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Active Immunization with Recombinant V Antigen from *Yersinia pestis* Protects Mice against Plague

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The gene encoding V antigen from *Yersinia pestis* was cloned into the plasmid expression vector pGEX-5X-2. When electroporated into *Escherichia coli* JM109, the recombinant expressed V antigen as a stable fusion protein with glutathione *S*-transferase. The glutathione *S*-transferase–V fusion protein was isolated from recombinant *E. coli* and cleaved with factor Xa to yield purified V antigen as a stable product. Recombinant V antigen was inoculated intraperitoneally into mice and shown to induce a protective immune response against a subcutaneous challenge with 3.74×10^6 CFU of virulent *Y. pestis*. Protection correlated with the induction of a high titer of serum antibodies and a T-cell response specific for recombinant V antigen. These results indicate that V antigen should be a major component of an improved vaccine for plague.

Plague is a zoonotic infection caused by the bacterium *Yersinia pestis* and is transmitted among natural animal reservoirs, usually by a flea vector. Humans are an occasional host in the natural cycle of the disease, and bubonic plague, characterized by the swelling of local lymph nodes, may occur following the bite of an infected flea. One of the complications of bubonic plague is secondary pneumonia, and in these cases, the disease is readily transmitted from person to person by airborne droplets.

Plague is endemic in regions of North and South America, Africa, China, and Asia (2). The current outbreaks are believed to be part of the fourth world pandemic of the disease, and there is clearly a need to protect individuals living or travelling in areas of endemicity and laboratory workers handling the bacterium.

A number of vaccines for plague have been developed, including the killed Cutter USP vaccine prepared in the United States and the live attenuated EV76 vaccine (8, 9). The Cutter USP vaccine was first manufactured in 1942 and consists of a formaldehyde-killed preparation of *Y. pestis* derived from a virulent strain (6). The vaccine is believed to be effective against bubonic plague, principally because there was a low incidence of the disease among inoculated U.S. troops in Vietnam (3). However, its ability to protect against pneumonic plague is doubtful, and cases of this form of the disease have been reported in vaccinated individuals (8). In addition, local side reactions and systemic effects, ranging from mild headaches to severe malaise and fever, were reported in 11 to 24% and 4 to 10%, respectively, of the individuals inoculated with the Cutter USP vaccine (6). The EV76 vaccine was tested extensively and used in the former Soviet Union from 1939, although its efficacy in evoking an immune response in humans is questionable (8, 9). It has been shown that the virulence of EV76 differs in several animal species, and nonhuman primates are particularly susceptible to a chronic infection with this strain (10). In the Western world, the vaccine is considered to be unsuitable for mass vaccinations because of the severity of the side effects.

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The identification of protective subunits of *Y. pestis* could enable an improved vaccine against plague to be developed. One of the potential protective subunits is V antigen, which is believed to have a bifunctional role in *Y. pestis*. It is thought to act as a regulator of the low calcium response (14), and it may also function as a virulence factor. The latter role was supported by the early observation that rabbit antiserum raised against partially purified V antigen provided passive protection to mice which had been challenged intraperitoneally with 100 50% lethal doses (LD_{50}) of *Y. pestis* (4). This result was later confirmed by raising antisera to purified V antigen, and it was shown that both monospecific polyclonal antisera (21, 22) and a monoclonal anti-V antibody (12, 18) provided passive immunity against a 5 to 10 minimum lethal dose intravenous challenge with *Y. pestis* KIM. Recently, it was demonstrated that polyclonal antisera raised against recombinant V antigen or a protein A-V antigen fusion (PAV) were also partially protective against *Y. pestis* KIM (11). By absorbing the antisera with truncates of PAV, it was deduced that at least one protective epitope lay between amino acids 168 and 275 of V antigen (11).

The role of V antigen in virulence is unknown, but Nakajima and Brubaker (12) suggested that it may be immunosuppressive, possibly by inhibiting cytokine synthesis, and so prevent the infiltration of host inflammatory cells into infected organs (20, 22). The passive protection conferred by anti-V antigen serum may therefore be attributed to the neutralization of this immunosuppressive activity (12).

