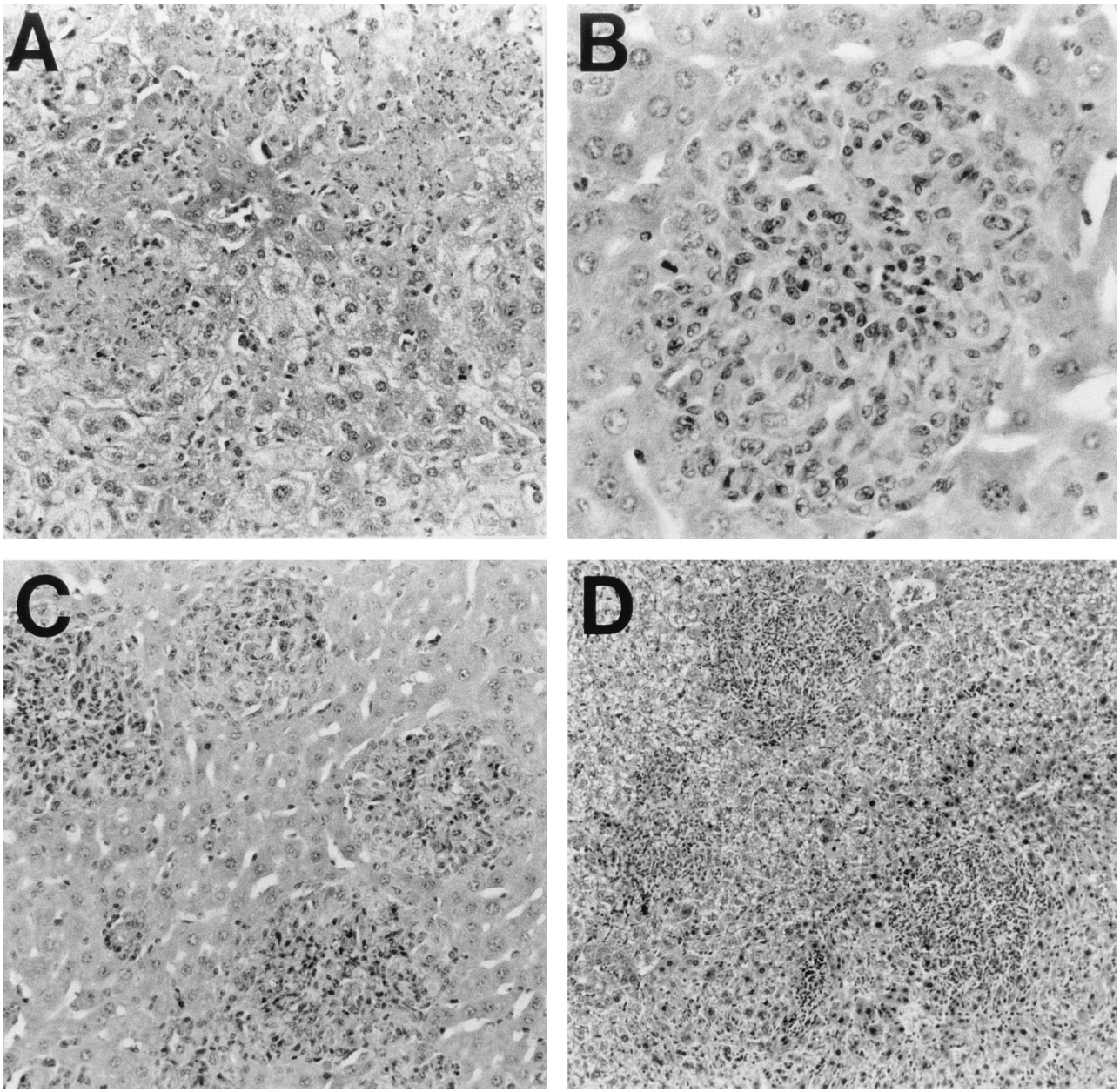

FIG. 5. Characteristic histopathological changes in liver caused by *Y. pestis* KIM on postinfection day 3. Hematoxylin and eosin stain was used. (A) Lcr^+ cells in control mouse actively immunized with PA, showing multiple focal necrotic lesions without inflammatory cell response (magnification, ×140); (B) Lcr^- cells in nonimmunized control mouse, exhibiting granuloma formation (magnification, ×280); (C) Lcr^+ cells in mouse actively immunized with PAV, showing protective granulomatous lesions (magnification, ×140); (D) Lcr^+ cells in mouse passively immunized with rabbit anti-PAV, showing lesions prompting accumulation of mononuclear cells (magnification, ×70).

against V antigen are protective (8, 9, 13, 14, 30, 34, 48, 49), an observation that is more consistent with a physiological role as an extracellular virulence factor than with a role as a cytoplasmic regulator of protein synthesis.

To help resolve this conundrum, we engineered the fusion protein PAV (33), which ensured both stability, a property entirely lacking in native V antigen (10), and homogeneity. It is generally known that fusion proteins often exhibit a surprising degree of biological activity. This point is illustrated by the present observation that PAV but not PA suppressed normal synthesis of IFN-γ and TNF-α in mice challenged with avirulent *lcrV* or Lcr^- yersiniae. This discovery provides a physiological role for V antigen that superficially appears to be inconsistent with regulation of bacterial growth but which accurately predicts the established histopathology of experimental plague in mice (44, 49). A full description of the mechanism whereby PAV prevents expression of these cytokines is, of course, beyond the scope of the present report, although it may be appropriate to note here that mice with suppressed cytokines exhibit symptoms consistent with those of endotoxin tolerance (17, 23). Additional work will also be required to determine why a peptide that suppresses cytokine synthesis induced by infection is also required for full expression of Yops. At present we favor the hypothesis that this effect re-

