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Regions of *Yersinia pestis* V Antigen That Contribute to Protection against Plague Identified by Passive and Active Immunization

JIM HILL,* SOPHIE E. C. LEARY, KATE F. GRIFFIN, E. DIANE WILLIAMSON, AND RICHARD W. TITBALL

Microbiology, CBD Porton Down, Salisbury, Wiltshire SP4 OJQ, United Kingdom

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V antigen of *Yersinia pestis* is a multifunctional protein that has been implicated as a protective antigen, a virulence factor, and a regulatory protein. A series of V-antigen truncates expressed as glutathione *S*-transferase (GST) fusion proteins (GST-V truncates) have been cloned and purified to support immunogenicity and functionality studies of V antigen. Immunization studies with GST-V truncates have identified two regions of V antigen that confer protection against *Y. pestis* 9B (a fully virulent human pneumonic plague isolate) in a mouse model for plague. A minor protective region is located from amino acids 2 to 135 (region I), and a major protective region is found between amino acids 135 and 275 (region II). In addition, analysis of IgG titers following immunization suggested that the major antigenic region of V antigen is located between amino acids 135 and 245. A panel of monoclonal antibodies raised against recombinant V antigen was characterized by Western blotting against GST-V truncates, and epitopes of most of the monoclonal antibodies were mapped to region I or II. Monoclonal antibody 7.3, which recognizes an epitope in region II, passively protected mice against challenge with 12 median lethal doses of *Y. pestis* GB, indicating that region II encodes a protective epitope. This is the first report of a V-antigen-specific monoclonal antibody that will protect mice against a fully virulent strain of *Y. pestis*. The combined approach of passive and active immunization has therefore confirmed the importance of the central region of the protein for protection and also identified a previously unknown protective region at the N terminus of V antigen.

The bacterium *Yersinia pestis* is the causative agent of bubonic plague, a zoonotic infection that is spread from natural animal reservoirs via the bite of an infected flea. The bubonic form of the disease can develop into a highly infectious pneumonic form which is spread from person to person in airborne droplets (see reference 17 for a review). Due to the high infectivity and mortality of pneumonic plague, there is a need to develop vaccines for people living in or travelling to areas in which plague is endemic as well as laboratory workers handling specimens from plague patients.

Current plague vaccines are based on whole-cell formulations. The Cutter USP vaccine, which is currently used in the Western world, is a suspension of a formalin-fixed virulent strain of *Y. pestis* (7). Transient side effects associated with the administration of this vaccine have been reported (7). Although it is believed to be protective against bubonic plague (3), protection against pneumonic plague is doubtful, as vaccinated individuals have been infected (8). EV76 is a live attenuated vaccine that has been used extensively in the former Soviet Union, but its efficacy has been questioned (9). The virulence of EV76 differs in several animal species, and nonhuman primates are particularly susceptible to this strain (10). Furthermore, the severity of the side effects of EV76 precludes its widespread use as a vaccine in humans. There is therefore a need for an improved plague vaccine that gives greater protection and produces fewer side effects than existing formulations.

Immunization with purified *Y. pestis* proteins has been considered as an alternative to whole-cell vaccines. Two subunits of *Y. pestis*, fraction 1 (F1) and V antigen, have been identified as vaccine candidates. The F1 protein, which forms a protein capsule-like structure on the bacterial surface that is believed to inhibit phagocytosis (26), protects mice against subcutaneous (s.c.) and aerosol challenge with virulent *Y. pestis* (1). Also, F1 expressed by an *aro*A strain of *Salmonella typhimurium* given intragastrically to mice has been shown to protect them against plague (16). The gene for V antigen (*lcrV*) is part of the *lcrGVH* operon (18, 19), which is located on a 70-kb low-calcium response (lcr) plasmid that is common to *Y. pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* (2). Early studies implicated V antigen as a protective antigen against plague in guinea pigs (4), and recently highly purified recombinant V antigen has been shown to protect mice against high challenge doses of *Y. pestis* GB (5). Similar protection given by a truncated form of V antigen fused to protein A indicates that the first 67 amino acids of V antigen are not necessary for protection against KIM, an attenuated strain of *Y. pestis* (14). Passive immunization with polyclonal V-antigen-specific serum adsorbed against truncated forms of V antigen indicated that the region from amino acids 168 to 275 contains at least one protective epitope (12). A monoclonal antibody (MAb) has also been shown to confer significant protection against *Y. pestis* KIM (15). Combined immunization with the V and F1 antigens has been shown to give mice higher levels of protection against plague than either subunit alone (27). The protection observed was several orders of magnitude greater than that of the killed Cutter USP vaccine (27).

