INFECTION AND IMMUNITY, Oct. 1996, p. 4313–4318 Vol. 64, No. 10
0019-9567/96/$04.00+0

V Antigen-Polyhistidine Fusion Peptide: Binding to LcrH and Active Immunity against Plague

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Received 12 June 1996/Returned for modification 10 July 1996/Accepted 27 July 1996

The structural gene for V antigen (*lcrV*) is known to be encoded within the *lcrGVH-yopBD* operon of the ~70-kb low-calcium-response or Lcr plasmid of *Yersinia pestis*. This 37-kDa monomeric peptide was reported to provide active immunity in mice, suppress inflammatory cytokines, and regulate expression of the low calcium response (Lcr^+). Here we describe pVHB62, encoding a polyhistidine-V antigen fusion peptide (V_h) and linked LcrH. V_h underwent degradation from both the C terminus and N terminus during classical chromatographic fractionation but remained intact within two compartments during Ni^{2+} affinity chromatography. The first was homogeneous, capable of active immunization (mouse intravenous 50% lethal dose, $>10^7$ bacteria), and stable at 4°C. The second remained bound to the affinity column but could be eluted as a mixture of V_h, LcrH, and low-molecular-weight material by application of 6 M guanidine · HCl. This mixture was dialyzed, denatured in 8 M urea, and again applied to the affinity column, which then bound V_h but not LcrH. The latter was recovered and renatured, and low-molecular-weight material was removed by biochemical fractionation. The resulting homogeneous LcrH bound protein A-V antigen fusion peptide but not protein A in a sandwich enzyme-linked immunosorbent assay, and this reaction was inhibited by V_h. These observations indicate that LcrH normally binds V antigen in bacterial cytoplasm and suggest that only free LcrH downregulates expression of the low calcium response.

The ~70-kb low-calcium-response or Lcr plasmid shared by *Yersinia pestis* and enteropathogenic *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* encodes exported virulence factors including V antigen and certain yersinia outer proteins termed Yops. Transcription of most if not all of these activities is coordinately mediated by the activator LcrF (VirF in *Y. enterocolitica*) (8, 14, 16, 38) and is regulated so that full induction occurs at 37°C in Ca^{2+}-deficient (≤1.0 mM) medium containing ≥20 mM Mg^{2+} (1, 6, 33, 34). This environment promotes bacteriostasis in vitro (13) unless Na^+ is also omitted (11) or exogenous nucleotides are present (40). The structural gene encoding V antigen (*lcrV*) is located within an *lcrGVH-yopBD* operon (4, 27). V antigen functions as a virulence factor by suppressing inflammatory cytokines (24, 25). Neutralization of this activity by specific antibody directed against at least one internal epitope present on a staphylococcal protein A-V antigen fusion protein (PAV) but not on protein A (PA) (22) provided significant protection against experimental plague in mice (17, 18, 22). *lcrV* isolates are avirulent and Ca^{2+} independent (i.e., do not require Ca^{2+} for growth at 37°C) and are unable to express maximum titers of Yops, suggesting that V antigen fulfills an additional role as regulator of the low calcium response (4, 26, 32). *lcrG* (31) and *lcrH* mutants (4, 28) are also avirulent but "Ca^{2+} blind" in that they fail to grow at 37°C in media containing sufficient Ca^{2+} (2.5 mM) to downregulate LcrF-mediated functions and thus promote vegetative growth of wild-type yersiniae. LcrH (SycD in *Y. enterocolitica*) was reported to function as a chaperone protein for YopD (37), which in turn mediates transfer of cytotoxic Yops into host cell cytoplasm (12, 29).

V antigen is a secreted (7, 17) 37-kDa monomeric peptide that undergoes marked degradation from the C-terminal end during purification by traditional chromatographic procedures (5, 22). The purpose of this report is to describe recombinant plasmid pVHB62, encoding a polyhistidine-V antigen fusion peptide (V_h) and linked LcrH. V_h underwent hydrolysis from both the C-terminal and N-terminal ends when subjected to classical methods of purification known to degrade recombinant V antigen. However, the peptide could be purified to homogeneity in two steps by metal-chelation chromatography, was stable in solution, and could protect mice against intravenous challenge with $>10^7$ Lcr^+ yersiniae. Coordinately induced LcrH remained tenaciously bound to V_h during metal-chelation chromatography but could be separated by additional affinity chromatography under denaturing conditions. After renaturation, LcrH bound PAV but not PA in a sandwich enzyme-linked immunosorbent assay (ELISA), and this reaction was inhibited by V_h. This finding indicates that LcrH and V antigen normally exist as a complex within bacterial cytoplasm.

