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Identification and Characterization of Vaccinia Virus Genes Encoding Proteins That Are Highly Antigenic in Animals and Are Immunodominant in Vaccinated Humans

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Vaccinia virus (VV) is a potent immunogen, but the nature of VV proteins involved in the activation of the immune response of the host is not yet known. By screening a lambda gt11 expression library of rabbitpox virus DNA with serum from humans vaccinated against smallpox or with serum from VV-immunized animals, we identified several VV genes that encode highly antigenic viral proteins with molecular masses of 62, 39, 32, 25, 21, and 14 kDa. It was found that VV proteins of 62, 39, 25, and 21 kDa are part of the virus core, while proteins of 32 and 14 kDa are part of the virus envelope. All of these proteins were synthesized at late times postinfection. Proteins of 62 and 25 kDa were produced by cleavage of larger precursors of 95 kDa (p4a) and 28 kDa, respectively. The 21-kDa protein was the result of a cleavage of p4a, presumably at amino acid Gly-697. DNA sequence analysis, in comparison with the known nucleotide sequence of VV, provided identification of the corresponding open reading frames. Expression of the viral genes in *Escherichia coli* was used to monitor which of the viral antigens elicit immunodominant responses and the location of antigenic domains. Three viral antigens of 62, 39, and 32 kDa exhibited immunodominant characteristics. The most antigenic sites of 62 and 39 kDa were identified at the N terminus (amino acids 132 to 295) and C terminus (last 103 amino acids), respectively. Immunization of mice with the 62-, 39-, or 14-kDa antigenic proteins conferred different degrees of protection from VV challenge. Proteins of 32 and 14 kDa induced cellular proliferative responses in VV-infected mice. Our findings demonstrate the nature of VV proteins involved in the activation of host immune responses after vaccination, provide identification of the viral gene locus, and define structural and immunological properties of these antigenic VV proteins.

Vaccinia virus (VV) has proved to be a very useful viral vector for potential vaccination purposes because of its broad host range, because of its prior use as a vaccine against smallpox in humans (8), and because of the ability to produce recombinant viruses that express a variety of foreign antigens in their native states that confer protection in immunized animals when challenged with the respective pathogen (21, 25). The success of live-recombinant VV as vaccines is based on the properties of limited replication with adequate stimulation of the appropriate elements of the immune system. Advantages include the opportunity for stimulation of the humoral and cell-mediated immune responses at critical local sites, longer duration of protection, broader antigenic coverage, and the reduced possibility of undesirable immunopathology (1, 21, 22).

Although VV is a proven potent immunogen and has the distinct ability of producing long-lasting immunity in humans, little is known about the nature of the viral proteins and corresponding epitopes involved in the activation of host immune responses at the B- and T-cell level that lead to protective immunity. Antibodies reactive against VV proteins of 58, 54, 34, 37, 32, 29, and 25 to 17 kDa have been found in sera from immunized animals (6, 24, 40). Monoclonal antibodies reactive against VV proteins of 30, 16, and 14 kDa had protective effects in mice (5). Only a few VV genes encoding antigenic proteins with roles in the immune response in experimental animals have been identified. These are envelope proteins, such as the hemagglutinin (33) and the 37-kDa acylated protein (12) present in extracellular virus, and 35-kDa (11), 32-kDa (19), and 14-kDa (26) proteins present in intracellular virus. The nature of the VV proteins involved in the activation of humoral and cellular immune responses in vaccinated humans is largely unknown (1, 8).

In this communication, we report the cloning and identification of VV genes encoding proteins that are highly antigenic in animals and are immunodominant in vaccinated humans. We also report the mode of expression of these proteins during virus infection, the localization of the most antigenic domains within some of these proteins, and the ability of some of these antigenic proteins to induce virus immunity and to activate cellular immune responses in vivo.

(This work was done in partial fulfillment of the requirements for the Ph.D. degrees of W. Demkowicz [1991] and J.-S. Maa [1988], Graduate School, State University of New York Health Science Center at Brooklyn.)

MATERIALS AND METHODS

Materials. Restriction endonucleases, modifying enzymes, isopropyl-β-D-thiogalactopyranoside (IPTG), and protein A were obtained from Bethesda Research Laboratories, Inc., New England Biolabs, or Boehringer Mannheim Biochemicals. The radioisotopes [^{3}H]thymidine, [^{35}S]methionine, ^{125}I, and [^{32}P]dATP were from Amersham or ICN. Goat anti-rabbit peroxidase and goat anti-human peroxidase were from Organon Teknika, Cappel.

Cloning, mapping, and DNA sequence analysis. DNA manipulations were carried out as previously described (31). DNA fragments obtained by digestion of viral and phage

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DNA were ligated into pUC19 or the prokaryotic expression vector pT7-7 (35, 36) and used for transformation into competent *Escherichia coli* JM83 or BL21 DE3 cells (35). DNA sequencing was performed by the method of Sanger et al. (32), using double-stranded DNA and the sequencing kit manufactured by United States Biochemical. Partial DNA sequence analysis of the DNA phage inserts was carried out after the phage *Eco*RI inserts were cloned into pUC19. Insert orientation was determined by endonuclease digestion, and sequencing was performed with universal primers. Deletions of a 2.7-kb *Sal*I DNA fragment (18) containing the entire 39-kDa open reading frame (ORF) in pUC19 were carried out after digestion with *Sph*I and *Hin*dIII, followed by exonuclease III treatment, removal of single-stranded DNA by S1 nuclease, blunt ending with Klenow fragment, and religation with DNA ligase. After transformation of *E. coli* cells, the insert size was determined by endonuclease digestion. Appropriate plasmids were selected, and DNA inserts were sequenced with universal primers. DNA sequence analysis of both strands of the 39-kDa gene was also performed with DNA fragments (*Sal*I-*Xba*I, *Xba*I-*Bam*HI, and *Bam*HI-*Xba*I) cloned in pUC19. Mapping of VV DNA was performed by blotting agarose gels containing digested DNA fragments to nitrocellulose filters and subsequent hybridization with specific nick-translated DNAs as probes.

Lambda gt11 rabbitpox virus DNA library screening and preparation of monospecific antibodies. The procedures used to screen the rabbitpox virus DNA lambda gt11 expression library and to selectively purify monospecific antibodies from immune sera have been described in detail elsewhere (18, 19, 26).

