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Expression of the *Bacillus anthracis* Protective Antigen Gene by Baculovirus and Vaccinia Virus Recombinants

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The gene encoding *Bacillus anthracis* protective antigen (PA) was modified by site-directed mutagenesis, subcloned into baculovirus and vaccinia virus plasmid transfer vectors (pAcYM1 and pSC-11, respectively), and inserted via homologous recombinations into baculovirus *Autographa californica* nuclear polyhedrosis virus or vaccinia virus (strains WR and Connaught). Expression of PA was detected in both systems by immunofluorescence assays with antisera from rabbits immunized with *B. anthracis* PA. Western blot (immunoblot) analysis showed that the expressed product of both systems was slightly larger (86 kilodaltons) than *B. anthracis*-produced PA (83.5 kilodaltons). Analysis of trypsin digests of virus-expressed and authentic PA suggested that the size difference was due to the presence of a signal sequence remaining with the virus-expressed protein. Immunization of mice with either recombinant baculovirus-infected *Spodoptera frugiperda* cells or with vaccinia virus recombinants elicited a high-titer, anti-PA antibody response.

Bacillus anthracis is the etiological agent of anthrax, a disease which affects domestic livestock and is frequently fatal. Humans can also become infected through contact with infected animals or animal products (4, 31). Two virulence factors have been described for *B. anthracis*, a tripartite exotoxin (1, 34, 37) and a poly-D-glutamic acid capsule (26, 40, 41, 49). The toxin and capsule have been shown to be encoded by separate plasmids contained in *B. anthracis*, pX01 and pX02, respectively (8, 27, 44). Anthrax toxin is composed of protective antigen (PA), lethal factor (LF), and edema factor (EF). PA combines with EF or LF to form edema toxin or lethal toxin, respectively, however, individually, each of these three components is not toxic. Lethal toxin may cause death in rats, guinea pigs, and mice (1, 5, 6). Intradermal injection of edema toxin causes edematous lesions in the skin (6, 36, 42). Although the biological role of PA as a component of the anthrax toxin is not completely understood, evidence has been obtained suggesting that PA (83.5 kilodaltons [kDa]) initially binds to a specific cell surface receptor and is subsequently proteolytically cleaved by a trypsin-like protease to produce a receptor-bound 63.5-kDa fragment and a 20-kDa fragment, which is released from the cell surface (16). The 63.5-kDa PA fragment is then bound competitively by LF or EF (16). The resulting toxin complex is thought to enter cells by receptor-mediated endocytosis (7), but the mechanism of cell death has not been determined. The poly-D-glutamic acid capsule contributes to disease virulence by inhibiting phagocytosis (14, 49).

Vaccines of variable efficacy and safety have been developed and used for many years. The current livestock vaccine contains the spores of an attenuated, toxigenic, noncapsulated strain of *B. anthracis*, originally developed by Sterne (38). In the United States, the licensed human vaccine consists of aluminum hydroxide-adsorbed supernatant material from fermentor cultures of V770-NP1-R, a toxigenic but noncapsulated strain of *B. anthracis* (32). Effective immunization requires an initial course of three doses followed by three additional doses given at 6, 12, and 18 months

(2). Subsequent annual boosters are necessary to maintain immunization. The immunization schedule is effective but inefficient, since multiple doses over long periods of time are required. More recently, the efficacy of the human vaccine against certain strains of *B. anthracis* has been questioned (17). PA appears to be the only essential component in an effective anthrax vaccine (6, 9, 10, 13). The protective epitopes on PA appear to be those also involved in recognition of cell surface receptors, as the only two anti-PA monoclonal antibodies that neutralize anthrax toxin (lethal toxin and edema toxin) also prevent PA from binding to cell surface receptors (18).

The primary objective of the studies described here was the development of vaccine candidates against *B. anthracis* which may be more efficacious and less reactogenic. Our approach has been to insert the PA gene into foreign vectors capable of expressing PA directly in animals or vectors capable of high expression rates in cell culture. The PA gene has been cloned, sequenced, and subsequently expressed in several procaryotic systems (12, 45). Certain eucaryotic viruses are currently being explored as foreign gene expression systems and expression vectors for potential vaccine use. *Autographa californica* nuclear polyhedrosis virus is an insect virus (baculovirus) which can express foreign genes to unusually high levels when the gene is inserted into the polyhedrin gene of the virus (19, 23, 25, 28, 33, 35). Vaccinia virus has been developed as a live, infectious expression vector for foreign genes inserted into the virus thymidine kinase gene (21, 22, 29, 30). Insertion of the PA gene into vaccinia virus provided a eucaryotic recombinant vector capable of PA expression in vivo, whereas a baculovirus-PA gene recombinant produced PA efficiently in cell culture. Demonstrated here are the expression of PA in both virus systems, the antigenicity of these products, and the immunogenicity of expressed PA in mice.