MATERIALS AND METHODS

Bacteria. Protease-deficient *Escherichia coli* BL21(DE3) [F^- *ompT lon* r_B^- m_B^- λ(DE3)] (Novagen, Madison, Wis.) carrying the T7 RNA polymerase gene under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lacUV5* promoter was used for expression of pVHB62-encoded $lcrV_h$-*lcrH* regulated by the linked T7 promoter. This strain was also utilized for production of pPAH10-encoded staphylococcal protein A-LcrH fusion peptide (PAH). Actively immunized mice were challenged with a nonpigmented mutant (15) of wild-type *Y. pestis* KIM (strain D27) which is fully virulent (50% lethal dose, ~10 bacteria) by intravenous injection (35).

Plasmids. Methods for preparation of plasmid DNA, its digestion with restriction endonucleases, treatment with Klenow enzyme, ligation, sequencing, and transformation into *E. coli* have been described (30). The vector pRSET C (Invitrogen, San Diego, Calif.) containing sequences encoding the T7 promoter and ATG translation initiation codon, six histidine residues, a transcript stabilizer from gene 10 of phage T7, and the enterokinase cleavage site was used for construction and expression of gene fusions. Recombinant plasmid pBVP5 containing the *lcrGVH-yopBD* operon of *Y. pseudotuberculosis* (23) was used as a source of *lcrV* and *lcrH*. pPAV13 containing the structural gene for staphylococ-

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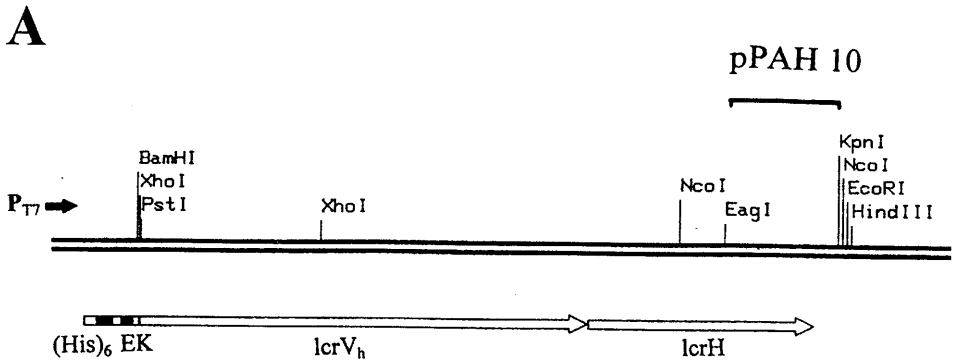

B

ATG CGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT
Met Arg Gly Ser His His His His His His Gly Met Ala

AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC
Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr

BamHI XhoI
GAC GAT GAC GAT AAG GAT CGA TGG ATC CGA CCT CGA GAT
Asp Asp Asp Asp Lys Asp Arg Trp Ile Arg Pro Arg Asp
(EK) ^

PstI Start of *lcrV* portion
CTG CAG ATC GAA TTG CTC AAG AAA ATC CTA . . .
Leu Gln Ile Glu Leu Leu Lys Lys Ile Leu

FIG. 1. (A) Portion of the physical map of the recombinant plasmid pVHB62 showing the hybrid gene $lcrV_h$ encoding V antigen fused with the polyhistidine sequence $(His)_6$ ligated to the T7 promoter of the vector pRSET C; *lcrV* (prior to fusion) and intact *lcrH* originated from the *lcrGVH-yopBD* operon of *Y. pseudotuberculosis* (23). The direction of transcription for these genes is shown by arrowheads, and the sequence used to express truncated LcrH by pPAH10 is designated. (B) Nucleotide sequence of 5′ end of $lcrV_h$ showing the enterokinase recognition site (EK) and cleavage site (^) of pRSET C; the sequence encoding the epitope recognized by commercial monoclonal anti-Xpress antibody is underlined.

cal PA fused in frame with *lcrV* (22) was used to construct a hybrid gene encoding a similar fusion between PA and LcrH, whose gene is located immediately downstream from *lcrV*. To engineer $lcrV_h$, the same 1.5-kb *Eco*RV fragment of plasmid pBVP5 previously used to construct pPAV13 was introduced into the *Pvu*II site of pRSET C and a recombinant plasmid containing an insertion in frame with the polyhistidine sequence was selected (pVHB62). Correctness of frame was verified by sequencing. The resulting fusion protein, V_h, possessed a 259-amino-acid C-terminal sequence originating from *lcrV* and a short leader peptide including the enterokinase cleavage site plus six tandem histidine residues that function as a metal binding domain during chelation chromatography (Fig. 1B). V_h thus exists as a 300-amino-acid monomeric peptide with a predicted molecular mass of 34.4 kDa and an isoelectric point of 6.1.