Generation of pT7-7 vectors for expression of VV genes. DNA fragments encoding p4a and the 39-, 32-, 25-, and 14-kDa proteins were cloned into the prokaryotic expression vector pT7-7 (36) and expressed in *E. coli* BL21 DE3 cells (35). Plasmid pWD4a was constructed by ligating a 2,572-bp fragment generated after digestion of VV *Hin*dIII-A with *Bam*HI and *Nar*I to vector pT7-7 digested with *Bam*HI and *Cla*I. This plasmid expresses a 834-amino-acid (aa) polypeptide corresponding to p4a but the 57 aa at its N terminus are replaced by 9 aa encoded by the vector. Plasmid pWD39K was generated by digestion of a 2.7-kb *Sal*I DNA fragment of *Hin*dIII-A that contains the 39-kDa-encoding sequence (18) with *Bam*HI, treated with Klenow fragment, and ligation into vector pT7-7 previously digested with *Eco*RI, treated with Klenow fragment, and digested with *Sal*I. The resulting plasmid expresses 254 of 281 aa encoded by the entire gene, and it contains 4 additional aa at the N terminus provided by the vector. Plasmid pWD25K was generated by digestion of VV *Hin*dIII-L DNA with *Hin*pI and *Hin*dIII and ligation of this 998-bp fragment into vector pT7-7 digested with *Acc*I and *Hin*dIII. The resulting plasmid expresses a polypeptide of 219 aa out of the 251 aa encoded by the entire gene in addition to an extra 13 aa at its N terminus encoded by the vector. The generation of plasmids expressing the entire 304-aa coding sequence of the 32-kDa protein (pT7EK32) and the 110 aa of the 14-kDa protein (pT7Nd14K) has been described previously (17, 19).

Plasmids containing defined subfragments of VV genes were generated as follows. Plasmid pWD21K was generated by ligation of a 674-bp *Hin*pI restriction fragment of pWD4a with pT7-7 digested with *Acc*I and treated with alkaline phosphatase. The resulting plasmid expresses a protein of 238 aa corresponding to the C terminus of p4a, with an additional 13 aa at the N terminus provided by the vector. Plasmid pWD62-100 was constructed by ligating an 812-bp fragment generated after digestion of plasmid pWD4a with *Bam*HI and *Pst*I to the vector digested with *Bam*HI and *Pst*I. The resulting plasmid expresses a protein of 287 aa which contains an additional 9 aa at its N terminus and 7 aa at its C-terminus encoded by the vector. Plasmid pWD62-101 was constructed by ligation of a 326-bp fragment generated by digestion of pWD4a with *Bam*HI and *Bgl*II into pT7-7 digested with *Bam*HI and treated with alkaline phosphatase. The resulting plasmid expresses a protein of 136 aa which contains an additional 9 aa at its N terminus and 18 aa at its C terminus encoded by the vector. Plasmid pWD62-103 was constructed by ligation of a 946-bp fragment of pWD4a generated by digestion with *Bgl*II and *Hin*pI into pT7-7 digested with *Bam*HI and *Cla*I. The resulting plasmid expresses a protein of 432 aa which contains an additional 9 aa at its N terminus and 12 aa at its C terminus encoded by the vector. Plasmid pWD62-105 was constructed by the ligation of a 316-bp fragment generated by digestion of pWD4a with *Bgl*II and *Nsi*I into pT7-7 digested with *Bam*HI and *Pst*I. The resulting plasmid expresses a protein of 132 aa with an additional 9 aa at the N terminus and 18 aa at the C terminus provided by the vector. Plasmid pWD62-106 was constructed by ligation of a 586-bp fragment generated by digestion of pWD4a with *Bgl*II, treatment with Klenow fragment, and insertion into pT7-7 cut with *Eco*RI and treated with Klenow fragment and alkaline phosphatase. The resulting plasmid expresses a protein of 228 aa containing an additional 4 and 29 aa at its N and C termini, respectively, which are encoded by the vector. Plasmid pWD62-107 was constructed by ligation of a 493-bp fragment generated by digestion of pWD4a with *Eco*RV and *Ssp*I into pT7-7 cut with *Eco*RI and treated with Klenow fragment and alkaline phosphatase. The resulting expressed protein of 168 aa contained an additional 4 aa at its N terminus encoded by the vector. Plasmid pWD39-201 was constructed by ligating a 453-bp fragment generated by digestion of 39K(S-S) with *Bam*HI, treatment with Klenow fragment, and further digestion with *Ssp*I into pT7-7 cut with *Eco*RI and treated with Klenow fragment, and alkaline phosphatase. The resulting expressed protein of 161 aa contained an additional 4 and 6 aa encoded by the vector at its N and C termini, respectively. Plasmid pWD-205 was constructed by ligation of a 770-bp fragment generated by digestion of 39K(S-S) with *Ssp*I into pT7-7 cut with *Eco*RI, followed by Klenow fragment and alkaline phosphatase treatment. The resulting expressed protein of 107 aa contained an additional 4 aa at its N terminus encoded by the vector. Plasmid pWD39-206 was constructed by ligating a 246-bp fragment generated by *Sca*I and *Ssp*I digestion of 39K(S-S) into pT7-7 cut with *Eco*RI and treated with Klenow fragment and alkaline phosphatase. The resulting expressed protein of 92 aa contained an extra 4 and 6 aa at its N and C termini, respectively, encoded by the vector.

Expression of VV proteins in *E. coli*. Cultures were grown at 37°C with vigorous shaking to an optical density at 600 nm of 0.6 in Luria broth containing ampicillin (50 mg/liter) and then induced with IPTG to a final concentration of 400 μM for 1 to 4 h. Cultures were centrifuged at 7,000 rpm for 8 min, washed once in HB buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 20 mM NaCl, 1 mM dithiothreitol), and resuspended in HB buffer containing 1% sodium dodecyl sulfate (SDS) and 100 mM NaCl. The resulting lysates were sonicated, cleared by centrifugation, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE and Western immunoblotting. Protein samples were boiled for 5 min in sample buffer (62.5 mM Tris [pH