MATERIALS AND METHODS

Viruses and cells. Vaccinia virus, strains Connaught (Con) and WR, and recombinants (Con-PA and WR-PA) were prepared, propagated, and assayed in Vero cells as described previously (3, 22). All viral infections were initiated

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in medium containing 10% fetal bovine serum. Vaccinia virus recombinations were performed by infection of Vero cells with 0.06 PFU per cell, and cells were incubated for 3.5 h at 37°C, followed by the addition of 30 µg of plasmid DNA by CaCl₂ precipitation. *A. californica* nuclear polyhedrosis virus and recombinant baculoviruses (1Bac-PA and 2Bac-PA) were prepared, propagated, and assayed in *Spodoptera frugiperda* (SF-9) cells as described previously (39).

Plasmids and bacteria. A phagemid vector that contained the PA gene within a 4.2-kilobase insert, pBLSCRPPA, was kindly supplied by J. Lowe, United States Army Medical Research Institute of Infectious Diseases (USAMRIID). The 4.2-kilobase fragment was excised from the plasmid pPA26 (47) with restriction enzymes, *Cla*I and *Bam*HI, and subcloned into the multiple cloning site of the phagemid vector, pBLUESCRIPT (Stratagene, La Jolla, Calif.). pBLSCRPPA plasmid DNA was transformed into competent *Escherichia coli* strain DH5 cells (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and amplified and purified as previously described (24). All other plasmid amplifications and extractions were performed according to the procedures of Maniatis et al. (24). Purified *B. anthracis*-produced PA was kindly supplied by Leppla (USAMRIID) and was prepared as previously described (15).

Site-directed mutagenesis. pBLSCRPPA DNA was transformed into competent *E. coli* CJ236 cells (UNG⁻ DUT⁻; Bio-Rad Laboratories, Richmond, Calif.). Transformed CJ236 cells were expanded, and single-strand pBLSCRPPA DNA template was generated by the method described in the Stratagene pBLUESCRIPT Exo/Mung DNA Sequencing System manual. Two different oligonucleotides were prepared (Applied Biosystems 381A DNA synthesizer, Foster City, Calif.) which were complementary, with the exception of one and two altered nucleotides corresponding to two sites on the single-strand DNA template located 5' to the putative PA gene promoter (47). Oligonucleotide no. 1 (3'-CATATTGG*TCTAGA*AAATAGGC-5') created a new *Bgl*II site 241 nucleotides upstream from the PA gene ATG initiation codon, and oligonucleotide no. 2 (3'-GGC TTGCC*TAGG*TGCATAA-5') created a new *Bam*HI site 224 nucleotides upstream from the PA gene ATG initiation codon (underlined sequences identify the new restriction endonuclease sites). The nucleotide sequence of the PA gene and certain flanking sequences have been reported previously (47). Site-directed mutagenesis was performed on pBLSCRPPA, according to the recommendations of the manufacturer. DNA synthesis products were transformed into competent *E. coli* MV1190 cells, and transformants were screened for the presence of mutations in pBLSCRPPA by differential hybridization with the appropriate oligonucleotides (48).

Fractionation of recombinant-infected cells, PAGE, and Western blot analysis. Recombinant-infected cells (Vero and SF-9 cells) were washed with ice-cold phosphate-buffered saline (Sigma Chemical Company, St. Louis, Mo.) and lysed by vigorous mixing in 1 ml of ice-cold cell lysis buffer (400 mM NaCl, 50 mM Tris hydrochloride, 1 mM EDTA, 1% Triton X-100, 10 µg of α-2-macroglobulin [Sigma] per ml, 10 µg of aprotinin [Sigma] per ml, 0.2% deoxycholate [pH 8.0]) per 1 × 10⁶ to 3 × 10⁶ cells. Cells were incubated on ice for 5 min and then centrifuged (10,000 × g) for 5 min at 4°C. The supernatant was stored at −70°C.