The evidence described above suggested that V antigen could induce an immune response which protects against challenge with *Y. pestis*. Previously, there was a brief report that guinea pigs immunized with a V antigen-rich fraction prepared from *Y. pestis* were protected against challenge with the bacterium (4). To examine this further, we have expressed V antigen as a fusion with glutathione *S*-transferase (GST) in *Escherichia coli*, purified the recombinant protein as a stable product, and tested its immunogenicity in vivo. Our results give the first detailed demonstration that V antigen, which is not contaminated with other compounds from *Yersinia* spp., is a highly effective protective antigen following active immunization and indicate that it should be investigated as a major component of an improved vaccine for plague.

MATERIALS AND METHODS

Enzymes and reagents. Materials for the preparation of growth media were obtained from Oxoid Ltd. or Difco Laboratories. All enzymes used in the manipulation of DNA were obtained from Boehringer Mannheim UK Ltd. and used as described in the manufacturer's instructions. All other chemicals and biochemicals were obtained from Sigma Chemical Co. unless otherwise indicated. Monospecific rabbit polyclonal anti-V and mouse anti-GST sera were prepared by R. Brubaker (Department of Microbiology, Michigan State University) and E. Williamson (Chemical and Biological Defence Establishment), respectively.

Bacterial strains and cultivation. *Y. pestis* GB was cultured aerobically at 28°C in a liquid medium (pH 6.8) containing 15 g of Proteose Peptone, 2.5 g of liver digest, 5 g of yeast extract, and 5 g of NaCl per liter, supplemented with 80 ml of 0.25% hemin dissolved in 0.1 M NaOH (blood base broth). *E. coli* JM109 was cultured and stored as described by Sambrook et al. (17).

Manipulation of DNA. Chromosomal DNA was isolated from *Y. pestis* by the method of Marmur (5). The gene encoding V antigen (*lcrV*) was amplified from *Y. pestis* DNA by PCR with 125 pmol of primers homologous to sequences from the 5′ and 3′ ends of the gene (15). The sequences of the 5′ primer (V/5′E: GATCGAATTC$^+$ATTAGAGCCTACGAACAA) and the 3′ primer (V/3′C: GGATCGTCGACTTA*CATAATTACCTCGTGTCA) also included 5′ regions encoding the restriction sites *Eco*RI and *Sal*I, respectively. In addition, the nucleotides C$^+$ and A* were altered from the published sequence of *lcrV* (15) to complete the *Eco*RI site and include an extra termination codon (TAA), respectively, in the amplified DNA. The PCR primers were prepared with a DNA synthesizer (model 392; Applied Biosystems). A DNA fragment was obtained after 30 cycles of amplification (95°C, 20 s; 45°C, 20 s; 72°C, 30 s [model 9600 GeneAmp PCR system; Perkin-Elmer]). The fragment was purified, digested with *Eco*RI and *Sal*I, ligated with suitably digested plasmid pGEX-5X-2, and transformed into *E. coli* JM109 by electroporation (17). A colony containing the recombinant plasmid (pVG100) was identified by PCR with 30-mer primers (5′ nucleotides located at positions 54 and 794 [15]) which amplified an internal segment of the *lcrV* gene. To confirm the nucleotide sequence of the cloned insert, sequencing reactions involving pVG100 and primers designed from the *lcrV* gene were performed with an automated *Taq* polymerase cycle sequencing protocol with fluorescently labelled dideoxy nucleotides (CATALYST Molecular Biology Labstation; Applied Biosystems). The reaction products were analyzed with an automated DNA sequencer (model 373A; Applied Biosystems).

Expression of recombinant V antigen (rV) in *E. coli*. Cultures of *E. coli* JM109/pVG100 were grown in LB medium (17) containing 100 μg of ampicillin ml^{-1} at 37°C until the A_{600} was 0.3. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to the culture to a final concentration of 1 mM, and growth was continued for a further 5 h. Whole-cell lysates of the bacteria were prepared as described by Sambrook et al. (17), and expression of the GST-V fusion protein was examined by staining 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels (Mini-Protean II; Bio-Rad) with Coomassie brilliant blue R250 and by Western blotting (immunoblotting) (17). Western blots were probed with rabbit anti-V serum at a dilution of 1/4,000 or mouse anti-GST serum at a dilution of 1/1,000, and protein bands were visualized with a colloidal gold-labelled secondary antibody (Auroprobe BLplus; Cambio).