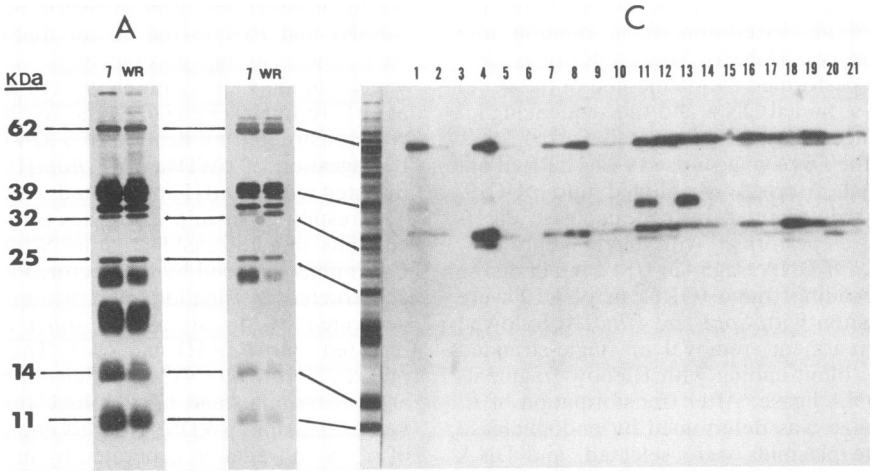

FIG. 1. Identification of structural proteins of VV that are antigenic in animals and humans by Western blot analysis. Rabbit polyclonal immune serum (A) was prepared after repeated inoculations with disrupted virions (16). For the preparation of immune mouse serum (B), 6-week-old BALB/c mice were inoculated i.p. with VV variant 48-7 (18) (10^8 PFU per mouse), and sera was collected after 4 weeks. Nitrocellulose strips containing 3 μg each of either purified wild-type VV virions (lane WR) or purified virions from variant 48-7 (lane 7) were reacted with a 1:500 dilution of rabbit serum (A) or with a 1:200 dilution of mouse serum (B). The pattern of antibody reactivity was visualized by immunoperoxidase staining (18). Serum from vaccinated humans was prepared as described in Materials and Methods (C). Nitrocellulose strips containing 5 μg of purified wild-type VV were reacted with human serum (1:50 dilution) obtained from different individuals, and immune reactivity was detected with ^{125}I-protein A after autoradiography (19). The ^{35}S-labeled VV polypeptides from purified virus are shown to the left of lane 1 in panel C. Each numbered lane represents serum from a different individual. Molecular masses of some of the structural VV polypeptides are indicated.

6.8], 3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue), loaded onto 12% SDS-polyacrylamide gels, and electrophoresed with the buffer system described by Laemmli (16). After electrophoresis, the proteins were transferred to nitrocellulose using the semidry blotting apparatus from Gelman Sciences. The filters were then incubated with antisera in BLOTTO overnight at 4°C with shaking, washed four times for 10 min each with phosphate-buffered saline (PBS), and further incubated with either goat anti-human (1:1,000) or goat anti-rabbit (1:500) antibody conjugated to horseradish peroxidase for 2 h at room temperature. After four more 10-min washes with PBS, the filters were developed with 0.02% 1-chloro-4-naphthol–0.006% hydrogen peroxide in PBS in order to visualize bound antibodies.

Preparation of rabbit and human immune sera from vaccinated animals and individuals. Preparation of the rabbit polyclonal antisera has been described previously (18). Human immune sera were collected from different individuals and preabsorbed twice with BL21 DE3 cell extracts prepared by resuspending pelleted cells in an appropriate volume of HB buffer, followed by three rounds of freeze-thawing and four sonications for 10 min each on a high setting. The extracts were then incubated with serum overnight at 4°C with rotation and centrifuged at high speed for 10 min. The antiserum was then used at the appropriate dilution in 5% BLOTTO (nonfat dry milk in PBS).

Computer analysis of protein sequences. Analyses were performed on an IBM PC computer. Amino acid sequence data were stored and analyzed by PC Gene programs, using the Protein Sequence Core Facility at the State University of New York Health Science Center at Brooklyn.

Gel elution of *E. coli* proteins and generation of specific antibodies. Protein gels were fixed in 50% (vol/vol) isopropanol–7% (vol/vol) glacial acetic acid for 2 h at room temperature and stained in 7% acetic acid with a 1:250 dilution of 2% Coomassie blue in methanol. The appropriate bands corresponding to proteins of 62, 39, 32, 25, and 14 kDa or subfragments of 62- and 39-kDa proteins were cut from the Coomassie-stained gels and placed inside washed dialysis tubing with 8 to 10 ml of elution buffer (0.05 M ammonium bicarbonate [pH 9.0] containing 0.1% [wt/vol] SDS). The tubing was immersed into a horizontal agarose gel electrophoresis tank containing 1 liter of elution buffer. The elution was carried out at a constant voltage of 50 V for 16 to 20 h. The sample was removed from the tubing, dried in a Savant Speed Vac concentrator (Savant, Farmingdale, N.Y.) overnight, resuspended in deionized water, and precipitated overnight at −20°C in 4 volumes of methanol-acetone (50:50, vol/vol). The samples were centrifuged at high speed for 30 min at 4°C, and the supernatants were discarded. The pellets were dried, resuspended in deionized water, and sonicated. Protein concentrations were determined by the BCA protein assay procedure, which was obtained commercially from Pierce. White male rabbits (4 months old; Charles River Laboratories) were immunized three times with the *E. coli*-expressed proteins. The first immunization was in Freund's complete adjuvant, while the other two immunizations were carried out in Freund's incomplete adjuvant. About 150 μg of protein was inoculated in three independent sites at time intervals of 3 weeks. Two weeks after the last immunization, blood was collected and serum was stored at −70°C. By this approach, we generated specific antibodies against the VV proteins of 62, 39, 32, and 14 kDa. Antibodies against the 25-kDa protein were generated by repeated immunization of mice by the intraperitoneal (i.p.) route with the *E. coli*-expressed protein.

Evaluation of mouse survival after challenge with VV by prior immunization with *E. coli*-expressed VV proteins. BALB/c mice (female, 6 weeks old, four per group; Charles River) were immunized with the *E. coli*-expressed VV proteins of 62, 39, and 14 kDa. The first injections were

performed i.p. with a mixture containing 50 μg of each protein in 150 μl of PBS mixed with 150 μl of Freund's complete adjuvant. Control animals were immunized with adjuvant alone. Three weeks later, mice were boosted i.p. with the same amount of protein but resuspended in Freund's incomplete adjuvant. Two weeks after the booster, mice were challenged i.p. with a lethal dose (40 times the 50% lethal dose [LD_{50}]) of wild-type VV. Survival following virus challenge was scored over a 3-week period.

Proliferative response to VV proteins. Proliferative responses to VV antigens were tested by a modification of the method of Chain et al. (3). Bulk spleen cells (5×10^5) were cultured with various dilutions of antigen in 0.2 ml of RPMI 1640 medium supplemented with glutamine (2 mM), 100 μg each of streptomycin and penicillin per ml, and 10% fetal calf serum (GIBCO/BRL, Grand Island, N.Y.) in 96-well round-bottom plates. After 4 days of incubation at 37°C, the cells were pulsed with 1.25 μCi of [^{3}H]thymidine per well for 16 to 18 h. Cells were harvested onto glass filter paper with a semiautomatic cell harvester (PhD Cell Harvester; Cambridge Tech Inc., Cambridge, Mass.), and [^{3}H]thymidine incorporation was measured in 1.0 ml of liquid scintillation and counted.