Polyacrylamide gel electrophoresis (PAGE) of lysed, infected cell samples was performed on 10% acrylamide-sodium dodecyl sulfate (SDS) gels. Polyacrylamide gels were either stained with Coomassie blue or transferred to nitrocellulose filters. All cell lysate samples subjected to SDS-PAGE contained 3 × 10⁴ to 9 × 10⁴ cells per lane. Western blot transfers were performed in 25 mM Tris, 192 mM glycine, and 20% methanol at 4°C for 12 to 16 h. Nitrocellulose filters were washed in 0.1% bovine serum albumin–0.05 M *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) buffer (GIBCO Laboratories, Grand Island, N.Y.)–0.1 M NaCl; preincubated 4 h at 4°C with 3% bovine serum albumin–0.05 M HEPES–0.1 M NaCl (blocking buffer); and incubated with rabbit anti-PA antibody (kindly supplied by S. Leppla) or PA-specific monoclonal antibodies (18) diluted in blocking buffer for 2 h at 37°C. Purified preparations of *B. anthracis* PA (15) were used to prepare polyclonal and monoclonal antibodies used for these studies. Filters were washed and incubated with protein A-horseradish peroxidase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) diluted in blocking buffer for 1 h at 37°C. Filters were then washed and reacted with 4-chloro-1-naphthol and hydrogen peroxide.

Trypsin digest conditions. A 10-µg sample of baculovirus recombinant-expressed PA or 20 µg of *B. anthracis*-produced PA was digested with trypsin (6.6 and 0.66 µg/ml, respectively) in 15-µl reaction volumes containing 0.25 M HEPES, 10 mM CaCl₂, and 5 mM EDTA (pH 5) at room temperature. A higher concentration of trypsin was necessary to digest baculovirus recombinant-expressed PA because this PA preparation also contained some nonspecific proteins which were still present after PA enrichment by affinity column chromatography.

ELISA. The quantity of vaccinia virus- and baculovirus recombinant-expressed PA was estimated by an antigen capture enzyme-linked immunosorbent assay (ELISA), as previously described (45). Recombinant-infected cells were disrupted by freezing and thawing and suspended in various amounts of phosphate-buffered saline. Cell suspension (100 µl) was added to 100 µl of dilution buffer (phosphate-buffered saline, 0.5% gelatin, 0.05% Tween 20) and sonicated on ice for 1 min. Samples were incubated at room temperature for 15 min, diluted in dilution buffer, and introduced into wells of flat-bottomed ELISA microtiter plates which had been coated with goat anti-PA antibody (kindly supplied by S. Leppla) from a goat hyperimmunized with purified *B. anthracis* PA.

The anti-PA titers of immunized mice were determined by an antibody capture ELISA as previously described (17).

Immunizations. ICR and C57BL/6 mice were inoculated with either baculovirus recombinant-infected SF-9 cells or vaccinia virus recombinants. Recombinant vaccinia virus inoculum was prepared by using the procedures described by Mackett et al. (22). Mice were given a single immunization with WR-PA or Con-PA (8 × 10⁶ and 2 × 10⁷ PFU, respectively) by tail scarification. Secondary immunizations consisted of intraperitoneal injection of either WR-PA or Con-PA (1.4 × 10⁷ and 4 × 10⁷ PFU, respectively) 21 days after the primary injection.