Quantification of GST-V fusion protein expression in vitro. Cultures of *E. coli* JM109 containing pVG100 or pGEX-5X-2 were grown as described above. One-milliliter aliquots were removed from the cultures in the logarithmic and stationary phases, and the number of viable cells was determined by inoculating the aliquots onto L agar containing 100 μg of ampicillin ml^{-1}. The cells were harvested from a second 1-ml aliquot by centrifugation and resuspended in 1 ml of phosphate-buffered saline (PBS). The cell suspension was frozen at −20°C for 1 h, thawed, and then sonicated on ice at 10% power three times for 30 s each (model XL2015 sonicator, 3.2-mm Microtip probe; Heat Systems Inc.). The sonicates and a standard solution of rV (5 μg ml^{-1}) were serially diluted in PBS in a microtiter plate and allowed to bind overnight at 4°C. The quantity of GST-V fusion protein in each sonicate was determined in a standard enzyme-linked immunosorbent assay (ELISA) with rabbit anti-V serum as the primary antibody. Antibodies were incubated in 1% skim milk powder in PBS.

Purification of rV. *E. coli* JM109/pVG100 was grown in five 100-ml volumes of LB as described above. The cells were harvested by centrifugation and resuspended in 3 ml of PBS. After the addition of 80 μl of lysozyme (10 mg ml^{-1}), the cell suspension was incubated for 10 min at 22°C. Triton X-100 was added to a final concentration of 1%, and the cells were frozen (−20°C), thawed, and sonicated on ice at 70% power three times for 30 s each (model XL2015 sonicator). The lysed cells were centrifuged, and the supernatant was made up to 30 ml with PBS and mixed with 5 ml of glutathione Sepharose 4B (Pharmacia Biotech), which had been washed three times with PBS plus 0.1% Triton X-100. The mixture was stirred for 18 h at 4°C, centrifuged and washed twice in 100 ml of PBS, and then packed into a chromatography column (Poly-Prep; Bio-Rad) as a 50% slurry. The GST-V fusion protein was eluted with 10 ml of 50 mM Tris (pH 8.0) containing 5 mM reduced glutathione (Pharmacia Biotech). After dialysis against PBS, the fusion protein was cleaved with factor Xa (Boehringer Mannheim UK Ltd.) for 18 h at 22°C at an enzyme/fusion protein ratio of approximately 1:200 by weight. Cleaved GST and excess uncleaved GST-V (but not factor Xa) were removed from the solution by affinity adsorption, as described above, to leave purified rV.

Immunization with rV. Six-week-old female BALB/c mice, raised under specific-pathogen-free conditions (Charles River Laboratories, Margate, Kent, United Kingdom) were used in this study. A group of 16 mice received intraperitoneally a 0.2-ml primary immunizing dose of 10 μg of rV, presented in a 1:1 water-in-oil emulsion with incomplete Freund's adjuvant. On days 14 and 34, each animal received booster intraperitoneal doses, prepared as described above. On day 64, six animals were sacrificed, and their tissues were removed for immunological analyses, as described below. The remaining animals were challenged with *Y. pestis*. An untreated control group of 16 age-matched mice was divided similarly into groups for tissue sampling and challenge. In a subsequent experiment to determine the degree of protection against higher challenge doses of *Y. pestis*, groups of five or six rV-immunized and control mice were used.