RESULTS

Animals primed with VV and humans vaccinated against smallpox develop antibodies reactive against a similar subset of viral proteins. When BALB/c mice are primed with VV and serum is collected 4 weeks later, anti-VV antibodies are largely reactive against virion polypeptides of 62, 59, 39, 36, 32, 27, 25, 21, 14, and 11 kDa, as measured by Western blot analysis. The same pattern was also observed with hyperimmune serum from a rabbit (Fig. 1A and B). Similar patterns of immunoreactive proteins have been described by other groups (23a, 40). When humans from different countries and various races are vaccinated with VV against smallpox and blood is collected several years later, circulating anti-VV antibodies are still present in their sera. Western blot analysis demonstrated that these antibodies reacted predominantly against a subset of virion polypeptides with molecular masses of 62, 59, 39, 36, and 32 kDa (Fig. 1C). Significantly, anti-VV antibodies in human sera were most frequently found to react against a 62- and a 36-kDa protein. There was no apparent correlation between antigen recognition and neutralizing antibody titers (Table 1). The fact that VV proteins of the same apparent molecular mass react with antibodies from human, rabbit, and mouse sera suggests that these viral proteins play a major role in the immune response of the host.

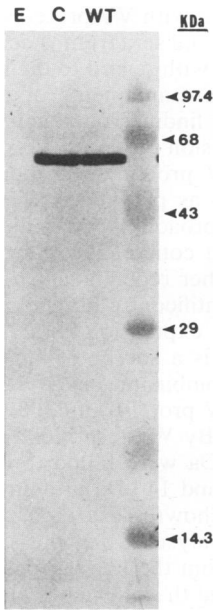

FIG. 2. Identification of a 62-kDa antigenic protein of VV as a core protein. Antibody screening, selection of monospecific antibodies from a lambda gt11 rabbitpox virus DNA library, and preparation of purified VV, envelopes, and cores were done as previously described (19). Western blots of about 5 μg of either envelope (lane E), core (lane C), or whole virions (lane WT) were reacted with a monospecific antibody, and the immunoreactive proteins were detected by immunoperoxidase staining. Molecular masses of marker polypeptides are given.

TABLE 1. Virus-neutralizing titers of human sera from individuals vaccinated against smallpox[a]

Sample no.	Sex	Age (yr)	Country origin	Neutralizing titer
1	M	26	American	1/16
2	M	37	American	1/8
3	F	24	American	1/8
4	M	36	Indian	1/256
5	M	22	American	1/16
6	M	44	Puerto Rican	1/32
7	M	24	American	1/8
8	M	48	American	1/8
9	M	24	American	1/16
10	F	27	Chinese	1/16
11	M	31	Indian	1/64
12	M	23	Chinese	1/16
13	M	36	American	1/16
14	M	25	American	1/8
15	M	24	Scottish	1/8
16	M	26	Chinese	1/8
17	M	32	Spanish	1/64
18	M	26	American	1/128
19	F	32	Iraqi	1/16
20	M	35	Spanish	1/32
21	M	42	Spanish	1/256

[a] The sex (M, male; F, female) age, and country origin of serum samples from humans are indicated. For neutralization titer, human serum was heat inactivated at 56°C for 30 min and incubated at different dilutions with 10^3 PFU of VV for 1 h at 37°C. Thereafter, BSC-40 cells grown in 60-mm dishes were infected with the virus, and after 1 h of adsorption at 37°C, the inoculum was removed and cells were overlaid with agar. After 4 days at 37°C, the overlay was removed, cells were stained with 1% crystal violet in 2% ethanol, and the number of plaques was counted. The neutralization titer is the reciprocal dilution of serum that results in 50% reduction of virus plaques.

Identification of VV genes encoding proteins that are highly antigenic in vaccinated humans and animals. To identify VV genes encoding antigenic proteins in vaccinated humans, a lambda gt11 expression library of rabbitpox virus DNA was screened with human sera from vaccinated individuals, and recombinant phage showing strong reactivity with the immune sera were selected (19, 26). These phage were used independently to infect *E. coli* cells; lysates from these IPTG-induced cells were transferred to nitrocellulose paper, and monospecific antibodies were selected after incubation with human or rabbit serum as described previously (19). The antibody specificity of the monospecific antibodies was established by Western blot analyses with filters containing lysates of purified VV. Recombinant phage selecting for

antibodies that reacted with VV proteins of the same apparent molecular mass as did sera from vaccinated humans were further characterized with regard to their distribution within the envelope or core components of the virus. Figure 2 shows representative findings obtained with a recombinant DNA phage whose fusion protein selects antibodies reactive against a 62-kDa VV protein. The monospecific antibody identified the protein as part of the viral core but not the envelope. By this approach, we identified various recombinant DNA phage that contained VV sequences encoding a 62-kDa protein. Further recombinant phage containing VV sequences were identified by screening the lambda gt11 rabbitpox virus DNA expression library with hyperimmune rabbit serum, which is a source of high-titer anti-VV antibodies. Several recombinant phage which contained sequences encoding VV proteins of 62, 39, 32, 25, 21, and 14 kDa were obtained. By Western blot analyses, proteins of 62, 39, 25, and 21 kDa were found to be part of the virus core, while the 32-, and 14-kDa proteins were found in the envelope (data not shown). We then performed Southern blot hybridization analyses using the specific phage DNA inserts as probes. From the known restriction maps of VV DNA (7), it was found that the genes encoding the proteins of 62, 39, 21, and 14-kDa mapped within the *Hin*dIII A fragment, the gene encoding the 32-kDa protein mapped in *Hin*dIII-D, and the gene encoding the 25-kDa protein mapped into the *Hin*dIII L fragment of the VV genome (data not shown).