Baculovirus recombinant-infected SF-9 cells were harvested 96 or 150 h (baculovirus recombinant no. 1 and 2, respectively), pelleted, suspended in PBS, and stored at −70°C. Mice were given a single immunization with 1Bac-PA- or 2Bac-PA-infected cells (1.66 × 10⁶ cells) intramuscularly. A second immunization was given intramuscularly with 3.32 × 10⁶ cells on day 21. On day 28, all animals were bled and sera were stored at −20°C.
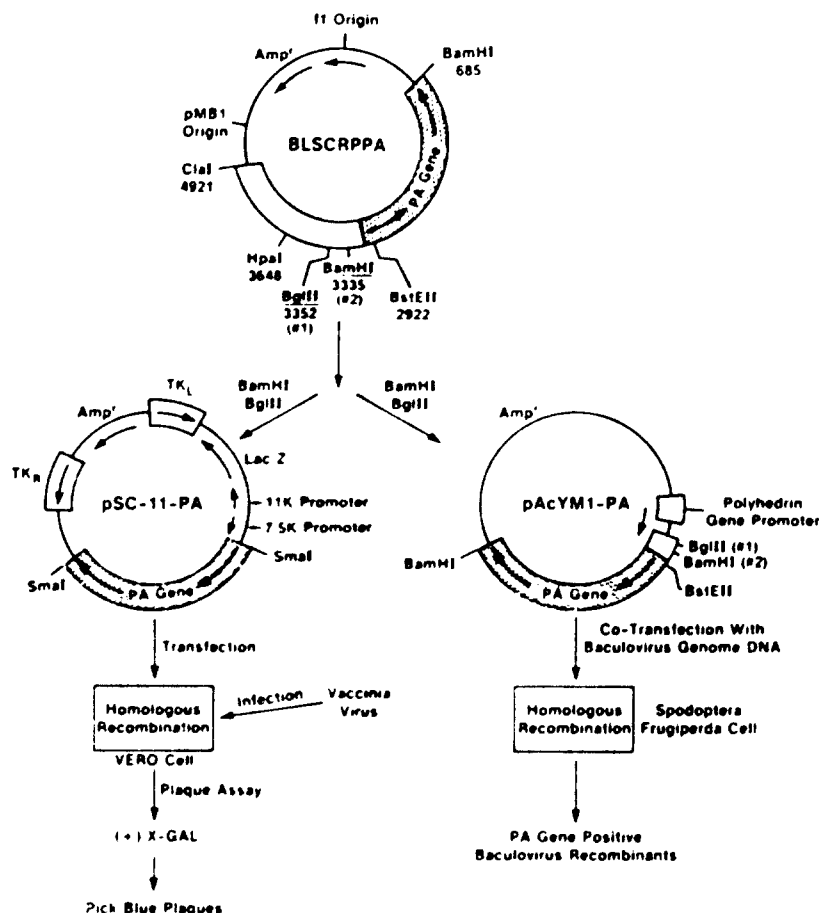
FIG. 1. A schematic diagram describing the procedures used to insert the PA gene of *B. anthracis* into vaccinia virus and baculovirus. TKL, thymidine kinase gene left; TKR, thymidine kinase gene right

RESULTS

Construction of baculovirus- and vaccinia virus-PA recombinants. Insertion of the PA gene into baculovirus and vaccinia virus required subcloning into the appropriate virus transfer vectors (pAcYM1 and pSC-11, respectively). Figure 1 describes schematically the general procedures for gene insertion and recombinant virus selection. To excise the PA gene from pBLSCRPPA, two restriction endonuclease sites, a *Bgl*II site (nucleotide 3352) and a *Bam*HI site (nucleotide 3335), were introduced by site-directed mutagenesis into two different pBLSCRPPA templates, pBLSCRPPA-1 and pBLSCRPPA-2, respectively. The PA gene was excised from pBLSCRPPA-1 with *Bgl*II and *Bam*HI or from pBLSCRPPA-2 with *Bam*HI and subcloned into the baculovirus transfer vector pAcYM1 (25) to produce plasmids pAcYM1-PA-1 and pAcYM1-PA-2. The PA gene was excised from pBLSCRPPA-1 with *Bgl*II and *Bam*HI, and blunt ends were generated with the large fragment of DNA polymerase I and subcloned into the *Sma*I site of the vaccinia virus transfer vector pSC-11 (3) to produce pSC-11-PA.

Baculovirus recombinants were prepared by cotransfection of pAcYM1-PA-1 or pAcYM1-PA-2 and the baculovirus genome DNA into SF-9 cells followed by homologous recombination between polyhedrin sequences contained in both the baculovirus genome and pAcYM1. The PA gene was inserted into the baculovirus polyhedrin gene under

control of the polyhedrin gene promoter. Baculovirus recombinants (1Bac-PA and 2Bac-PA) were selected by terminal dilution of the initial recombinant virus mixture and hybridization to PA-specific nucleic acid probes. Vaccinia virus recombinants were generated by infection of Vero cells with vaccinia virus (strains WR or Connaught) followed by transfection of pSC-11-PA. The PA gene was inserted into the vaccinia virus thymidine kinase gene, under control of the vaccinia virus 7.5-kDa promoter, by homologous recombination between the thymidine kinase sequences in pSC-11 and vaccinia virus. Vaccinia virus recombinants (WR-PA and Con-PA) were selected from plaques that were positive for β-galactosidase expression. The *lacZ* gene is unique to the transfer vector pSC-11 (3).