Measurement of serum antibody titer. Blood was sampled by cardiac puncture from mice anesthetized intraperitoneally with a 0.1-ml cocktail containing 6 mg of Domitor (Norden Laboratories) and 27 μg of ketamine hydrochloride (Ketalar; Parke-Davis). The samples were pooled, and the serum was separated. The serum antibody titer was measured by a modified ELISA (23). Briefly, a microtiter plate was coated with rV (5 μg ml^{-1} in PBS), and the test sera were serially diluted in duplicate on the plate. Bound antibody was detected with peroxidase-labelled conjugates of anti-mouse polyvalent immunoglobulin. The titer of specific antibody was estimated as the maximum dilution of serum giving an absorbance reading of greater than 0.1 U, after subtraction of the absorbance due to nonspecific binding detected in the control sera.

Isolation of purified T cells from the spleen. A crude suspension of mixed spleen cells was prepared by gently grinding the spleen on a fine wire mesh. The cells were flushed from the splenic capsule and connective tissue with 2 ml of tissue culture medium (Dulbecco modified Eagle medium [DMEM] with 20 mM L-glutamine, 10^5 U of penicillin liter^{-1}, and 100 mg of streptomycin liter^{-1}). A population of mixed lymphocytes was separated from the spleen cell suspension by density gradient centrifugation on Ficoll-Hypaque (lymphocyte separation medium; ICN Flow). A mixed acridine orange (0.0003% [wt/vol]) and ethidium bromide (0.001% [wt/vol]) stain was used to determine the percentage of viable cells in the preparation.

The mixed lymphocytes were incubated with sheep anti-mouse immunoglobulin G-coated Dynabeads (M450, Dynal UK) at a ratio of 1:3 (numbers of cells/numbers of beads) for 30 min at 4°C. The Dynabead-linked B cells were removed by magnetic separation, and the remaining T cells were resuspended in DMEM, supplemented with antibiotic and 10% (vol/vol) fetal calf serum (FCS), at the desired cell density for seeding onto microtiter plates.

In vitro proliferation of crude spleen cells and purified T cells against rV. Doubling dilutions of rV or concanavalin A (positive control) in DMEM (range, 0 to 50 μg ml^{-1}) were made in the wells of a microtiter plate such that 0.1 ml remained in each well. Negative controls consisted of 0.1 ml of DMEM alone. An equal volume of the crude spleen cell suspension or purified T-cell suspension was seeded into each well at a minimum density of 4.9×10^4 cells and incubated for 72 h at 37°C (5% CO_2). One microcurie of [^{3}H]thymidine ([*methyl*-^{3}H]thymidine, 74 GBq mmol^{-1}; Amersham) in 30 μl of DMEM supplemented with 10% FCS was aliquoted into each well, and incubation was continued for 24 h. The well contents were harvested onto glass fiber filters with a cell harvester (Titertek), and discs representing each well were punched from the filter mats into 1.5 ml of scintillation fluid (Cytoscint; ICN Biomedicals Inc.) to measure the incorporation of [^{3}H]thymidine into cells. The cell stimulation index was calculated from four replicates as the mean of the counts per minute of the stimulated cells divided by the counts per minute of the negative control cells.

Challenge with *Y. pestis*. Groups of 5 to 10 mice from the immunized and control groups were challenged subcutaneously with 0.1-ml aliquots of *Y. pestis* GB at various cell densities (16). Strain GB was isolated from a fatal human case of plague and has a median lethal dose of <1 CFU in BALB/c mice by the subcutaneous route (16). The mice were observed for 14 days, and where appropriate, the time to death was recorded. A postmortem was carried out on all animals. The livers and spleens were examined for enlargement. To test for the presence of *Y. pestis*, samples of blood, liver, and spleen were also smeared onto Congo red agar and incubated at 28°C for 48 h.

RESULTS

Cloning and expression of *lcrV* in *E. coli*. The *lcrV* gene was amplified by PCR from the chromosomal DNA of *Y. pestis* GB and cloned downstream of the GST gene encoded by the plasmid pGEX-5X-2 to form an in-frame fusion. An IPTG-induced culture of *E. coli* JM109, containing the recombinant plasmid pVG100, produced a unique protein which was detected by Western blotting with rabbit anti-V and mouse anti-GST sera (Fig. 1). The molecular weight of this protein was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and was similar to the predicted value of 63,700 for the GST-V fusion. An equivalent protein was not expressed in IPTG-induced cultures of *E. coli* JM109/pGEX-5X-2.