The phage inserts were sequenced to define the primary structure of the VV proteins recognized by the monospecific serum. The DNA inserts from the recombinant phage that

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                                    -30                                   0
ATAATATCGTCTGTGTCTGCCATTTAATCTTATAATCGCAATTCAATTTTAAAGCCTTAA
                                    30                                   60
ATGGACTTCTTTAACAAGTTCTCACAGGGGCTGGCAGAATCCTCTACACCAAAGTCGTCA
MetAspPhePheAsnLysPheSerGlnGlyLeuAlaGluSerSerThrProLysSerSer
                                    90                                  120
ATCTATTATTCTGAAGAAAAGGATCCGGATACGAAAAAGGATGAAGCGATTGAAATAGGA
IleTyrTyrSerGluGluLysAspProAspThrLysLysAspGluAlaIleGluIleGly
                                   150                                  180
CTAAAGTCTCAAGAGTCGTATTATCAAAGACAGTTGCGAGAACAACTAGCTAGAGATAAT
LeuLysSerGlnGluSerTyrTyrGlnArgGlnLeuArgGluGlnLeuAlaArgAspAsn
                                   210                                  240
ATGACGGTCGCCAGCAGACAGCCTATCCAACCGCTACAACCAACTATTCATATAACTCCA
MetThrValAlaSerArgGlnProIleGlnProLeuGlnProThrIleHisIleThrPro
                                   270                                  300
CAGCCGGTTCCAACAGCTACACCGGCTCCTATTCTTCTACCTAGTAGTACTGTTCCTACA
GlnProValProThrAlaThrProAlaProIleLeuLeuProSerSerThrValProThr
                                   330                                  360
CCAAAACCACGACAACAAACTAATACATCATCTGATATGTCTAATCTTTTTGATTGGCTG
ProLysProArgGlnGlnThrAsnThrSerSerAspMetSerAsnLeuPheAspTrpLeu
                                   390                                  420
TCTGAAGATACTGATGCGCCGGCGAGTTCACTCCTTCCAGCGTTGACGCCGAGCAATGCT
SerGluAspThrAspAlaProAlaSerSerLeuLeuProAlaLeuThrProSerAsnAla
                                   450                                  480
GTTCAGGATATTATCTCTAAATTTAATAAAGATCAAAAGACGACGACACCGCCATCTACC
ValGlnAspIleIleSerLysPheAsnLysAspGlnLysThrThrThrProProSerThr
                                   510                                  540
CAACCTTCTCAGACGTTACCAACAACTACATGTACACAACAATCGGATGGAAATATTTCT
GlnProSerGlnThrLeuProThrThrThrCysThrGlnGlnSerAspGlyAsnIleSer
                                   570                                  600
TGTACTACTCCAACGGTTACACCTCCTCAACCTCCTATTGTGGCCACTGTATGTACTCCT
CysThrThrProThrValThrProProGlnProProIleValAlaThrValCysThrPro
                                   630                                  660
ACACCTACTGGTGGTACAGTATGTACAACAGCACAACAAAATCCAAATCCAGGAGCAGCA
ThrProThrGlyGlyThrValCysThrThrAlaGlnGlnAsnProAsnProGlyAlaAla
                                   690                                  720
TCTCAACAAAATCTAGACGATATGGCCCTTAAGGATCTCATGTCGAATGTTGAAAGAGAT
SerGlnGlnAsnLeuAspAspMetAlaLeuLysAspLeuMetSerAsnValGluArgAsp
                                   750                                  780
ATGCACCAACTTCAGGCCGAAACAAACGATCTGGTGACGAACGTATATGATGCAAGGGAG
MetHisGlnLeuGlnAlaGluThrAsnAspLeuValThrAsnValTyrAspAlaArgGlu
                                   810                                  840
TATACGCGTAGGGCAATAGATCAAATTCTACAACTAGTCAAAGGTTTTGAACGATTCCAA
TyrThrArgArgAlaIleAspGlnIleLeuGlnLeuValLysGlyPheGluArgPheGln
                                   870                                  900
AAGTAATAAGATTGGATATTAAAATCACGCTTTCGAGTAAAAACTACGAATATAAATAATG
Lys***
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FIG. 3. Nucleotide sequence and deduced amino acid sequence of the VV gene encoding the 39-kDa protein. DNA sequence analysis of the entire 39-kDa gene was carried out as described in Materials and Methods. The nucleotide sequence encodes a protein of 281 aa. The nucleotides are numbered above the sequence in the 5′-to-3′ direction, starting with nucleotide 1 in the A of the ATG codon for the initiator methionine. Amino acids are numbered beginning at the initiator methionine.

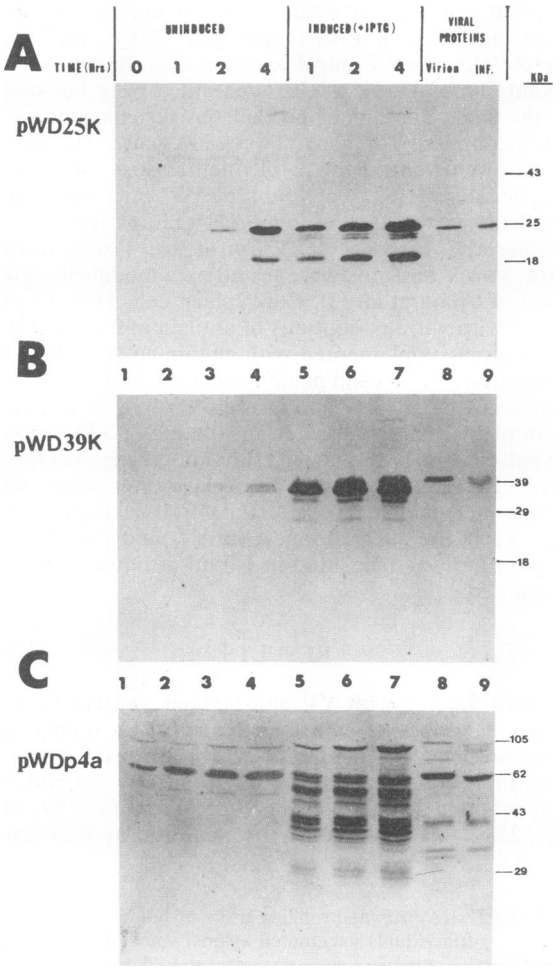

FIG. 4. Expression of antigenic VV proteins in *E. coli*. Generation of plasmids containing VV genes encoding antigenic proteins and their expression in *E. coli* were carried out as described in Materials and Methods. Samples were removed at different times from uninduced and induced cultures with 400 μM IPTG, analyzed by 12% SDS-PAGE and Western blotted, and protein profiles were revealed by immunoperoxidase staining after reactivity with monospecific antiserum. Protein profiles from cells transformed with plasmids pWD25K (A), pWD39K (B), and pWD4a (C) are shown. Lane Virion, lysate of purified VV; lane INF, lysate of VV-infected BSC-40 cells prepared at 24 h postinfection. The monospecific antiserum selected in each case was from a lysate of *E. coli* cells infected with a recombinant DNA phage that contains specific VV DNA sequences for the 25-kDa, 39-kDa, and p4a proteins.