Characterization and antigenicity of recombinant-expressed PA. Expression of the PA gene was detected by immunofluorescent antibody staining in both baculovirus recombinant-infected SF-9 cells harvested 72 h postinfection and vaccinia virus recombinant-infected Vero cells harvested 24 h postinfection. PA appeared to accumulate in the cytoplasm in both SF-9 and Vero cells, but the precise subcellular localization was not determined. Baculovirus recombinant-infected SF-9 cells were also examined by using 36 monoclonal antibodies against *B. anthracis* PA (18). All PA epitopes defined by a battery of monoclonal antibodies were detected on baculovirus recombinant-expressed PA.
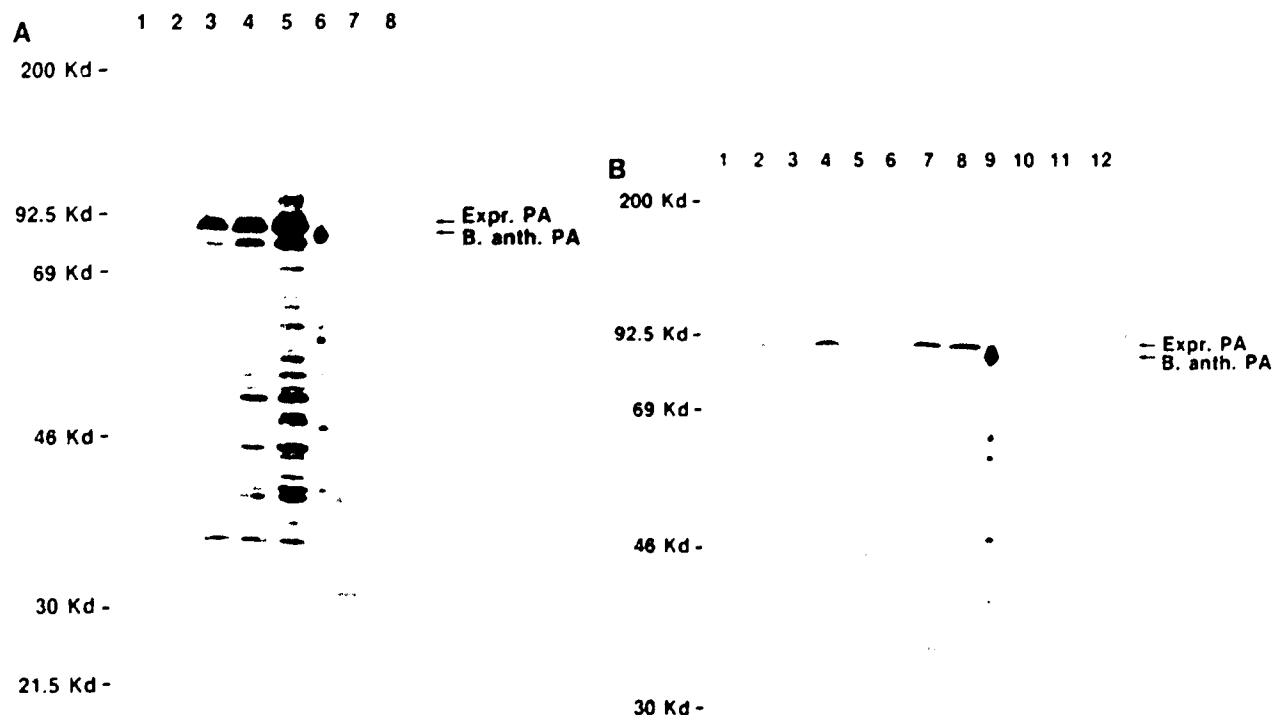
A   1   2   3   4   5   6   7   8