To construct the gene encoding PAH, pPAV13 was digested by restriction endonucleases *Bam*HI and *Eag*I, ends were filled by treatment with Klenow enzyme, and DNA was self-ligated. pPAV13 possesses distinct recognition sites for these two enzymes, which, as a consequence of this process, removed *lcrV* and about half of *lcrH*. The resulting selected plasmid, termed pPAH10, thus possessed an in-frame fusion between the gene encoding PA and a part of *lcrH* (Fig. 1A). The ensuing peptide contained PA fused with 58 C-terminal amino acid residues of LcrH and, after purification by affinity chromatography, was used to raise anti-LcrH in rabbits.

Purification of soluble V_h. Cells of *E. coli* BL21(DE3)/pVHB62 were aerated at 37°C in 10-liter fermentor vessels containing M9ZB medium (1% Sheffield NZ Amine Type A [Kraft Inc., Memphis, Tenn.], 0.1% NH_4Cl, 0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.5% NaCl, 1 mM $MgSO_4$, 6 mM D-glucose, and ampicillin [50 μg/ml]). V_h was induced by addition of 0.1% IPTG when the cultures achieved an optical density of ~1.2 at 620 nm. After an additional 4 h of incubation, the bacteria were harvested by centrifugation (10,000 × *g* for 30 min) and suspended in 0.025 M Tris · HCl buffer (pH 7.8). Preparations of this type were utilized in experiments concerned with degradation as described previously (5, 20). The bacteria were subjected to disruption in the French press (SLM Instruments, Inc., Urbana, Ill.), centrifuged again to remove insoluble debris, and used for hydrophobic interaction chromatography on phenyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) with 0.05 M Tris · HCl buffer (pH 7.8) or ion-exchange chromatography on DEAE-cellulose (Whatman, Inc., Clifton, N.J.) with a 0 to 0.5 M gradient of NaCl in 0.05 M Tris · HCl buffer (pH 7.8).

Metal affinity chromatography was undertaken with samples prepared with pelleted IPTG-induced bacteria suspended in 0.05 M Tris · HCl–0.15 M NaCl, pH 8.0. This suspension received lysozyme (5 mg/ml) followed in 1 h by Triton X-100 (0.1%). After overnight incubation (22), 500-ml samples of the resulting solubilized proteins were diluted against an eightfold-concentrated stock of binding buffer to yield a final concentration of 0.5 M NaCl, 5 mM imidazole, and 0.1% Triton X-100 in 20 mM Tris · HCl, pH 8.0, and applied to a column (2.5 by 20 cm) containing an 80-ml packed volume of chelating Sepharose (Pharmacia) charged with Ni^{2+}. The column was first washed with 10 void volumes of binding buffer and then 10 void volumes of wash buffer (0.5 M NaCl and 60 mM imidazole in 20 mM Tris · HCl [pH 8.0]). V_h was eluted with 6 void volumes of elution buffer (0.5 M NaCl, 0.8 M imidazole, and 0.2% Triton X-100 in 20 mM Tris · HCl [pH 8.0]) and dialyzed against 0.05 M sodium phosphate buffer, pH 8.0, containing 0.3 M NaCl and 0.2% Triton X-100. After dialysis, 500-ml samples were applied to a column (2.5 by 10 cm) containing a 30-ml packed volume of Ni-NTA agarose (Qiagen, Chatsworth, Calif.). The column was washed with