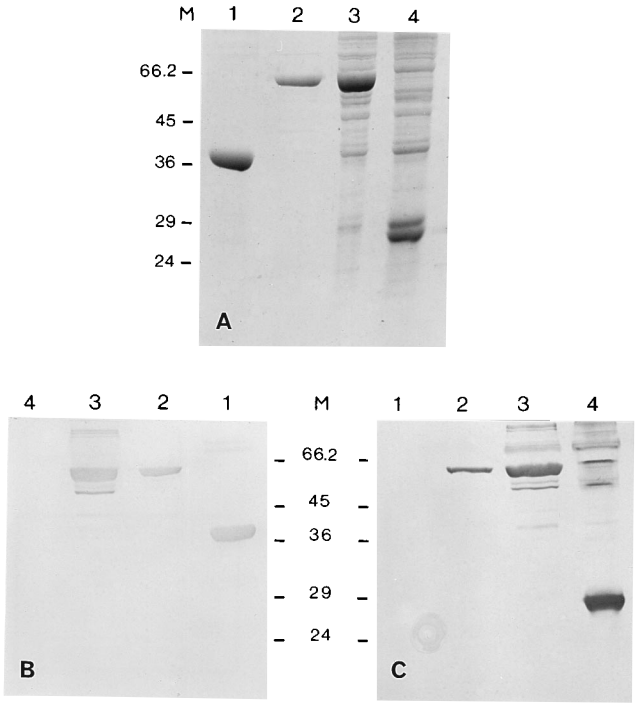

FIG. 1. Coomassie brilliant blue R250-stained proteins separated by SDS-PAGE (10% polyacrylamide gel) (A) and Western blots of duplicate gels probed with a monospecific rabbit polyclonal anti-V serum (B) or a mouse polyclonal anti-GST serum (C). Lanes: M, positions of protein molecular weight markers (10^3); 1, purified rV; 2, purified GST-V fusion; 3, *E. coli*/pVG100 cell lysate (plus IPTG); 4, *E. coli*/pGEX-5X-2 cell lysate (plus IPTG).

Purification of rV. The GST-V fusion protein was isolated from 0.5 liter of an *E. coli* JM109/pVG100 culture and induced with IPTG, and rV was cleaved from the carrier protein with factor Xa. From the amino acid sequence of the GST-V fusion (translated from the nucleotide sequence), it was apparent that the residues Gly-Ile-Pro-Gly were located downstream of the factor Xa cleavage site, and it was predicted that these residues were added to the N terminus of rV. When examined by SDS-PAGE, Coomassie brilliant blue R250 stained only one band in the rV preparation (Fig. 1A), and by Western blotting, breakdown products of the purified protein were not detected with rabbit anti-V serum (Fig. 1B). The molecular weight of rV was estimated to be 36,000 by SDS-PAGE.

Quantification of GST-V expression in vitro. To determine the level of expression of GST-V in vitro, sonicates were prepared from logarithmic- and stationary-phase cultures of *E. coli* JM109/pVG100 and analyzed by ELISA with rabbit anti-V serum as the primary antibody. Expression of GST-V from logarithmic- and stationary-phase cultures occurred at a low level (<10 μg ml^{-1}) without induction with IPTG. However, in the presence of IPTG, expression was increased more than 10-fold, reaching levels of 130 μg ml^{-1} in the logarithmic-phase culture and 400 μg ml^{-1} in the stationary-phase culture. This increase in expression was observed despite 4-fold and 50-fold reductions in the viable cell densities in the logarithmic- and stationary-phase cultures, respectively. The GST-V fusion protein was not detected by ELISA in cultures of *E. coli* JM109/pGEX-5X-2.

Titer of serum antibodies in rV-immunized animals. Mice were inoculated intraperitoneally with three 10-μg doses of rV. On day 64, blood was sampled from six animals and pooled, and the titer of rV-specific serum antibodies was determined.