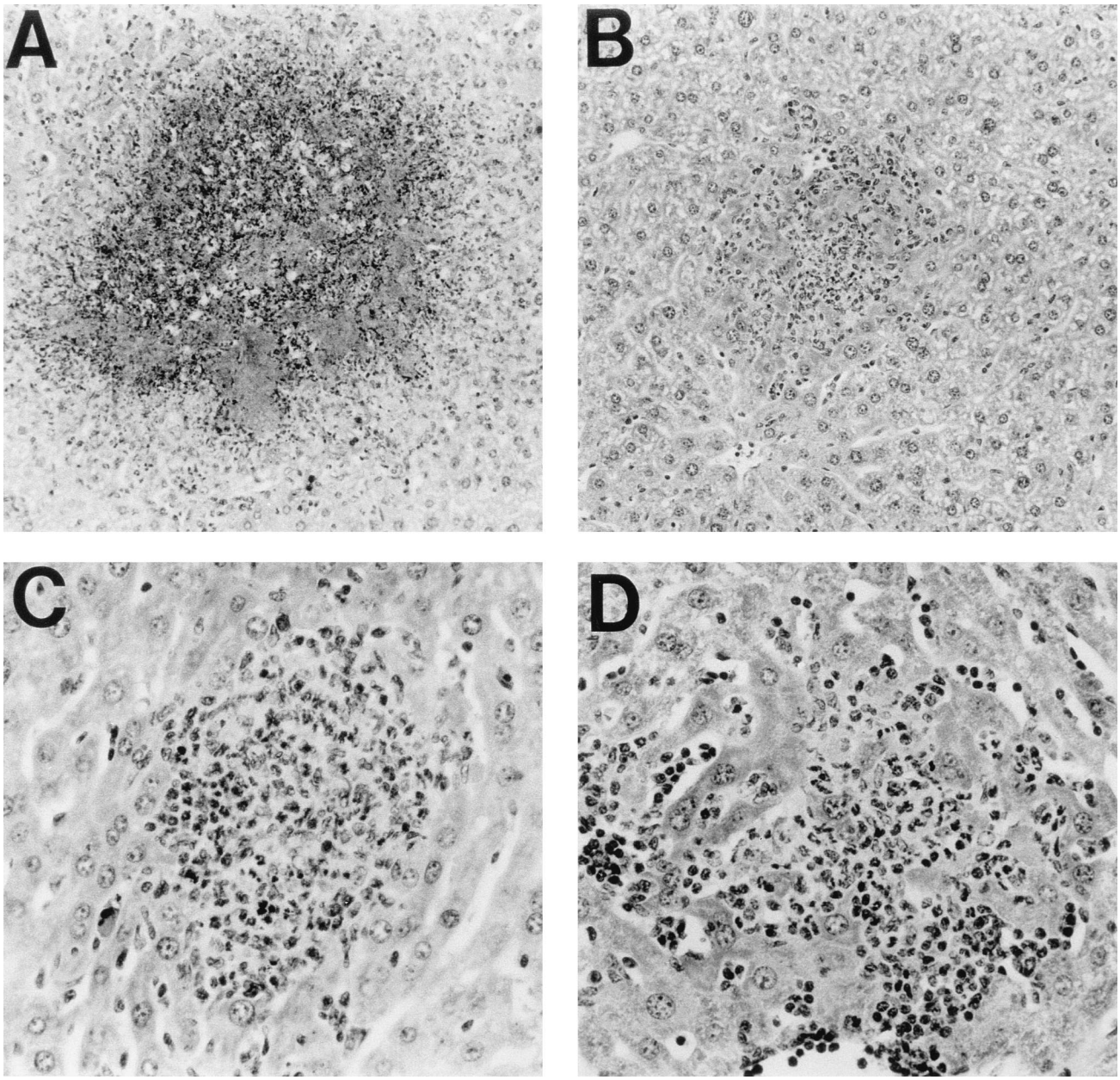

FIG. 6. Characteristic histopathological changes in liver caused by *Y. pseudotuberculosis* PB1 on postinfection day 3. Hematoxylin and eosin stain was used. (A) Lcr^+ cells in control mouse actively immunized with PA, showing central abscesses provoking massive infiltration of neutrophils (magnification, ×140); (B) Lcr^- cells in nonimmunized control mouse, exhibiting modest lesions with slight infiltration of inflammatory cells composed of neutrophils and mononuclear cells (magnification, ×280); (C) Lcr^+ cells in mouse actively immunized with PAV, showing infiltration of neutrophils and mononuclear cells without abscess formation (magnification, ×280); (D) Lcr^+ cells in mouse passively immunized with rabbit anti-PAV, showing inhibition of abscess formation (magnification, ×280).

flects enhancement of Mg^{2+}-mediated signal transduction at the cell surface instead of internal regulation at the level of transcription (22).

The kinetics of growth reported here for actively immunized mice resembled patterns previously found to occur after passive immunization except that bacterial multiplication in the liver and spleen was halted almost immediately. As a consequence, both processes prevented spillover of bacteria from visceral organs into the vascular system, thereby averting colonization of the lung. Similarly, results of histopathological studies performed with passively and actively immunized mice showed that curtailed bacterial growth reflected prompt infiltration of inflammatory cells to initial sites of colonization. In both systems this immediate chemotactic response resulted in the generation of protective granulomas indistinguishable from those formed by normal mice in response to infection by Lcr^- yersiniae (49). The most straightforward explanation for these observations is that they reflect the consequences of cytokine suppression mediated by V antigen.