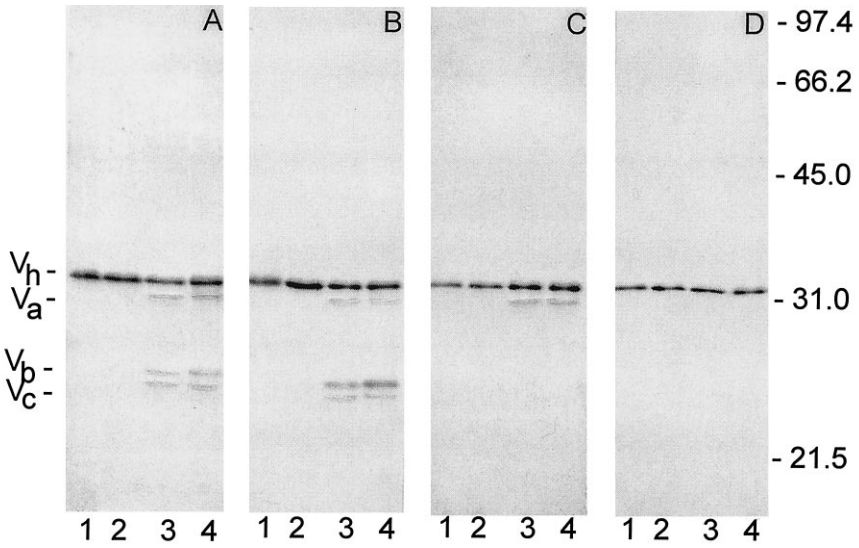

FIG. 2. Immunoblots prepared with rabbit polyclonal anti-V antigen (A), mouse monoclonal anti-V antigen 15A4.8 directed against an internal epitope (B), mouse monoclonal anti-V antigen 17A4.6 directed against a C-terminal epitope (C), and commercial mouse monoclonal anti-Xpress antibody directed against the N-terminal polyhistidine stretch (D) showing reactions against whole cells of *E. coli* BL21(DE3)/pVHB62 (lanes 1), clarified cytoplasmic extracts (lanes 2), fraction recovered after chromatography on phenyl-Sepharose CL-4B (lanes 3), and fraction recovered after chromatography on DEAE-cellulose (lanes 4). Bands corresponding to V_h and the degradation products V_a, V_b, and V_c are designated; numbers on the right indicate molecular mass in kilodaltons.

10 void volumes of 0.05 M sodium phosphate buffer, pH 8.0, and then with 10 void volumes of 0.05 M sodium phosphate buffer, pH 6.0. Addition of 0.05 M sodium phosphate buffer, pH 3.5, resulted in prompt elution of essentially homogeneous V_h, which was immediately adjusted to pH 7 by dropwise addition of 0.5 M sodium phosphate buffer, pH 9.2, and stored at −20°C prior to immunization.

Denaturing Ni-NTA chromatography. V_h not released by 0.05 M sodium phosphate buffer, pH 3.5, from the Ni-NTA column described above was recovered by application of 6 M guanidine · HCl, pH 3.0. The resulting preparation was dialyzed against 0.025 M Tris · HCl, pH 8.0, and then solid urea was added to achieve a concentration of 8 M. This sample was applied to the Ni-NTA column equilibrated with Tris-urea buffer (8 M urea in 0.025 M Tris · HCl [pH 8.0]), and eluted material was dialyzed against 0.025 M Tris · HCl, pH 8.0, prior to analysis. The column was washed with 10 void volumes of Tris-urea buffer; remaining bound V_h was then eluted with Tris-urea buffer adjusted to pH 3.5 and dialyzed against 0.025 M Tris · HCl, pH 8.0. All samples were stored at −20°C prior to analysis.

Purification of LcrH and LcrH-sandwich ELISA. Material eluted from the Ni-NTA column during denaturing chromatography in 8 M urea was collected, renatured by dialysis against 0.025 M Tris · HCl, pH 8.0, and applied to a column (1.0 by 10 cm) containing DEAE-cellulose (Whatman). Proteins were eluted with a linear gradient (0 to 0.35 M NaCl in 0.05 M Tris · HCl [pH 7.8]), and those fractions that reacted with anti-LcrH antibodies by immunoblotting were pooled. This procedure permitted isolation of essentially homogeneous LcrH, which was used in ELISA experiments.

Immulon 4 microtiter plates (Dynatech, Chantilly, Va.) were coated with 100 μl of a solution (5 μg/ml) of either LcrH or bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS). The plates were sealed and incubated overnight at 4°C, and then the wells were washed with PBS containing 0.1% Tween 20 and 0.25% BSA (PBSTB). To prevent nonspecific binding, blocking solution consisting of 1% BSA in PBSTB was applied and the plates were incubated for 2 h at room temperature. Twofold antigen dilutions in PBSTB were then added to the wells, and the plates were incubated for 0 to 30 h at 4°C. To perform competitive analyses, homogeneous V_h (50 μg/ml) was added together with antigen consisting of purified PAV or PA at concentrations of 2 to 16 μg/ml. These preparations were incubated with mouse immunoglobulin G2a (IgG2a) (Calbiochem, San Diego, Calif.), capable of binding to the PA domain. After addition of antigen, goat anti-mouse IgG (Fc specific) conjugated with horseradish peroxidase (Sigma) was added to each well at a dilution of 1:5,000 in PBSTB and the plates were incubated for 1 h at room temperature. The presence of these anti-mouse Fc antibodies was detected by reaction with *O*-phenylenediamine hydrochloride in stable peroxidase buffer (Pierce, Rockford, Ill.). The reaction was stopped by addition of 1 M H_2SO_4, and A_{490} was determined within 30 min.