Studies suggest that V antigen is also a virulence factor (23) that reduces local expression of the host cytokines tumor necrosis factor alpha and gamma interferon in response to yersinia infection (14, 15, 25), thus allowing the bacterium to

* Corresponding author. Mailing address: Microbiology, CBD Porton Down, Salisbury, Wiltshire SP4 OJQ, United Kingdom. Phone: 44 (1980) 613903. Fax: 44 (1980) 613284. E-mail: 100432.3200@compuserve.com.

TABLE 1. PCR primers used to clone regions of V antigen from *Y. pestis GB*

Primer[a]	Sequence	Enzyme[b]
5V2	ATGCGGATCCGAGCCTACGAACAAAACCCACAAC	*Bam*HI
5V74	ATCGGGATCCTAGCTTATTTTCTACCCGAGG	*Bam*HI
5V135	ATCGGGATCCTGAAAGTGATTGTTGATTCAATGAAT	*Bam*HI
5V215	ATCGGGATCCTCGAGAAAATGCCTCAAACCAC	*Bam*HI
5V186	ATCGGGATCCATGATAAATCCATTAATCTCATG	*Bam*HI
3V135	GGAAGTCGACTAAATATCATCATCGATACGATCGGC	*Sal*I
3V213	TTTTCTCGAGAATTTAGTACTCTGCG	*Xho*I
3V245	GGAAGTCGACTCATTTATTCTCACTTCCAAGAAAG	*Sal*I
3V275	ATCGATGTCGACTAATCCGAGCAGGTGGACCG	*Sal*I
3V326	GGATCGTCGACTTACATAATTACCTCGTGTCATTTACC	*Sal*I

[a] The 5 and 3 prefixes denote forward and reverse primers, respectively; the last number indicates the first or last primer-encoded V-antigen amino acid.
[b] In the sequences, restriction sites used for cloning are underlined.

become established. V antigen also has a role in the regulation of the low-calcium response (11, 18, 23).

We report here the cloning and purification of truncated forms of V antigen expressed as glutathione *S*-transferase (GST) fusion proteins to aid characterization of antigenic and protective regions of V antigen. Active immunization with GST fusion proteins has identified two distinct immunogenic regions. We have also localized the epitopes of V-antigen-specific MAbs to regions of the protein and identified a protective MAb. Both passive- and active-immunization data identify amino acids 135 to 275 as the major protective region of V antigen.

MATERIALS AND METHODS

Reagents. Plasmid pGEX-5X-2 and reagents required for GST fusion protein production were purchased from Pharmacia Biotech, St. Albans, United Kingdom. Other reagents were purchased from Boehringer unless stated otherwise.

Bacterial strains and cultivation. *Escherichia coli* JM109 was cultured in L broth (22) supplemented, if required, with ampicillin at a final concentration of 50 μg/ml (LB-Amp). *Y. pestis* GB was grown aerobically as described previously (5). *Y. pestis* GB is a human pneumonic plague isolate with a median lethal dose (MLD) of approximately 1 CFU given by the s.c. route in BALB/c mice and an outbred mouse strain (21).

DNA cloning. Chromosomal DNA was isolated by a standard method (6). Regions of the *lcrV* gene, which encodes V antigen (19), were amplified from *Y. pestis* GB DNA by PCR (22). The primers used for PCR are listed in Table 1. A *Bam*HI restriction site was introduced at the 5′ end of all forward primers to facilitate cloning into the *Bam*HI site of pGEX-5X-2. The *Bam*HI site (GGAT CC) of pGEX-5X-2 encodes the last two residues of glycine (XGG), all of isoleucine (ATC), and the first base of a proline residue (CXX) of GST. Codons that begin with a C encode amino acids leucine, proline, histidine, glutamine, and arginine. The forward primer was therefore designed to encode a region of V antigen with isoleucine followed by leucine, proline, histidine, glutamine, or arginine near the desired start site for the truncated protein. The reverse primer was designed to incorporate a stop codon at the desired residue and a restriction site compatible with the multiple cloning site of pGEX-5X-2. PCR was performed with a Perkin-Elmer model 9600 GeneAmp PCR system (35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min). DNA fragments were visualized on 1.5% agarose gels with a 100-bp ladder as a standard (Pharmacia Biotech). DNA bands of the expected size were excised and purified (Sephaglas BandPrep kit; Pharmacia Biotech). PCR products and pGEX-5X-2 digested with the appropriate restriction enzymes were ligated by using T4 DNA ligase and transformed into *E. coli* JM109 by electroporation (22). Ampicillin-resistant colonies were screened by PCR for *Y. pestis* DNA of the expected size. Purified plasmid DNA (Qiagen-tips Maxi-prep; Qiagen) was digested with restriction enzymes and sequenced across the multiple cloning site of pGEX-5X-2 to verify insertion of the PCR product.