selected monospecific antibodies reactive against the 62-kDa core protein of VV (data not shown) corresponded to the sequence of p4a from VV(WR) (37). The phage DNA insert whose sequence encodes the 21-kDa protein was found to be identical to the sequence present at the carboxy-terminal region of p4a (data not shown). DNA sequence analyses of phage DNA inserts for the 25-kDa core protein revealed identity in sequence with a 28-kDa VV core protein previously described (39). DNA sequence analyses of the phage corresponding to the 32-kDa envelope protein revealed identity in sequence with the 32-kDa cell surface-binding protein of VV (19). DNA sequence analyses of the phage insert encoding the 14-kDa envelope protein revealed identity in sequence with the 14-kDa fusion protein (26). The

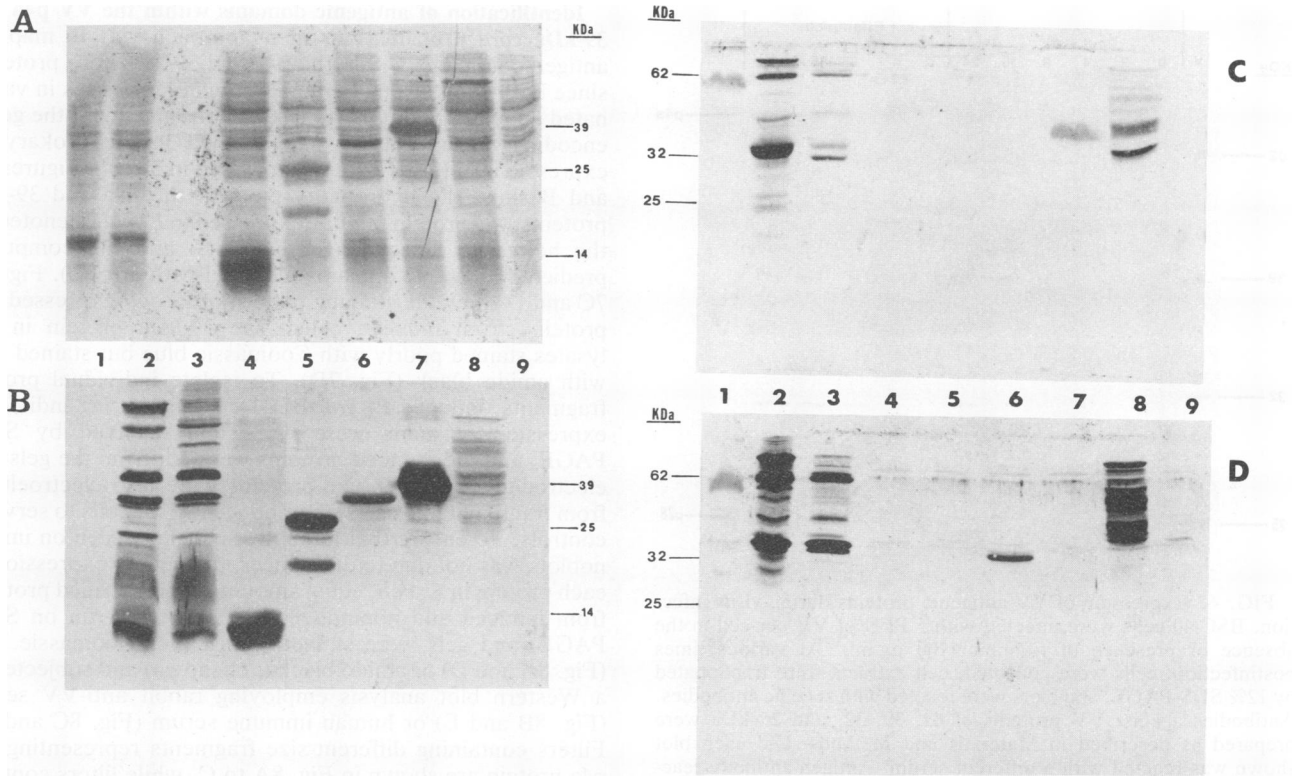

FIG. 5. Rabbit and human antibody reactivity to VV proteins produced in *E. coli*. Samples were removed 4 h after induction with 400 μM IPTG, analyzed by 12% SDS-PAGE, and blotted, and protein profiles were revealed by amido black staining (A), by immunoperoxidase staining after reactivity with rabbit anti-VV serum (B), or after reactivity with two different human immune sera (C and D). Lanes: 1, lysates of uninfected BSC-40 cells; 2, lysates from VV-infected BSC-40 cells at 24 h postinfection; 3, lysates of purified VV. Other lanes corresponded to *E. coli* BL21 cells transformed with pT7Nd14K (lane 4), pWD25K (lane 5), pT7EK32 (lane 6), pWD39K (lane 7), and pWD4a (lane 8). Lane 9 represents lysates of uninduced cells. Each lane was loaded with about 40 μg of protein except for lanes 3 (10 μg).

nucleotide sequence of the insert corresponding to a 39-kDa protein of VV(WR) did not correspond to any previously identified gene. We previously mapped this gene between the two major VV core polypeptides p4a and p4b (18) and therefore sequenced the entire gene. The complete DNA sequence and predicted amino acid sequence of the 39-kDa protein are given in Fig 3. The 39-kDa gene encodes a protein of 281 aa with a predicted pI of 4.71. The 5′ end of the 39-kDa gene contains a characteristic TAAATG motif of late genes (20). The gene encoding the 39-kDa protein immediately precedes the ORF encoding the p4b major core polypeptide (30), which starts 52 nucleotides downstream of the 39-kDa termination codon; the two genes have the same leftward direction of transcription.

Identification of VV genes encoding immunodominant proteins. To determine whether the VV genes identified above encoded immunodominant proteins in vaccinated humans, we first expressed each of the genes in *E. coli*, using the prokaryotic expression vector pT7-7. As shown in Fig. 4A, cells harboring the expression plasmid pWD25K synthesized a polypeptide of about 25 kDa and another protein of about 19 kDa which could result from a cleavage of the 25-kDa protein or from internal initiation. Cells harboring the expression plasmid pWD39K synthesized a single polypeptide of about 37 kDa, due to the removal of 27 aa at the N terminus of the 39-kDa protein and addition of 4 aa from the vector as a result of cloning (see Materials and Methods). Cells harboring the expression plasmid pWD4a, containing 835 aa of the p4a precursor protein, synthesized a polypeptide of about 95 kDa (the unprocessed precursor protein) together with a mature protein of about 62 kDa and smaller products, presumably originating from proteolytic cleavages, internal initiation, or both. Cells harboring the expression plasmid pT7Nd14K synthesized a polypeptide of about 14 kDa together with a processed 12-kDa protein (17). Cells harboring the expression plasmid pT7EK32 synthesized a single polypeptide of about 32 kDa (19). The ability of sera from immune animals and vaccinated humans to recognize the *E. coli*-expressed proteins was tested by immunoblotting. Representative findings with rabbit anti-VV serum and human immune sera from two vaccinated individuals are shown in Fig. 5. All of the *E. coli*-expressed VV proteins revealed by staining (Fig. 5A) showed reactivity with rabbit anti-VV serum (Fig. 5B). Two representative human sera, however, showed reactivity only against the p4a, 39-kDa, and 32-kDa *E. coli*-expressed proteins (Fig. 5C and D). The extent of reactivity was evaluated with 10 different human sera. It was found that the majority (9 of 10) of the human immune sera tested from vaccinated individuals contained antibodies reactive against p4a. Five human sera showed significant reactivity against the 32-kDa protein, and three human sera demonstrated reactivity with the 39-kDa protein. Interestingly, none of the 10 human sera tested reacted significantly with either the 25- or 14-kDa protein. Sera from nonvaccinated humans showed no reactivity with any of the VV proteins (data not shown). We conclude that after