Purification of PAH. PAH was purified to near homogeneity by affinity chromatography on IgG-Sepharose 6FF (Pharmacia) exactly as described for PAV (22).

Antibodies. Monoclonal antibodies (MAbs) recognizing nonconformational epitopes of V antigen have been described previously (22). Of these, MAb 17A4.6 reacted with an epitope present on the last 50 C-terminal amino acids and MAb 15A4.8 recognized an internal epitope within amino acid residues 168 to 275. Commercial MAb anti-Xpress (Invitrogen), recognizing the sequence Asp-Leu-Tyr-Asp-Asp-Asp-Lys-Asp, was used to detect the leader peptide of V_h containing the polyhistidine stretch. Rabbit anti-egg white lysozyme was purchased from Chemicon (Temecula, Calif.). Anti-LcrH was raised in rabbits injected with PAH in TiterMax adjuvant (CytRx Corp., Norcross, Ga.), as described for PAV (22). The resulting IgG was purified and absorbed with an excess of disrupted and lyophilized cells of *E. coli* BL21 in order to remove naturally occurring antibodies directed against this species (22).

Active immunization with V_h. Female Swiss-Webster mice (Charles River Laboratories, Wilmington, Mass.) 5 to 6 weeks of age were immunized with V_h in TiterMax adjuvant according to the protocol used for PAV (22). Titers of anti-V antigen in actively immunized mice were determined by ELISA as previously described (22). Mice were challenged intravenously 14 days after the last immunization and observed daily for 3 weeks to ascertain 50% lethal dose and mean survival time.

Miscellaneous. Calf intestine enterokinase (Boehringer Mannheim, Indianapolis, Ind.) was used to eliminate the polyhistidine-containing sequence of V_h. Incubation was carried out in 0.05 M Tris · HCl, pH 8.0, for 20 h at 37°C at an enzyme-to-substrate ratio of 1:40 (wt/wt). Peptides were located by silver staining (21) following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to protocols defined previously (22). Alkaline phosphatase conjugated with anti-rabbit or anti-mouse IgG (Sigma) was used as a secondary antibody in immunoblots performed by established methods (22). Protein was determined by the method of Lowry et al. (19). Fisher's exact probability test was used to determine the statistical significance of observed active immunity.

RESULTS

Degradation of V_h. The ability of crude V_h to undergo degradation during chromatography on DEAE-cellulose and phenyl-Sepharose was determined with cytoplasm obtained from disrupted IPTG-induced cells of *E. coli* BL21(DE3)/pVHB62. Both of these procedures generated three major degradation products, termed V_a (32 kDa), V_b (25 kDa), and V_c (24 kDa), that were visible in immunoblots prepared with polyclonal anti-V antigen (Fig. 2A) or MAb 15A4.8 raised against an internal epitope (Fig. 2B). MAb 17A4.6, directed against an epitope located at the C terminus, also recognized V_a (Fig. 2C) as opposed to the anti-Xpress MAb specific for the N-terminal polyhistidine stretch (Fig. 2D). These findings indicate that V_a arose via loss of an N-terminal portion of V_h. In contrast, V_b