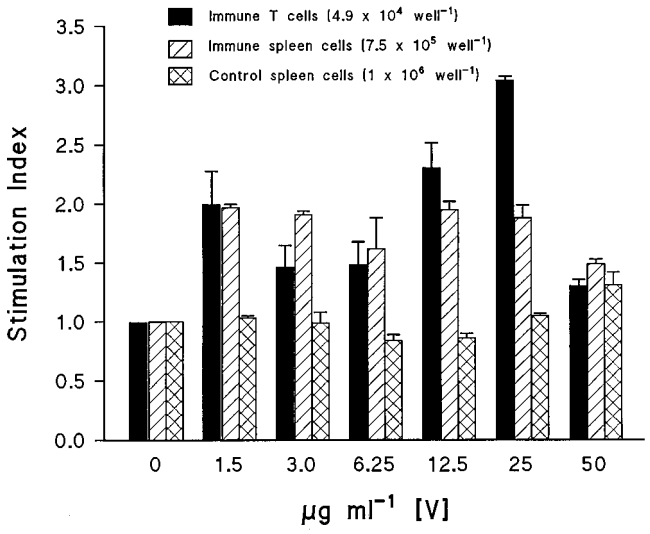

FIG. 2. Proliferation of crude spleen cells and purified T cells, isolated from rV-immunized or control mice, against rV. The results show the means of four replicates + standard errors of the means.

Immunized animals developed a high titer (1:128,000) of circulating immunoglobulins with specificity for rV.

In vitro proliferation of crude spleen cells and purified T cells from rV-immunized mice. The ability of the rV antigen to stimulate a T-cell response was determined by assaying in vitro the proliferation of crude spleen cells and purified T cells upon reexposure to rV. Proliferation was observed in both cell populations, although the response was greatest in the purified T cells, with a peak stimulation index at 25 μg of rV ml^{-1} in the tissue culture medium (Fig. 2). As expected, a significant 26-fold increase in the response to 1.5 μg of concanavalin A ml^{-1} was also observed in the T-cell population, which confirmed the efficiency of the purification process. Cell staining methods also indicated that the T-cell population was more than 95% pure (data not shown).

Protection against challenge with *Y. pestis*. In initial experiments to investigate the protective efficacy of rV, groups of 10 rV-immunized or control mice were challenged subcutaneously with 122 CFU of *Y. pestis* GB. All of the rV-immunized animals survived the challenge, although the controls succumbed with a mean time to death of 119 ± 4.9 h. Subsequently, groups of five rV-immunized mice were challenged with *Y. pestis* GB to determine the degree of protection at higher doses (Table 1). All of the immunized animals were fully protected and survived without development of externally

TABLE 1. Protection of rV-immunized mice against challenge with *Y. pestis*[a]

Group	Challenge dose (CFU)	No. of survivors/ total no.	Time to death (h)[b]
rV i.p.	3.74×10^6	5/5	
	3.74×10^5	5/5	
	3.74×10^4	4/5	84 ± 0
	3.74×10^3	5/5	
	374	5/5	
Control	37.4	0/6	132 ± 5.7

[a] Groups of five to six rV-immunized and control mice were challenged subcutaneously with a range of doses of *Y. pestis* GB and observed for 14 days. Where necessary, the mean time to death was estimated.

[b] Values are means ± standard errors of the means.

visible symptoms for the 14-day observation period. The only exception occurred in the group which received 3.74×10^4 CFU, in which one of five mice died at 84 h postchallenge. This mouse was assumed to be less hyperimmune than other animals in the group. In contrast, all six mice in the control group, which received a low dose of *Y. pestis*, succumbed to the challenge.

Postmortem analysis of challenged animals. A postmortem was conducted on animals challenged with *Y. pestis* either at the time of death or after the 14-day observation period. By inoculating tissue samples onto Congo red agar, *Y. pestis* was detected in the blood, livers, and spleens of all mice in the control groups, which succumbed to the challenge with the bacterium, confirming that plague was the cause of death. However, tissue samples removed from surviving rV-immunized animals on day 14 postchallenge did not contain *Y. pestis* cells. There were no visible morphological changes to the livers of the rV-immunized mice, although the spleens were enlarged in 90% of the animals, indicating that there had been a recent acute infection. In the majority of the affected animals (approximately 80%), the spleens were only slightly larger than normal. Morphological changes to the livers and spleens of the control animals were not observed, mainly because the animals succumbed to the infection before these effects were noticeable. A postmortem was not conducted on the rV-immunized mouse which died 84 h after the challenge, because of the onset of rigor mortis.