Purification and analysis of recombinant proteins. GST-V fusion proteins were purified as previously described, with some modifications (5). Briefly, a stationary-phase culture containing *E. coli* JM109 plus recombinant plasmid was subcultured into 1 liter of LB-Amp. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM at an optical density at 600 nm of 0.8. The culture was grown for at least a further 5 h and then harvested at 4,500 × *g* and 4°C for 10 min (Beckman J2-HS centrifuge). The pellet was resuspended in 5 ml of phosphate-buffered saline (PBS) and frozen at −20°C. The cells were thawed, and 320 μl of lysozyme (10 mg/ml) was added. After 10 min at 22°C, Triton X-100 was added to a final concentration of 1%. The cells were sonicated for three 30-s bursts, with 1 min on ice between bursts (Braun Labsonic 2000 sonicator; large probe, low setting, 70% power). The sonicate was centrifuged at 12,000 × *g* and 4°C for 30 min (Beckman J2-HS), and then the supernatant was mixed with glutathione-Sepharose 4B (Pharmacia Biotech) for 1 h at 22°C with continuous agitation. The glutathione-Sepharose 4B was pelleted at 500 × *g* and 4°C for 5 min (Beckman GS-6R centrifuge) and then washed four times with 10 volumes of PBS. The matrix was incubated for 30 min at 22°C with an equal volume of 1 mM glutathione with agitation. The slurry was loaded onto an Econo-Pac chromatography column (Bio-Rad), and the eluate was collected. The bed was then washed with an equal volume of 1 mM glutathione, and the two eluates were pooled. After analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the presence of GST fusion protein, the sample was dialyzed extensively against PBS. The protein concentration was determined by bicinchoninic acid protein assay [Pierce & Warriner (UK) Limited] against a bovine serum albumin standard curve. Protein was concentrated by centrifugation through a 10,000-molecular-weight CO (10,000-MWCO) membrane (Centriplus; Amicon) as required.

SDS-PAGE was performed with precast 12% acrylamide Ready Gels (Bio-Rad). Proteins were electroblotted onto Immobilon P membranes (Millipore) and probed with V-antigen-specific rabbit polyclonal antiserum. Immunogold-labelled secondary antibodies were detected with Auroprobe reagents as recommended by the supplier (Cambio, Cambridge, United Kingdom).

V-antigen-specific polyclonal antibody. Polyclonal antibody was produced from a female New Zealand White rabbit injected with highly purified recombinant V antigen (rV antigen) (5). The rabbit received three 250-μg doses of rV antigen in incomplete Freund's adjuvant and PBS intradermally over a 12-week period. Serum was collected 21 weeks after the initial dose of rV antigen.

Immunization with recombinant proteins. Six-week-old female BALB/c mice reared under specific-pathogen-free conditions (Charles River, Margate, United Kingdom) were used in this study. Six animals per group were immunized intraperitoneally (i.p.) with 100 μl of recombinant protein in Alhydrogel (Superfos Biosector a/s, Vedback, Denmark). Antigen was administered at a molar concentration equivalent to 10 μg of rV antigen (267 pmol), and mice were given booster injections on day 21 as for the priming dose. The mice were tail bled, and sera were analyzed for V-antigen-specific immunoglobulin G (IgG) by enzyme-linked immunosorbent assay prior to challenge. Briefly, a microtiter plate was coated with 100 μl of rV antigen (5 μg of rV antigen per ml diluted in PBS). After the plates were blocked with 1% skim milk powder in PBS, the test serum was serially diluted in duplicate. Horseradish peroxidase conjugates against mouse polyvalent IgG and mouse IgG1 and IgG2a (Harlan Sera-lab, Loughborough, United Kingdom) were used at 1:2,000 as secondary antibodies. IgG1 and IgG2a secondary antibodies have been shown to be specific for the homologous subclass and to have equivalent sensitivities of detection (data not shown). Titers were calculated as the maximum dilution of serum to give a 414-nm absorbance reading 0.1 U greater than background and are presented as the reciprocal of the dilution.