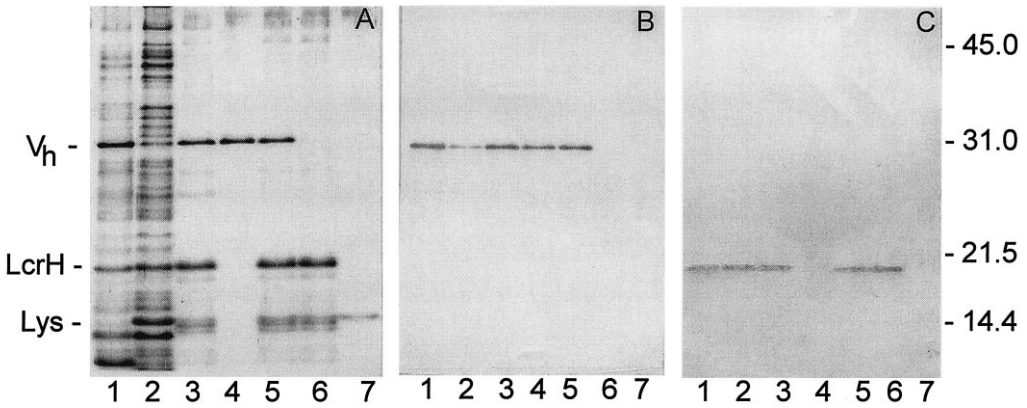

FIG. 3. Silver-stained (SDS-PAGE, 10 to 16%) gel (A) and corresponding immunoblots developed with mouse monoclonal anti-V antigen 15A4.8 (B) or rabbit polyclonal antiserum raised against staphylococcal PA-LcrH fusion peptide (C). Lanes 1, washed whole cells of *E. coli* BL21(DE3)/pVHB62 after induction with IPTG; lanes 2, corresponding clarified cell-free extract; lanes 3, eluent from the Ni^{2+}-charged Sepharose column; lanes 4, essentially homogeneous V_h eluted from the Ni-NTA agarose column; lanes 5, residue stripped from Ni-NTA column with 6 M guanidine · HCl, pH 3.0; lanes 6, dialyzed residue from the Ni-NTA column after reapplication in and elution with 8 M urea; lanes 7, commercial egg white lysozyme used for bacterial lysis. Positions for V_h, LcrH, and lysozyme (Lys) are shown on the left, and molecular masses of standards in kilodaltons are given on the right.

and V_c failed to react against either MAb 15A4.8 or the anti-Xpress MAb, showing that they had lost segments of both the N-terminal and C-terminal ends of V_h. These results were unexpected because only portions of the C terminus were removed during similar treatment of whole recombinant V antigen (22).

Affinity chromatography of recombinant V_h. Significant V_h appeared as a major peptide of 34.4 kDa in samples of whole IPTG-induced cells of *E. coli* BL21(DE3)/pVHB62 (Fig. 3A, lane 1; Fig. 3B, lane 1). This remarkable expression, supported by the powerful T7 promoter, resulted in partial insolubility, with loss of ~80% of total activity upon lysis of the cells and clarification of the resulting extract (Fig. 3A, lane 2; Fig. 3B, lane 2). Nevertheless, the resulting concentration of soluble V_h typically exceeded that of V antigen in cell-free extracts of Ca^{2+}-starved yersiniae (5). The fusion peptide could be purified to near homogeneity in one step by metal chelate affinity chromatography on Ni^{2+}-charged chelating Sepharose (Fig. 3A, lane 3; Fig. 3B, lane 3). An additional step of purification on higher-affinity Ni-NTA agarose resulted in recovery of essentially pure V_h (Fig. 3A, lane 4; Fig. 3B, lane 4). However, only ~15% of the peptide added to the Ni-NTA agarose column was retrieved during this step, resulting in final yields of ~1 mg of V_h per liter of fermentor medium. V_h did not undergo detectable degradation during storage (either frozen or at 4°C) but did tend to form insoluble aggregates upon repeated freezing and thawing. V_h prepared by this process was frozen once and then used for active immunization. Digestion of V_h by enterokinase to remove hexahistidine did not significantly improve its solubility (data not shown).

Interaction between V_h and LcrH. As noted above, elution of V_h from the Ni-NTA agarose column was incomplete. To determine the nature of the retained material, the column was stripped by application of 6 M guanidine · HCl, pH 3.0. This process resulted in elution of two major peptides with molecular masses of ~34 and ~18 kDa, shown by immunoblotting to correspond to V_h (Fig. 3B, lane 5) and LcrH (Fig. 3C, lane 5), respectively. In addition, denatured complexes containing lysozyme, which was initially added in great excess (5 mg/ml), were eluted as aggregates of 12 to 14 kDa (Fig. 3A, lanes 5 to 7). The Ni-NTA column is capable of binding polyhistidine residues of denatured proteins. Accordingly, the material eluted with 6 M guanidine · HCl was dialyzed, brought to 8 M with respect to urea, and again passed through the Ni-NTA column. This process again resulted in all V_h being bound but permitted elution of now dissociated LcrH (Fig. 3A, lane 6; Fig. 3C, lane 6).