DISCUSSION

When extracted from *Y. pestis*, V antigen degrades during the purification process, yielding five antigenic fragments (1). A similar pattern of degradation was observed for recombinant V antigen purified from a protease-negative strain of *E. coli* containing the *lcrGVH* operon (11). These observations led to the concepts that V antigen may undergo autocatalytic hydrolysis or, during purification, become susceptible to physical stress or contaminating proteases (1, 12). The difficulty in obtaining sufficient quantities of the purified protein has hindered a full investigation of the role of V antigen in the regulation of the low calcium response, virulence, and in particular, immunosuppression (12).

In this study, rV was purified in a stable form from the cytoplasm of *E. coli*, thus suggesting that the protein lacks autoproteolytic activity. This supports the previous finding that V antigen from *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is not prone to degradation (1). The fusion of V antigen to N-terminal carrier proteins, such as GST or protein A (11), may also protect the protein from physical or exogenous proteolytic degradation during cell disruption and the initial stages of purification.

We have reported that rV, produced as a GST fusion, is expressed at a high level in *E. coli* (400 μg ml of stationary-phase culture^{-1}), and that the fusion was easily isolated in one step by affinity chromatography to yield a stable product. In this respect, our results reflect those of Motin et al. (11), who produced a fusion of protein A with a V truncate lacking 67 amino acids at the N terminus (i.e., PAV). However, the GST-V fusion described here can be cleaved specifically with the enzyme factor Xa, thus avoiding the need for the stringent acid hydrolysis required for the cleavage of PAV (11), and the apparent yield of rV is higher than that indicated by Motin et al. (11). In addition, the product cleaved from GST-V represents the complete V antigen molecule. Therefore, we have developed a means of producing a stable form of V antigen, which can be purified in large quantities and is free from other contaminating *Yersinia* antigens, thus providing a reliable source of the protein for further investigation of its role in virulence and the regulation of the low calcium response.

The molecular weight of rV was estimated to be 36,000 by SDS-PAGE, which is similar to the values of 37,000 reported for native V antigen (1) and 37,200 predicted from the amino acid sequence. Recombinant V antigen was also recognized in Western blots by monospecific polyclonal antiserum raised against native V antigen, indicating that the antigenicity of the recombinant protein had been preserved.

When used to inoculate mice, purified rV elicited solid protective immunity against a subcutaneous challenge with up to 3.74×10^6 CFU of *Y. pestis* GB. Protection was correlated with the induction of a high titer of specific circulating antibodies (1:128,000) in the immunized animals and the induction of a significant proliferative response in purified T cells when restimulated with rV in vitro. Thus, we have demonstrated that rV is a highly protective immunogen which is able to stimulate effectively both serum antibody and T-cell responses.

The protection offered by active immunization with rV supports a number of previous studies showing that anti-V serum confers passive immunity in mice against low challenge doses of 10 minimum lethal doses or 100 LD_{50} of *Y. pestis*. Passive protection was first demonstrated with antisera raised against partially purified V antigen (4). Subsequently, improved techniques for the purification of V antigen allowed anti-V sera of greater specificity to be developed, and these preparations were further purified by absorption with cells and outer membranes from *Yersinia* species and by isolation of the gamma globulin (1, 12, 21). Finally, an unambiguous indication of the role of anti-V sera in protection was shown by passively administering monoclonal anti-V antigen (12) and polyclonal antisera raised against purified PAV (11), although immunity was not complete in all animals challenged with 10 minimum lethal doses. Our results complement these findings by demonstrating the ability of highly purified rV, which is not contaminated with other compounds from *Yersinia* spp., to provide protection in an active immunization schedule. In addition, immunization with rV elicited solid protection against a *Y. pestis* dose of more than 10^6 median lethal doses, which is approximately 10^4-fold higher than that shown previously (4, 11, 18, 21, 22), and against the highly virulent strain GB (16). In many protection studies (11, 18, 21, 22), the challenge organism was *Y. pestis* KIM, a nonpigmented mutant, which is virulent only by intravenous injection (11).