Challenge with *Y. pestis*. Groups of six mice were challenged with approximately 10^5 or 10^7 MLD of *Y. pestis* GB (a fully virulent human pneumonic plague isolate) in a 100-μl volume by s.c. injection. Dilutions of the inoculum were plated onto Congo red agar plates and grown for 2 days at 28°C to enumerate the CFU per dose (1 CFU is equivalent to 1 MLD [21]). The animals were closely monitored throughout the working day over a 14-day period. The symptoms of plague infection in our model of infection are (i) starry or ruffled coat, (ii) lethargy, (iii) hunched back, (iv) reluctance to move, and (iv) incapacitance. In our model for plague, incapacitated animals that have been inoculated with *Y. pestis* GB do not recover and were therefore euthanized to alleviate suffering. More than 90% of the deaths reported in this study were unassisted, however, as most animals died between monitoring periods. Blood, liver, and spleen samples from a representative sample of animals that survived challenge were streaked onto Congo red agar plates as described above to check for the presence of *Y. pestis.*

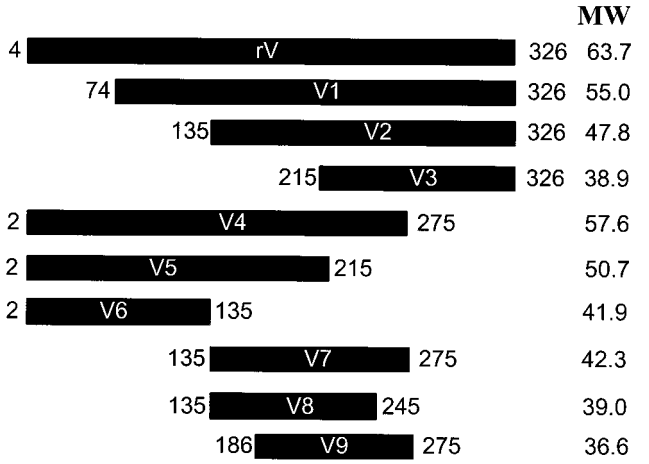

FIG. 1. Schematic representation of the regions of V antigen expressed as C-terminal GST fusion proteins. The first and last V-antigen amino acids are shown. The predicted molecular weight (MW) of each GST-V truncate is expressed in thousands.

Passive immunization. Tissue culture supernatants from mouse hybridomas expressing MAbs were raised against native rV antigen or SDS-denatured rV antigen. Tissue culture supernatant of MAbs shown below (see Table 3) was provided by T. J. Easterbrook, CAMR, Porton Down, Salisbury, Wiltshire, United Kingdom. MAb was concentrated from tissue culture supernatant with a 10,000-MWCO Centriplus concentrator (Amicon) and then diluted to 3.5 mg/ml with PBS. A 100-μl volume of MAb was administered i.p. 24 h prior to s.c. challenge with *Y. pestis* GB, as recommended by George Anderson, U.S. Army Medical Research Institute for Infectious Diseases, Ft. Detrick, Md. The challenge dose was calculated as 12 MLD, as described above. Mice were monitored for 16 days, and the time to death was recorded.

RESULTS

Production and purification of GST-V fusion proteins. Regions of the *lcrV* gene (4) were amplified by PCR from *Y. pestis* GB genomic DNA with primers containing restriction enzyme sites (Table 1). The PCR products were digested with restriction enzymes and cloned into pGEX-5X-2 (Pharmacia Biotech). The regions of V antigen cloned into pGEX-5X-2 and expressed as a GST fusion protein are shown in Fig. 1. Cloned regions were chosen on the basis of previous studies (12), the availability of cloning sites (see Materials and Methods), and computer-generated secondary-structure and antigenicity predictions for V antigen (DNASTAR software; results not shown). GST fusion proteins purified by affinity chromatography were analyzed by SDS-PAGE and Western blotting against rabbit polyclonal anti-V-antigen antibody (data not shown). Purified proteins were of the predicted molecular weight given in Fig. 1, and all proteins were stable, except for GST-V3 and GST-V9, which showed some degradation. Although all the proteins reacted with rabbit polyclonal anti-V-antigen antibody in Western blots, GST-V3 and GST-V9 produced weaker bands than the other proteins.