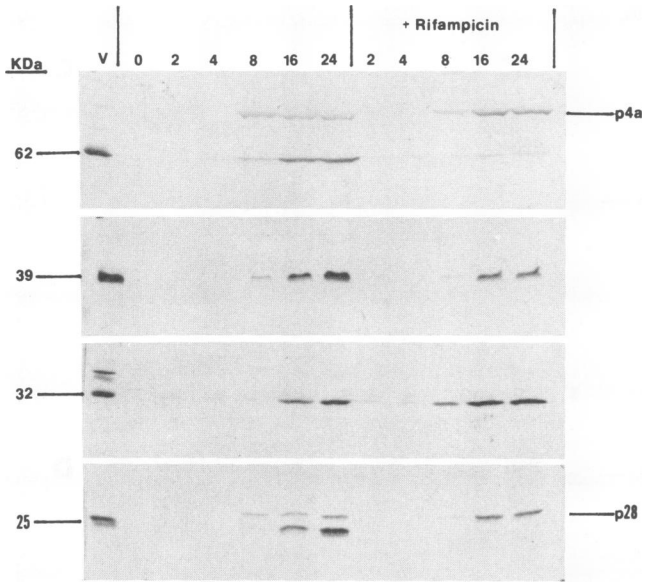

FIG. 6. Expression of VV antigenic proteins during virus infection. BSC-40 cells were infected with 5 PFU of VV per cell in the absence or presence of rifampin (100 μg/ml). At various times postinfection, cells were collected, cell extracts were fractionated by 12% SDS-PAGE, and blots were reacted with specific antibodies. Antibodies against VV proteins of 62, 39, 32, and 25 kDa were prepared as described in Materials and Methods, and each blot shown was reacted with a different serum. Antigen-antibody reactivity was detected by immunoperoxidase staining. The times (hours) postinfection are indicated at the top. Lane V, lysate of purified virions. The molecular mass is indicated for each protein.

vaccination against smallpox, most humans have acquired the ability to maintain the long-lasting production of antibodies reactive against the VV p4a and 39-kDa core and 32-kDa envelope proteins, with the p4a antigen being the most immunodominant.

Expression of VV antigenic proteins during virus infection. To characterize the time and mode of expression of VV antigenic proteins during virus infection, we carried out immunoblot analysis. BSC-40 cells were infected with VV in the absence or presence of rifampin, an inhibitor of virus assembly; at various times postinfection cell extracts were fractionated by SDS-PAGE, and the proteins were blotted and reacted with specific antibodies. Figure 6 shows an immunoblot of fractionated proteins reacted with antibodies against VV proteins of 62, 39, 32, and 25 kDa. It is clear that all of the VV proteins analyzed belong to the late class of viral genes, since their appearance was not observed until about 8 h postinfection. By inspection of individual proteins, we found that the 62- and 25-kDa proteins are derived by cleavage of larger precursors. Antibodies against the 62-kDa protein reacted with proteins of 95 and 62 kDa, while antibodies against the 25-kDa protein reacted with proteins of 28 and 25 kDa. The cleavage of both precursors, 95 and 28 kDa, was inhibited by rifampin (Fig. 6). Because rifampin is a known inhibitor of cleavage of VV p4a and 28-kDa proteins (14, 38), this result confirms the identity of the VV antigenic gene products as p4a and 28-kDa proteins. Only a single polypeptide of expected size, as deduced from the DNA sequence, was observed in Fig. 6, suggesting that synthesis of VV proteins of 62, 39, 32, and 25 kDa begins at the first ATG codon.

Identification of antigenic domains within the VV p4a and 39-kDa core proteins. Our next approach was to map the antigenic domains within the p4a and 39-kDa core proteins, since both elicit strong humoral immune responses in vaccinated animals and humans. Thus, subfragments of the genes encoding these proteins were cloned in the prokaryotic expression vector pT7-7 and expressed in *E. coli*. Figures 7A and B show the hydropathy plots of the p4a and 39-kDa proteins, the protein regions expressed in *E. coli* (denoted by the horizontal lines above the plots) and the computer-predicted antigenic domains (denoted with arrows). Figures 7C and D show the staining pattern of *E. coli*-expressed VV proteins from induced cells. The 39-kDa protein in the lysates stained poorly with Coomassie blue but stained well with amido black (Fig. 7D). To isolate individual protein fragments, induced *E. coli* BL21 cells harboring individual expression plasmids were lysed and resolved by SDS-PAGE, and the induced proteins were cut from the gels and electroeluted. Similar-size proteins were also electroeluted from fractionated lysates of uninduced BL21 cells to serve as controls. To ensure that antibody specificity seen on immunoblots was not due to differences in levels of expression of each protein in *E. coli*, equal amounts of gel-purified proteins from induced and noninduced cultures were run on SDS-PAGE, and gels were stained with either Coomassie blue (Fig. 8A and D) or amido black (not shown) and subjected to a Western blot analysis employing rabbit anti-VV serum (Fig. 8B and E) or human immune serum (Fig. 8C and F). Filters containing different-size fragments representing the p4a protein are shown in Fig. 8A to C, while filters containing different-size fragments corresponding to the 39-kDa protein are shown in Fig. 8D to F. Inspection of the pattern of antibody reactivity in Fig. 8B, using rabbit anti-VV serum, revealed that the strongest antigenic determinant of p4a is contained within aa 132 to 295 (as defined by plasmids p106 and p107). Inspection of the pattern of antibody reactivity in Fig. 8E by using rabbit anti-VV serum revealed that the antigenic domains for the 39-kDa protein are contained at both the N and C termini but not in the middle region of the protein. The antibody reactivity observed in Fig. 8B and E to proteins of higher molecular mass than the expected-size fragments is probably due to protein aggregation after electroelution and may represent protein dimers (note that dimers are barely visible in the stained gel of Fig. 8A and D). When human serum was reacted with subfragments of p4a, antibody reactivity was observed only within aa 132 to 295 (Fig. 8C; defined by p106 and p107). When human immune serum was reacted with subfragments of the 39-kDa protein, antibody reactivity was observed only against the last 103 aa at the C terminus (Fig. 8F).