Although the median lethal dose of *Y. pestis* GB in rV-immunized mice has not been calculated, rV provided protection against a challenge dose which was more than 10^6-fold higher than the median lethal dose of *Y. pestis* GB in nonimmunized mice (16). It may be significant that an equivalent decrease of at least 10^6-fold was observed in the lethality of an *lcrV*-negative mutant of *Y. pestis* (14). The ability of rV to induce protective immunity emphasizes the idea that V antigen plays an important role in the virulence of *Y. pestis.*

Russell et al. (16) reported that Porton Outbred mice inoculated intramuscularly with a single dose of the live vaccine strain EV76 were protected against a challenge with 5.75×10^6 CFU of *Y. pestis* GB. In contrast, mice which received two intramuscular doses of the Cutter USP vaccine were protected fully up to a challenge dose of only 5×10^3 CFU (16). We have demonstrated that immunization with the subunit rV confers at least a similar level of protection against *Y. pestis* as these whole-cell vaccines. Furthermore, rV-immunized mice did not display external symptoms of disease, even after challenge with the highest dose of *Y. pestis*, and at postmortem, the only indication of infection was a slightly enlarged spleen.

The absence of *Y. pestis* in the livers and spleens of vaccinated mice and the lack of macroscopic lesions suggested that the immune response mounted against rV prevented the bacterium from colonizing these organs and forming characteristic areas of necrosis (20, 22). In vivo, Lcr^+ *Y. pestis* has been shown to suppress the production of the cytokines tumor necrosis factor alpha and gamma interferon in the tissues of infected mice (12). The passive inoculation of monospecific polyclonal anti-V sera has been linked to the inhibition of this immunosuppressive activity, the promotion of *Y. pestis* clearance from the liver and spleen, and protection in animals challenged with *Y. pestis* (12, 21, 22). Direct administration of exogenous tumor necrosis factor alpha and interferon gamma has also been shown to promote clearance of Lcr^+ *Y. pestis* from the spleens of infected mice (12). It is possible that the absence of *Y. pestis* in the organs of rV-vaccinated mice, despite the large challenge doses, is due to the effective induction of circulating antibodies to V antigen and the consequent development of antibacterial responses, which include the production of these cytokines.

Other subunits of *Y. pestis* which are important in eliciting an immune response to plague have been identified. For example, a single intramuscular dose of purified F1 capsular antigen provided protection against an intraperitoneal challenge with 10^5 CFU of *Y. pestis* (19), and immunization with recombinant *Salmonella typhimurium aroA* expressing F1 protected mice against a subcutaneous challenge with approximately 50 CFU (13). However, the high degree of protection conferred by rV in vaccinated animals indicates that this protein is one of the most important immunogenic subunits of *Y. pestis*. A subunit vaccine incorporating rV has some advantages over the current whole-cell vaccines: rV is simple to prepare and free from other toxic components of *Y. pestis*. As a result, it is likely to be safer and less reactogenic than either the live EV76 vaccine or the killed Cutter USP vaccine.

A requirement of an improved plague vaccine would be to induce mucosal and systemic immune responses, since immunity at the lung surface would be an important factor in protecting against the pneumonic form of plague, which is transmissible from person to person. The efficacy of the current whole-cell vaccines in protecting against pneumonic plague is largely unknown, but because they are administered by parenteral routes, they are unlikely to elicit a mucosal immune response (7). This is supported by reports of pneumonic plague occurring in individuals inoculated with the Cutter USP vaccine (8). We are investigating a number of delivery systems for V antigen which are designed to induce mucosal and systemic immune responses. If these delivery systems are able to present V antigen effectively and stimulate protective immunity, they will form the basis of novel vaccines which have the potential to protect against both bubonic and pneumonic plague.

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