200 Kd –

92.5 Kd –   — Expr. PA
             — B. anth. PA

69 Kd –

46 Kd –

30 Kd –

21.5 Kd –

B   1   2   3   4   5   6   7   8   9   10   11   12

200 Kd –

92.5 Kd –   — Expr. PA
            — B. anth. PA

69 Kd –

46 Kd –

30 Kd –

FIG. 2. Western blots of cell lysates from recombinant-infected cell cultures. (A) 1Bac-PA-infected SF-9 cells were harvested at various times postinfection (PI), lysed with cell lysis buffer, subjected to SDS-PAGE through 10% acrylamide gels, and transferred to nitrocellulose and reacted with rabbit anti-PA antibody. Lanes: 1, 0 h PI; 2, 24 h PI; 3, 36 h PI; 4, 48 h PI; 5, 60 h PI; 6, purified bacterial PA (100 ng); 7, wild-type baculovirus-infected cells, approximately 72 h PI; 8, uninfected SF-9 cell lysates. (B) Vaccinia virus recombinant (WR-PA and Con-PA)-infected Vero cell lysates were harvested at various times PI, lysed with cell lysis buffer, subjected to SDS-PAGE through 10% acrylamide gels, and transferred and reacted with rabbit anti-PA antibody. Lanes: 1, Con-PA 6 h PI; 2, Con-PA 12 h PI; 3, Con-PA 18 h PI; 4, Con-PA 24 h PI; 5, WR-PA 6 h PI; 6, WR-PA 12 h PI; 7, WR-PA 18 h PI; 8, WR-PA 24 h PI; 9, purified bacterial PA (100 ng); 10, uninfected Vero cell lysates; 11, wild-type vaccinia virus (Connaught)-infected cells 24 h PI; 12, wild-type vaccinia virus (WR)-infected cells 24 h PI. Molecular masses to the left of each panel indicate the migration locations of protein standards not shown. Expr., Expressed; B. anthr. *B. anthracis*.

Recombinant virus-expressed PA was characterized further by Western blot analysis. Baculovirus recombinant-expressed PA was detected as early as 24 h postinfection (Fig. 2A, lane 2) and accumulated through 60 h postinfection (lanes 3 through 5). PA production in SF-9 cells infected at a multiplicity of infection of 10 PFU per cell was estimated to plateau at 96 h postinfection (data not shown). Expressed PA, the most prominent band in lanes 2 through 5, appears slightly larger (approximately 86 kDa) than *B. anthracis*-produced PA (83.5 kDa [lane 6]). In addition, there were a number of PA-specific fragments (lanes 2 through 6). Rabbit anti-PA recognized expressed PA (lanes 2 through 6) and not cell-specific proteins (lane 8). However, the antibody reacted with a baculovirus protein of approximately 32 kDa (lane 7). The entire battery of anti-PA monoclonal antibodies (18) also recognized baculovirus recombinant-expressed PA bound to nitrocellulose. The PA-specific signal varied depending upon the monoclonal antibody used.

Vaccinia virus recombinant-infected Vero cells were harvested at 6-h intervals during a 24-h incubation. At 24 h postinfection, >90% of the cells showed morphological signs of viral infection. Recombinant-expressed PA was detected 6 h postinfection with both WR and Connaught strain recombinants (Fig. 2B, lanes 1 and 5) and continued to be produced through 24 h (lanes 2 through 4 and 6 through 8). As seen with the baculovirus recombinant-expressed PA, the apparent molecular mass of the PA produced by the

vaccinia virus recombinants was slightly larger (approximately 86 kDa) than that of *B. anthracis*-produced PA (lane 9). A number of PA-specific fragments were also observed (lanes 1 through 8); however, these fragments were different in size and quantity from those of baculovirus origin. Nonspecific binding of rabbit anti-PA antibody to wild-type WR- or Connaught-infected Vero cell lysates or to uninfected Vero cell lysates was not observed (lanes 10 through 12).

Baculovirus-expressed proteins have been reported to represent up to 25% of the proteins detectable in infected SF-9 cells (19) monitored by Coomassie staining. Expressed PA in recombinant-infected SF-9 cells could be detected by Coomassie staining protein lysates subjected to SDS-PAGE (data not shown). Vaccinia virus recombinant-expressed PA was not detectable in cell lysates examined by these procedures (data not shown). The amount of expressed PA produced per recombinant-infected cell was estimated by antigen capture ELISA. Baculovirus recombinants produced up to 6 pg of PA per cell, and each vaccinia virus recombinant produced approximately 0.2 pg of PA per cell.