Although the demonstration that the V antigen domain of PAV can suppress cytokine synthesis induced by attenuated yersiniae strongly indicates that native V antigen fulfills this function in vivo, the observation does not exclude the possibility that Yops or other Lcr^+-specific activities may also pre-

TABLE 2. Levels of TNF-α and IFN-γ in spleens of mice actively immunized with PA or PAV after challenge with 10 MLD (100 bacteria) of Lcr$^+$ *Y. pestis* KIM

Cytokine	Vaccine	Concn (ng/g)[a] of cytokine in spleen on postinfection day:					
		0	1	2	3	4	5
TNF-α	PA	<4.0	<4.0	<4.0	<4.0	12.1 ± 4.9	21.0 ± 12.9
	PAV	<4.0	30.5 ± 6.8	28.6 ± 10.2	15.1 ± 9.6	6.0 ± 2.1	<4.0
IFN-γ	PA	<1.5	<1.5	<1.5	<1.5	<1.5	<1.5
	PAV	<1.5	120.4 ± 23.1	79.4 ± 12.6	42.1 ± 14.9	21.0 ± 9.6	10.5 ± 6.1

[a] Each value represents the mean ± standard deviation for four mice.

vent cytokine biosynthesis (5). The finding that the same levels of IFN-γ and TNF-α were induced in mice challenged with either *lcrV* or Lcr$^-$ mutants does not necessarily indicate that only V antigen promotes significant suppression because, as noted above, *lcrV* cells produce a lower level of Yops than do cells of their Lcr$^+$ parent. However, a convincing demonstration of alternative suppressors will now require direct experimental evidence analogous to that provided in this report for V antigen. The necrotic lesions observed in this study and previously in livers and spleens of nonimmunized control mice (9, 44, 49) arise as a function of both necrosis of nonprofessional phagocytes and inhibition of neutrophil and mononuclear cell chemotaxis. We suspect that necrosis may be mediated by the cytotoxic Yops and that inhibition of chemotaxis is caused by V antigen. In this case, mutational loss of some critical component of the cytotoxic Yop system would block the occurrence of necrosis and thereby prevent invading yersiniae from obtaining necessary nutrients and electrolytes originating from host cell cytoplasm. As a result, the initial rate of bacterial growth of mutants lacking salient Yops would be sufficiently slow to enable the host to mount an effective specific immune response. Viewed in this context, any mutation that denies yersiniae access to Ca^{2+}-deficient but Mg^{2+}-enriched host cell cytoplasm within focal necrotic lesions would also prevent induction of V antigen, thereby enabling the host to mount a normal inflammatory response.

Further effort will be required to determine why Lcr$^+$ cells of *Y. pseudotuberculosis* formed abscesses surrounded by professional phagocytes as opposed to the stark necrotic lesions generated by *Y. pestis*. This distinction may reflect diffusion of some additional cytotoxic or antichemotactic activity unique to *Y. pestis* (e.g., murine exotoxin or plasminogen activator) or a chemotactic response to Yops which are released and accumulate in intact form by cells of *Y. pseudotuberculosis* as opposed to *Y. pestis*, where most of these peptides undergo posttranslational degradation (31, 32, 42) in the absence of polarized transfer following bacterium-host cell contact (20, 26, 41). In addition, more work will be required to determine why anti-PAV fails to protect against infection with *Y. enterocolitica* WA. Results of preliminary studies indicate that this isolate readily colonizes the mouse lung, thus causing death before cytokine-mediated defenses can effectively curtail the infection (22).

The results presented in this report indicate that V antigen suppresses the normal inflammatory system, thereby enabling the invading organisms to achieve a lethal cellular burden before an effective specific immune response can be initiated. This interpretation was favored by the demonstration that the same regimen of PAV that prevented significant induction of cytokines by *lcrV* yersiniae also promoted marked survival of these mutants. Analogous findings obtained with salmonellae and especially *L. monocytogenes*, with which injected PAV prompted a lethal outcome, clearly illustrate that PAV-induced suppression is not specific to yersiniae; thus, V antigen may possess the potential for development as a generalized anti-inflammatory agent.

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