Antigenicity of GST-V1 to GST-V9. Mice immunized i.p. with GST-V and GST-V1 to GST-V9 were tail bled prior to challenge with *Y. pestis* GB. The sera from individual animals were pooled, and V-antigen-specific total IgG, IgG1, and IgG2a titers were determined by enzyme-linked immunosorbent assay (Fig. 2). An anti-V-antigen response was measured in all groups, and in all cases the IgG1 response was at least fourfold higher than the IgG2a response. The IgG1 isotype in the mouse is commonly associated with a cytokine profile indicative of a Th2 response (24).

The highest anti-V IgG titers (>100,000) were achieved with

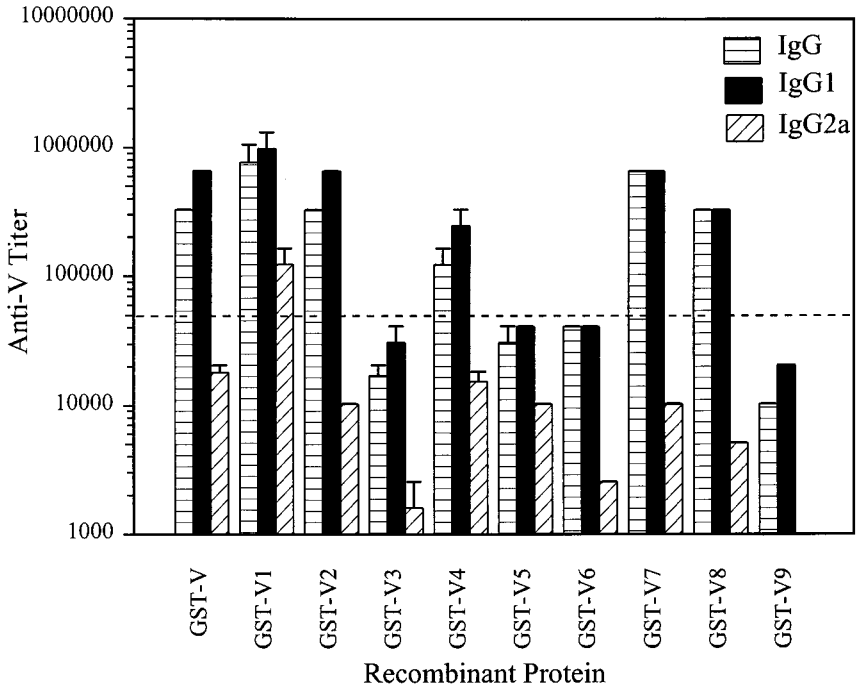

FIG. 2. Antibody titers of animals prior to challenge. The IgG, IgG1, and IgG2a titers of mice immunized with GST-V fusion protein are shown. Equal volumes of serum from individually bled mice were pooled and analyzed in duplicate. The corresponding protection is seen in Table 2. Results are expressed as the reciprocal of the titer (means ± standard errors of the means). The dotted line indicates a titer of 50,000. Truncates were deemed to be highly antigenic (titer > 100,000) or moderately antigenic (titer < 50,000).

TABLE 2. Protective efficacy of GST fusion proteins

Immunizing protein	Amino acids of V antigen	Challenge dose of *Y. pestis* GB (MLD)	Survivors per group
GST-V	4–326	1.1×10^5	6/6
		1.1×10^7	6/6
GST-V1	74–326	1.1×10^5	6/6
		1.1×10^7	6/6
GST-V2	135–326	1.1×10^5	6/6
		1.1×10^7	6/6
GST-V3	215–326	1.1×10^5	0/6
		1.1×10^7	0/6
GST-V4	2–275	1.1×10^5	6/6
		1.1×10^7	3/6
GST-V5	2–213	1.1×10^5	2/6
		1.1×10^7	0/6
None (untreated)	None	1.1×10^5	2/6
		1.1×10^7	0/6
GST-V6	2–135	1.0×10^5	5/6
		1.0×10^7	ND[a]
GST-V7	135–275	1.0×10^5	5/6
		1.0×10^7	ND
GST-V8	135–245	1.0×10^5	0/6
		1.0×10^7	ND
GST-V9	186–275	1.0×10^5	0/6
		1.0×10^7	ND
GST	None	1.0×10^5	0/6
		1.0×10^7	ND

[a] ND, not determined.

truncates containing amino acids 135 to 245. Truncates lacking all or part of this region (GST-V3, GST-V5, GST-V6, and GST-V9) produced comparatively reduced titers, which suggests that amino acids 135 to 245 encode a major antigenic region. Moderate titers (<50,000) generated against truncates GST-V3, GST-V5, and GST-V6 indicate that regions of V antigen other than amino acids 135 to 245 contribute to antigenicity but to a lesser extent.