The size difference observed between the recombinant-expressed PA and the *B. anthracis*-produced PA (Fig. 2) could be the result of a number of different co- or posttranslational processing events. Trypsin cleaves native PA of *B. anthracis* preferentially at one location to yield 63.5- (carboxy-terminal) and 20- (amino-terminal) kDa fragments (16). We digested baculovirus recombinant-expressed PA with
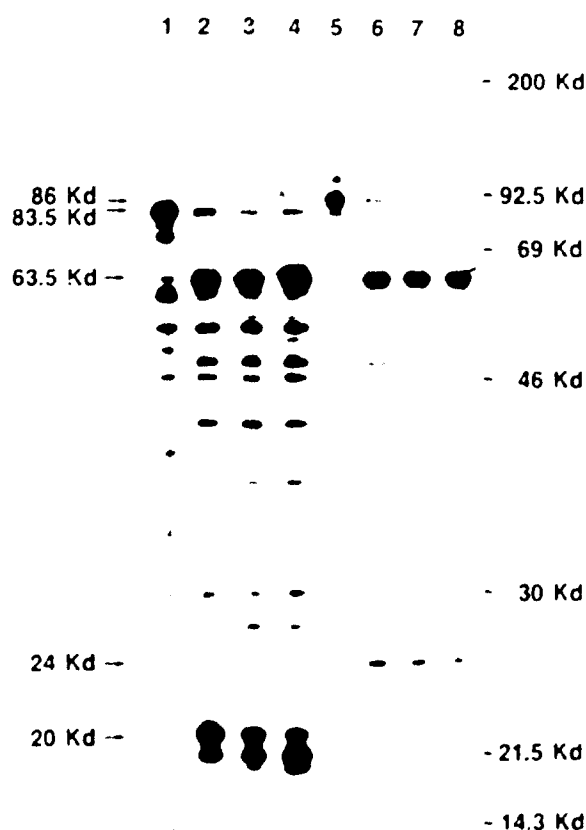
FIG. 3. Trypsin digest of baculovirus recombinant-expressed PA. Expressed PA and purified samples of bacterial PA were treated with trypsin. Samples were subjected to SDS-PAGE through 12.5% acrylamide, transferred to nitrocellulose filters, and reacted with rabbit anti-PA antibody. Lanes: 1, bacterial PA (undigested), 2, bacterial PA (5-min digest), 3, bacterial PA (10-min digest), 4, bacterial PA (20-min digest), 5, recombinant-expressed PA (undigested), 6, recombinant-expressed PA (5-min digest), 7, recombinant-expressed PA (10-min digest), 8, recombinant-expressed PA (20-min digest). Molecular masses to the right of the panel indicate the migration locations of protein standards not shown. Arrows and molecular masses to the left of the panel indicate the migration locations of PA fragments produced by treatment with trypsin.

trypsin in an attempt to associate the PA size difference with one or both primary fragments. Baculovirus recombinant-expressed PA was concentrated by immunosorbent chromatography (data not shown) with a PA-specific monoclonal antibody (18, 20). B. anthracis-produced PA was cleaved efficiently from 83.5 (Fig. 3, lane 1) to 63.5- and 20-kDa fragments (lanes 2 through 4). Baculovirus recombinant-expressed PA (lane 5) was also sensitive to digestion with trypsin and produced predominantly a 63.5-kDa fragment and a second fragment which was approximately 24 kDa (lanes 6 through 8). Therefore, the size difference between bacterial and baculovirus recombinant-expressed PA would appear to be associated with the amino-terminal 20-kDa protein fragment.

Immunogenicity of recombinant-expressed PA in mice. High anti-PA titers are found in animals immunized with the Sterne vaccine and several PA-producing bacterial vectors (10–12, 46). Anti-PA antibody confers some degree of protection in these animals. To determine whether recombinant-

expressed PA was immunogenic, mice were injected with baculovirus recombinant-infected SF-9 cells or infected with vaccinia virus recombinants. Mouse serum was collected and screened by antibody capture ELISA with B. anthracis-produced PA. The results of these experiments are presented in Table 1. Animals given only a single immunization as well as those given a second immunization with vaccinia virus recombinants displayed anti-PA antibody; however, animals receiving a second immunization exhibited consistently higher titers than animals given a single immunization. In addition, animals immunized with WR-PA displayed higher antibody responses than those immunized with Con-PA.

Baculovirus recombinant-infected SF-9 cells also elicited a PA-specific immune response in mice; however, the anti-PA titers were generally lower than those observed for vaccinia virus recombinant-immunized animals. A second immunization with recombinant 2Bac-PA induced high titers, similar to those observed in mice immunized twice with the vaccinia virus recombinants.