LcrH sandwich ELISA. LcrH obtained during this step from the Ni-NTA column was renatured by dialysis, and low-molecular-weight material was removed by chromatography on DEAE-cellulose. The resulting highly purified LcrH was then used as a probe for PAV in a sandwich ELISA. In these assays, LcrH (attached to the surface of microtiter wells) was exposed to PAV, which, after binding, was determined by its ability to couple mouse IgG2a. The latter was estimated colorimetrically by interaction with horseradish peroxidase-conjugated Fc-specific anti-mouse IgG. Binding of PAV to LcrH at 4°C as a function of time is shown in Fig. 4A; saturation was complete in 30 h. This reaction was markedly inhibited by competition with V_h for binding sites on LcrH (Fig. 4B).

Active immunization with V_h. Mice were immunized with V_h

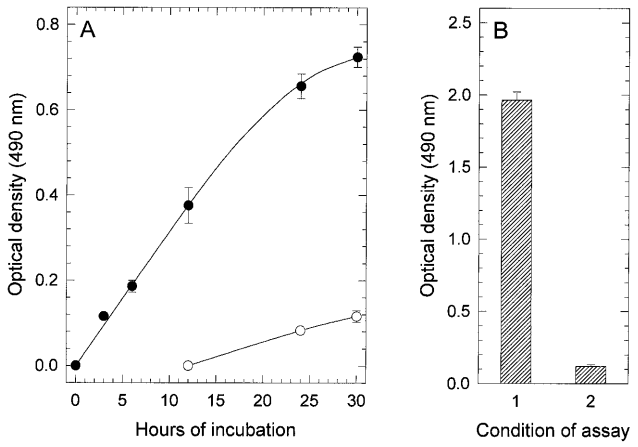

FIG. 4. (A) Binding of staphylococcal PAV (●) and PA (○) to LcrH at 4°C in a sandwich ELISA as a function of time; the concentration of both peptides was 4 μg/ml. (B) Binding of PAV to LcrH without added V_h (bar 1) and with excess V_h (bar 2); the concentration of PAV was 16 μg/ml, and the time of incubation was 29 h (wells coated with LcrH were initially treated with 50 μg of V_h per ml of PBS for 6 h at 4°C, and the solution of added PAV contained the same concentration of V_h (bars indicate standard errors of triplicate samples).

TABLE 1. Ability of V_h to actively immunize mice against intravenous challenge with Lcr^+ cells of *Y. pestis* KIM

No. of injected bacteria	Bacterial MLD[a]	Immunogen	No. of mice surviving on postinfection day(s):														
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14–21
10^2	10^1	None[b]	9	9	9	8	7	6	3	2	2	2	1	1	0	0	0
10^3	10^2	None	10	10	10	8	6	0	0	0	0	0	0	0	0	0	0
10^4	10^3	None	10	10	10	9	4	3	2	0	0	0	0	0	0	0	0
10^2	10^1	V_h	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10^c
10^3	10^2	V_h	10	10	10	10	10	9	9	9	9	9	9	9	9	9	9^c
10^4	10^3	V_h	10	10	10	10	9	9	9	9	9	9	9	9	9	9	9^c
10^5	10^4	V_h	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10^c
10^6	10^5	V_h	10	10	10	9	9	9	9	9	9	9	9	9	9	9	9^c
5×10^6	5×10^5	V_h	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10^c
10^7	10^6	V_h	10	10	10	10	8	7	7	7	7	6	6	6	6	6	6

[a] MLD, minimal lethal dose.
[b] None indicates immunization with adjuvant alone.
[c] $P < 0.001$.

according to the protocol described for PAV, and the resulting concentrations of antibodies were similarly determined by ELISA (22). Results showed that titers after injection of V_h were about 100 times greater than those previously determined for mice actively immunized with PAV (data not shown). Control mice previously injected with adjuvant alone and mice injected with V_h emulsified in adjuvant were challenged intravenously with Lcr^+ cells of *Y. pestis*. As shown in Table 1, the 50% lethal dose for immunized mice was $>10^7$ organisms.