GST-V1 to GST-V5 protection. Six animals per immunization group were challenged s.c. with approximately 1.1×10^5 or 1.1×10^7 MLD of *Y. pestis* GB and observed for 14 days (Table 2). GST-V-, GST-V1-, and GST-V2-immunized animals were protected at both challenge doses, suggesting that the first 134 amino acids are not necessary for protection and that the N-terminal boundary of a protective region lies at amino acid 135. GST-V4 provided complete protection against the lower dose, indicating that the C-terminal boundary of the protective region lies at amino acid 275. Although amino acids 135 to 275 clearly contain one or more protective epitopes, full protection against the higher challenge dose requires amino acids 276 to 326, as seen from the GST-V2 and GST-V4 protection data (Table 2). The contribution of amino acids 135 to 275 to protection was confirmed by immunization studies, since GST-V3 and GST-V5, which contain only part of this region, failed to protect fully against either challenge dose. There is therefore an overlap between the most antigenic region of V antigen and the region most important for protection.

GST-V6 to GST-V9 protection. In the previous challenge experiment, the use of a lower challenge dose (1.1×10^5 MLD) allowed GST-V3 and GST-V5 to be distinguished in terms of protection. Therefore, in order to define further the protective regions of V antigen, mice immunized with GST-V6 to GST-V9 were challenged with 10^5 MLD (Table 2).

GST-V7, GST-V8, and GST-V9 were used to probe the central region of the protein. Partial protection was noted only in the GST-V7 group, confirming the importance of amino acids 135 to 275 for immunity. Truncates GST-V8 and GST-V9, encoding only part of this region, did not confer protection on immunized mice, despite the fact that both are at least moderately antigenic.

Protection against 10^5 MLD was given by GST-V6 (amino acids 2 to 135), indicating that there are one or more minor protective epitopes at the N terminus of V antigen. This is the first indication of a protective region at the N terminus of V antigen. The limited protection seen with GST-V5 (Table 2) supports this observation.

Mapping MAbs to regions of V antigen. A set of MAbs had been raised against rV antigen in PBS, and a second set had been raised against SDS-denatured protein. Table 3 shows the degree of recognition of selected GST fusion proteins by MAbs in Western blotting studies. The MAbs fell into three main categories in terms of their binding to truncates. The group I MAbs did not recognize V antigen with deletions at the N terminus (GST-V1 to GST-V3) but did recognize GST-V4 and GST-V5, indicating that a significant part of their epitope is located within the first 73 amino acids. Group II MAbs strongly recognized GST-V1, GST-V2, and GST-V4 but not GST-V3 and GST-V5, which indicates that their epitope is located between amino acids 135 and 275. Previously, the epitope of one or more protective antibodies present in polyclonal antiserum had been mapped to a similar region (amino acids 168 to 275 [12]). The group III MAb could not be localized to a region of V antigen on the basis of its recognition pattern.

Passive protection with MAbs. Representative MAbs from group I (MAb 101.3) and group II (MAb 7.3) were chosen for studies of passive protection against plague. MAb (350 μg each) was administered i.p. to mice 24 h prior to s.c. challenge with 12 MLD of *Y. pestis* GB, and the animals were monitored for 16 days. MAb 7.3 provided complete protection when given alone or combined with MAb 101.3 (six of six mice in each group survived). There was no significant difference in protection between MAb 101.3 and nonimmune mouse serum (zero of six mice in each group survived). rV antigen-specific rabbit polyclonal antiserum provided limited protection, with four of six animals surviving to the end point. Passive-protection studies were necessarily performed with a relatively low challenge dose (12 MLD) compared with active-immunization challenges (10^5 and 10^7 MLD). The ability to passively protect against a challenge with 10 MLD of *Y. pestis* KIM has been reported as significant (12). Although MAb 7.3 may prove to be protective against higher challenge doses, protection against 12 MLD indicates that we have identified a protective MAb that recognizes an epitope located between amino acids 135 and 275.

DISCUSSION

As part of a program to analyze the structure of V antigen and relate it to function, we have immunized mice with truncated forms of V antigen expressed as GST fusion proteins.