DISCUSSION

Baculovirus recombinants and vaccinia virus recombinants have been used to express foreign, immunologically significant proteins of pathogenic agents (19, 29). The vaccinia virus and baculovirus expression systems have been used here successfully to produce PA. As far as can be determined, baculovirus recombinant-expressed PA is identical antigenically to bacterial PA, because all 36 antigenic determinants, as defined by monoclonal antibodies (18), are present. PA can be safely produced free of trace amounts of LF and EF for potential use in vaccine development and biological function and structure studies of anthrax toxin. Preliminary results indicate that purification of PA from baculovirus recombinant-infected SF-9 cells is possible by immunoaffinity chromatography (unpublished results).

The unit size of baculovirus recombinant-expressed PA appears slightly larger than that of the bacterial PA. B. anthracis PA is secreted from bacterial cells with the aid of a 29-amino-acid hydrophobic signal sequence located at the amino terminus of the nascent polypeptide (16, 47). One possibility for the size difference is that the eucaryotic expression systems do not recognize this procaryotic signal sequence efficiently in the nascent PA polypeptide and thus do not remove it. This would produce a PA approximately 3 kDa larger than the mature bacterial PA. Amino-terminal sequencing of full-size baculovirus recombinant-expressed PA was attempted, but the amino-terminal amino acid was blocked. Trypsin digests of B. anthracis-produced PA and baculovirus recombinant-produced PA showed that the amino-terminal fragment from recombinant-expressed PA was 24 instead of 20 kDa. These data suggest that the PA signal sequence was not removed in the recombinant-expressed product.

Our results indicate that PA production in vaccinia virus recombinant-infected Vero cells is significantly less than that of baculovirus recombinant-infected SF-9 cells. Antigen detection data suggested that either the PA expression levels differed in the two eucaryotic systems or that the expressed PA products in the two eucaryotic systems were different enough antigenically to result in differential anti-PA antibody recognition. In support of the former possibility, expressed PA was detectable in baculovirus recombinant-infected SF-9 cell lysates by Coomassie blue-stained acrylamide gels but not in vaccinia virus recombinant-infected Vero cell lysates.

TABLE 1. Antibody titers in mice after immunization with PA-producing vaccinia virus recombinants or baculovirus recombinant-infected *S. frugiperda* cells

Vaccine	Anti-PA titer[a]			
	ICR		C57BL/6	
	One immunization	Two immunizations	One immunization	Two immunizations
Vaccinia virus				
WR-PA	9,856 (1,280–16,000)	128,000 (128,000)	3,936 (800–12,800)	40,000 (8,000–128,000)
Con-PA	226 (0–400)	31,333 (30,000–32,000)	1,056 (320–1,600)	29,240 (6,400–64,000)
Baculovirus				
1Bac-PA	20 (0–100)	3,330 (10–12,800)	66 (10–320)	5,280 (640–12,800)
2Bac-PA	1,760 (800–3,200)	56,960 (12,800–128,000)	976 (400–1,280)	58,880 (12,800–128,000)

[a] Average geometric mean (n = 5) reciprocal for animals as determined by ELISA. The range is indicated in parentheses. The first immunization was given on day 0, the second immunization was given on day 21, on day 28, all animals were bled.

Therefore, we suggest a difference in the PA concentration per cell.

PA expressed by these recombinants was immunogenic for mice. Serum titers were determined by antibody capture ELISA with bacterial PA. Therefore, the titers in animals reflected antibody that recognized authentic, toxin component PA. Anti-PA antibody titers were comparable to anti-PA antibody titers reported in other animals immunized with PA or PA-producing bacteria (10–13, 17, 43). PA produced by 2Bac-PA appeared to immunize the animals better than did 1Bac-PA; however, an antigen capture ELISA demonstrated that 2Bac-PA-infected cells used to immunize animals contained six to eight times more PA per cell than the 1Bac-PA-infected cells (data not shown). Thus, the lower antibody titers presumably reflect a lower immunizing dose rather than a qualitative difference in the expressed immunogens. These results suggest that the anti-PA antibody titers are directly related to the amount of PA used to immunize the animals and demonstrate that baculovirus recombinant-expressed PA and vaccinia virus recombinants are capable of inducing high anti-PA titers in mice. The ability of these anti-PA antibodies to neutralize toxin and protect animals from a *B. anthracis* spore challenge is being explored currently.

ACKNOWLEDGMENTS

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