DISCUSSION

The course of plague in mice is now known to reflect suppression of tumor necrosis factor alpha and gamma interferon. This inhibition enables Lcr^+ cells to form necrotic lesions in liver and spleen that expand and eventually coalesce, resulting in loss of organ function followed by death. In contrast, similar infection with Lcr^- or *lcrV* mutants results in prompt synthesis of inflammatory cytokines which mediate infiltration of inflammatory cells with attendant generation of protective granulomas (25, 36). Proof that this anti-inflammatory effect is caused by V antigen was provided by demonstrating that antiserum raised against the fusion protein PAV prevented disease by permitting normal production of tumor necrosis factor alpha and gamma interferon and that injected PAV markedly inhibited synthesis of these cytokines in mice infected with Lcr^- or *lcrV* mutants (24, 25). However, this interpretation is difficult to reconcile with a putative additional regulatory function for V antigen because instances of single procaryote proteins capable of catalyzing two unrelated physiological processes are uncommon. Furthermore, it is generally recognized that products of genes contained within the same operon tend to solely contribute to the accomplishment of a single physiological function which, in this case, is suppression of inflammation.

It is probably significant that native V antigen underwent degradation upon subjection to processes that promoted its removal from LcrH (20). This observation is in accord with the present finding indicating that these peptides can exist as a stable complex. To further explore this possibility, we constructed the recombinant plasmid pVHB62 encoding V_h (a truncated V antigen separated from hexahistidine by the enterokinase cleavage site) and linked *lcrH*. V_h and LcrH were produced in abundance following induction of *E. coli* BL21(DE3)/pVHB62 with IPTG. After disruption, the fusion peptide underwent degradation from both the C-terminal and N-terminal ends during fractionation by chromatographic methods previously shown to promote separation of V antigen and LcrH (20). It is of interest that only the C-terminal end underwent hydrolysis when whole recombinant V antigen was subjected to these treatments (22). This difference may reflect protection against degradation provided by the native N-terminal end, which was replaced in V_h by the polyhistidine stretch and enterokinase cleavage site. This substitution also resulted in reduced solubility of the V_h-LcrH complex discussed below as compared to the putative native V antigen-LcrH complex expressed in wild-type yersiniae.

V_h could be purified to homogeneity in two steps by Ni^{2+}-chelation chromatography, and the resulting product was stable at 4°C or when frozen. However, much of the material added to the high-affinity Ni-NTA agarose column remained firmly bound unless denatured with 6 M guanidine · HCl and eluted as a mixture of V_h and LcrH. Application of this mixture to Ni-NTA agarose after dialysis and solubilization in 8 M urea again resulted in retention of V_h with elution of LcrH. This observation provides strong evidence indicating that native LcrH can tightly bind V antigen. Further evidence favoring specific recognition was provided by showing that LcrH bound PAV as a function of time in a sandwich ELISA and that this capability was lost upon competition with excess V_h.

This observation may have important implications in understanding the phenotypes of *lcrV* and *lcrH* mutants. An obvious interpretation is that LcrH serves as a chaperone protein for V antigen. Since the products of these two genes are normally complexed in cytoplasm, nonpolar *lcrV* mutants would accumulate unbound LcrH in excess. This free LcrH might then interfere or even compete with other chaperone proteins reported to be necessary for secretion of Yops (37), thus preventing their release. It is generally known that inhibition of Yop export can at least indirectly result in feedback inhibition resulting in reduced *yop* transcription (2, 3, 9, 10, 39). Similarly, nonpolar *lcrH* mutants would accumulate free V antigen in excess, which might be toxic to the bacteria. Many other possibilities exist. We anticipate that further study of this interaction will demonstrate that the salient "regulatory" protein encoded within the *lcrGVH-yopBD* operon is LcrH rather than V antigen.

V_h known to be free of bound LcrH could actively immunize mice against intravenous challenge with $>10^7$ Lcr^+ cells of *Y. pestis* (50% lethal dose, ~10 bacteria in control mice [35]). It is significant that a similar 10^6-fold decrease in virulence accompanies loss of the Lcr plasmid (7) or mutation to *lcrV* (26). This dramatic protection exceeded that reported for the V antigen moiety of the glutathione transferase fusion protein (50% lethal dose, ~10^6 bacteria) (18). We attribute this modest dif-

ference to use of the effective adjuvant TiterMax rather than to any unique property of V_h or difference in challenge organism. Unlike the glutathione transferase fusion, however, preparation of V_h did not require treatment with commercial enterokinase, thus avoiding an extra cumbersome and expensive step of purification. V_h shared the ability of PAV (25) to suppress inflammatory cytokines (unpublished observations). A description of this activity is beyond the scope of this report and will be reported in a separate communication.

ACKNOWLEDGMENTS

This research was sponsored by Department of Defense contract DAAA15-93-K0012 from the U.S. Army Research, Development, and Engineering Command and by a generous gift from the Bayer Corporation, West Haven, Conn.

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Editor: V. A. Fischetti