TABLE 3. Recognition of truncates by anti-V-antigen MAbs in Western blots

MAb (recognition site)[a]	Recognition of the indicated fusion protein[b]					
	GST-V (aa 4–326)	GST-V1 (aa 74–326)	GST-V2 (aa 135–326)	GST-V3 (aa 15–326)	GST-V4 (2–275)	GST-V5 (aa 2–213)
Group 1 (aa 2–74)						
109.3[c]	+++	−	−	−	+++	+++
107.3[c]	+++	−	−	−	+++	+++
102.3[c]	+++	−	−	−	++	+
45.3[d]	+++	−	−	−	+	+
101.3[c]	+++	−	+/−	−	++	+
17.3[d]	+++	−	−	−	+	+/−
108.3[c]	+++	−	−	−	+	−
104.3[c]	+++	+/−	+/−	+/−	++	+
Group II (aa 135–275)						
7.3[d]	+++	+++	+++	−	+++	−
15.3[d]	+++	+++	+++	−	+++	−
51.3[d]	+++	+++	+++	−	+++	−
47.3	+++	+++	+++	−	+++	+/−
1.3[c]	+++	+++	+++	−	++	−
103.3[d]	+++	++	++	+	++	−
131.3[d]	+++	++	++	−	++	+
Group III (undefined)						
11.3[d]	+++	−	−	−	−	−

[a] MAbs are grouped according to their recognition pattern. aa, amino acids.
[b] Recognition was assessed qualitatively as strong (+++), moderate (++), weak (+), equivocal (+/−), or nonexistent (−). Numbers in parentheses indicate amino acids of V antigen.
[c] MAb was produced from native rV.
[d] MAb was produced from denatured rV.

Immunization with fusion proteins containing amino acids 74 to 326 (GST-V1) and 135 to 326 (GST-V2) protected mice against 1.1×10^7 MLD of *Y. pestis* GB, the highest challenge dose tested. Protection at this level of challenge compares with previously reported protection data for V antigen, where mice survived a challenge of 2×10^7 MLD of *Y. pestis* GB but some breakthrough occurred at 2×10^8 MLD (27). GST-V2 therefore provides a level of protection similar to that of full-length rV antigen against s.c. challenge with *Y. pestis*. The N terminus of a protective region has thus been mapped to amino acid 135. This is consistent with previous protection data showing that a protein A fusion protein lacking 67 amino acids from the N-terminal end of V antigen protected mice against 10^4 MLD of *Y. pestis* KIM (14).

Mice immunized with GST-V4 were completely protected against a challenge dose of 1.1×10^5 MLD of *Y. pestis* GB, but there was only 50% survival at the higher challenge dose (1.1×10^7 MLD). The results indicate that the C terminus of a protective region lies at amino acid 275. However, the last 51 amino acids are important for protection and may encode additional epitopes, or their omission might cause conformational changes that affect immunogenicity.

Reduced protection in mice immunized with GST-V3 and GST-V5 supports the theory that a protective region lies between amino acids 135 and 275, as these truncates contain only part of this region. Five of six mice immunized with GST-V7 were protected against 10^5 MLD, which confirmed the presence of one or more protective epitopes within this central region of V antigen. The results agree with those of a previous passive-protection study, in which immune sera adsorbed with truncated forms of V antigen were used to protect mice passively against 10 MLD of *Y. pestis* KIM (12). Although the challenge dose was necessarily low, the results clearly demonstrated the presence of one or more protective epitopes between amino acids 168 and 275.

All of the immunized mice produced either a high (>100,000) or a moderate (<50,000) titer of V-antigen-specific IgG. All of the truncates containing amino acids 135 to 245 induced a high titer, whereas those containing none or only part of this region had moderate titers, thus suggesting that the major antigenic determinants of V antigen are located between amino acids 135 and 245. The putative major antigenic region therefore overlaps with the major protective region of V antigen.

Attempts to define further the central protective region in this model failed, as GST-V8 and GST-V9 were not protective. Analysis of the V-antigen-specific IgG responses shows that GST-V8 elicited an antibody response similar to that of GST-V, indicating that loss of protection cannot be attributed to a reduction in the V-antigen-specific IgG titer. The results indicate that amino acids 135 to 186 encode all or part of one or more protective epitopes or that these residues are important for the conformation of a more centrally located epitope. Combination of the results of this study with the results of Motin and coworkers, who localized one or more protective epitopes to amino acids 168 to 275 (12), suggests that residues 168 to 186 are crucial for the formation of one or more protective epitopes. Again, these residues may encode part of the epitope or may be important for protein folding. Similar conclusions for the role of amino acids 246 to 275 were drawn on the basis of GST-V9 protection data. It is possible that protection offered by GST-V8 and GST-V9 against plague might be distinguished if immunized animals are challenged with a lower dose of *Y. pestis.*

Protection against 10^5 MLD by active immunization with GST-V6 demonstrated the presence of a protective region at

the N terminus (amino acids 2 to 135). GST-V5 also conferred a degree of protection. As GST-V5 lacks a large portion of the central protective region, and small deletions within amino acids 135 to 275 (Table 2) abolished protection against 10^5 MLD, it is likely that protection also derives from the N-terminal residues of V antigen. Differences observed between GST-V5 and GST-V6 in the level of protection might be due to differences in the challenge dose (1.1×10^5 versus 1.0×10^5 MLD). Alternatively, the addition of amino acids 136 to 213 might mask, destabilize, or alter the conformation of protective epitopes located within the first 135 amino acids of GST-V5.

Results of Western blotting experiments suggested that a group of MAbs recognized epitopes located wholly or partly within the N-terminal 74 amino acids (group I) and that another group recognized epitopes within the central protective region (group II). It is interesting that six of the eight MAbs that bound to the N terminus were generated against nondenatured rV antigen. In contrast, six of seven MAbs that recognized an epitope found between amino acids 135 and 275 were generated against SDS-denatured rV antigen. Denaturation of rV antigen might alter protein folding to such an extent that centrally located B-cell epitopes were exposed.

Passive immunization with MAb 7.3 (group II) resulted in protection against an s.c. challenge with 12 MLD of *Y. pestis* GB. This result confirms the importance of amino acids 135 to 275 for protection and indicates the presence of at least one protective B-cell epitope. MAb 101.3, a representative of the group I MAbs, did not protect against plague. However, other group I MAbs, might prove to be protective in this model, although preliminary analysis of the antibodies described in Table 3 (except for MAbs 11.3, 102.3, and 131.3) has failed to identify any further protective group I or group II MAbs (data not shown).

Sequence analysis of the *lcrV* gene from strains of *Y. pestis* and *Y. pseudotuberculosis* showed that V antigen is highly conserved (13). Sequence variability between strains was noted, however, between amino acids 177 and 256 of *Y. pestis* KIM V antigen, with a highly variable region centered at amino acid 227 (13). Roggenkamp et al. (20) have recently shown that two forms of *lcrV* exist in yersiniae. One form is found in *Y. enterocolitica* O8 strains (LcrV-Yen O8), and another form is typically found in *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* O3, O9, and O5,27 strains (LcrV-Yps). DNA sequencing has indicated that the main differences between LcrV-Yen O8 and LcrV-Yps reside in a hypervariable region (amino acids 225 to 232). Furthermore, serum raised against LcrV-Yen O8 and LcrV-Yps passively protected mice against challenge with yersiniae, but only if the immunizing V antigen was the same group as that derived from the challenge organism (20). The investigators attributed the difference in protection between species to this hypervariable region, suggesting that it encodes a major protective epitope. The proposed location of such an epitope is consistent with the active- and passive-immunization data presented in this study.

In summary, the existence of two distinct regions of V antigen that protect mice against high levels of a fully virulent strain of *Y. pestis* has been demonstrated by active immunization. The major protective region is located between amino acids 135 and 275 and overlaps the major antigenic region (amino acids 135 to 245). We have also shown that a protective MAb recognizes an epitope located within this region. Protection was also conferred by an N-terminal portion of V antigen (amino acids 2 to 135). Previous studies have shown that a protein A–V-antigen fusion peptide containing amino acids 68 to 326 of V antigen has immunosuppressive properties (14). Amino acids 25 to 40 have also been implicated as important for virulence function by the generation of nonpolar deletions within *lcrV* of *Y. pestis* (23). It is therefore possible that two regions of V antigen are involved in virulence. Immunization with GST-V6 and GST-V7 might generate separate pools of neutralizing antibodies that reduce the virulence function of V antigen. It would therefore be interesting to compare the level of protection achieved in mice coimmunized with GST-V6 and GST-V7 with that of singly immunized animals and to define the upper limit of GST-V2 protection.

Work to identify functionally important regions of V antigen is in progress, making use of the truncates and MAbs described in this report. A combination of data describing protective and functional properties of V antigen will assist the development of safe and effective new-generation vaccines against plague.

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