From the results shown in Fig. 8, we conclude that vaccinated humans and animals develop antibodies predominantly against a specific domain of p4a (aa 132 to 295) and against the C-terminal 103 aa of the 39 kDa protein. These antibodies persist for years.

The antigenic domain of p4a (aa 132 to 295) markedly boosts antibodies in humans after revaccination. If the antigenic domain of p4a (aa 132 to 295) is immunodominant, it would be expected to boost specific antibodies in previously vaccinated individuals upon revaccination. Thus, we compare the extent of antibody reactivity in serum from an individual before and 3 weeks after revaccination with VV. The serum was reacted with subfragments of p4a (as for Fig. 8); the results of the Western blot are shown in Fig. 9. The patterns of antibody recognition observed before (Fig. 9A) and after (Fig. 9B) revaccination are identical, but the extent of

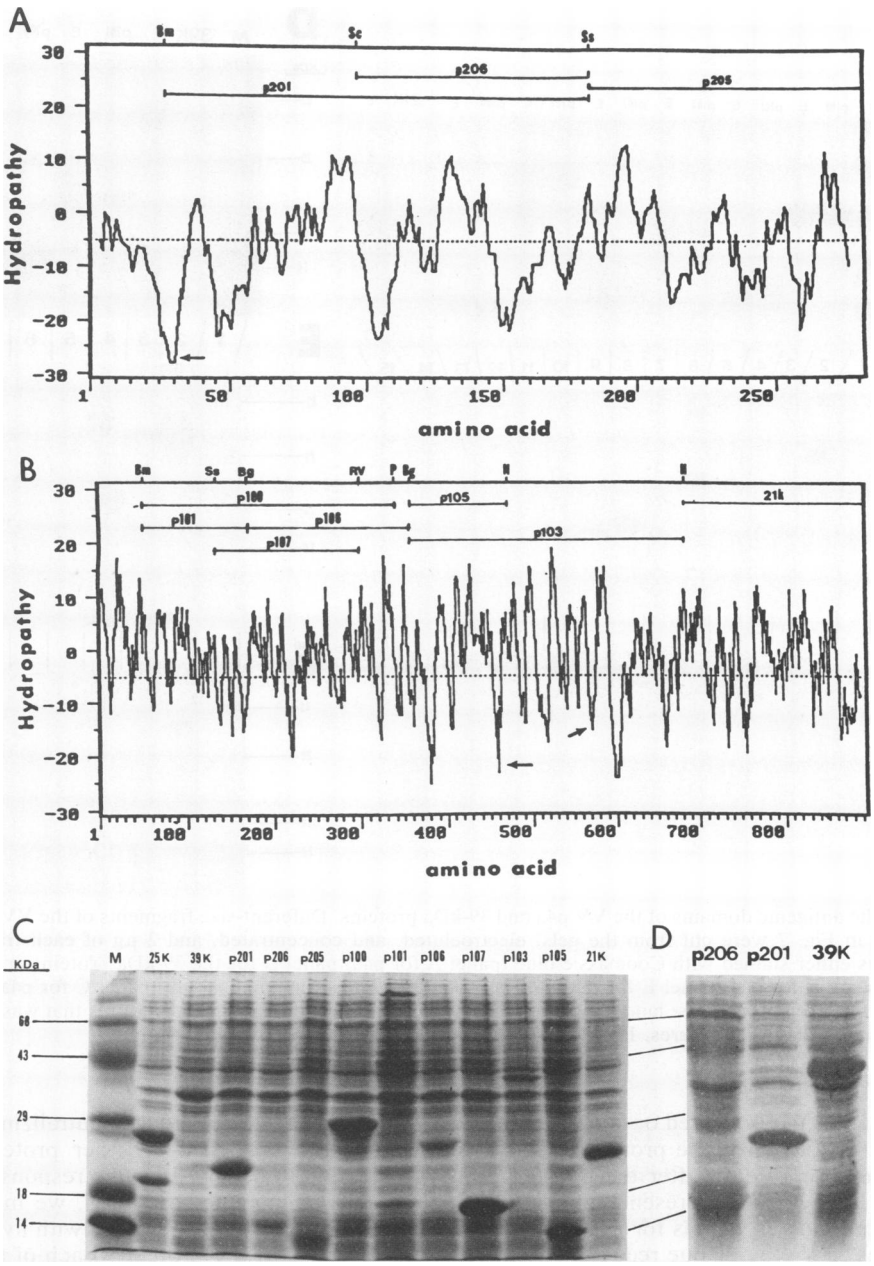

FIG. 7. Generation and expression of subfragments of VV proteins in *E. coli*. Generation of plasmids containing different domains of the 39-kDa and p4a VV proteins is described in Materials and Methods. (A and B) Hydropathy plots of the VV 39-kDa (A) and p4a (B) proteins as deduced from the DNA sequence by using the PC Gene program SOAP. Hydrophilic regions are below the axis, and hydrophobic regions are above. The gene fragments expressed in pT7-7 are represented by the horizontal lines above the plot. The strongest computer-predicted epitopes are represented by the arrows. Restriction enzymes: *Bgl*II (Bg), *Bam*HI (Bm), *Hin*pI (H), *Nsi*I (N), *Pst*I (P), *Ssp*I (Ss), *Eco*RV (RV), and *Sca*I (Sc). (C and D) Expression of different VV protein subfragments in *E. coli*. Samples were removed 4 h after induction with IPTG and analyzed by 12% SDS-PAGE, and protein profiles were revealed by Coomassie blue (C) or amido black (D) staining. Plasmid origins in abbreviated form are given above the lanes and are described in Materials and Methods.

antibody reactivity was markedly increased after revaccination (also confirmed by enzyme-linked immunosorbent assay; [ELISA]; data not shown). Because antibody specificity was mainly directed against aa 132 to 295 of p4a, this result further attests that this is the most immunogenic domain of p4a. A similar type of analysis was also carried out with the same serum against subfragments of the 39-kDa protein and against the entire 32-, 25-, and 14-kDa proteins. There was no significant increase in antibody reactivity against any of these VV proteins with this serum from a revaccinated individual (data not shown).

Immunization of mice with antigenic VV proteins protects against VV infection. To determine whether the 62- and 39-kDa immunodominant proteins could elicit protective immune responses in vivo, BALB/c mice were immunized with these proteins as described in Materials and Methods.

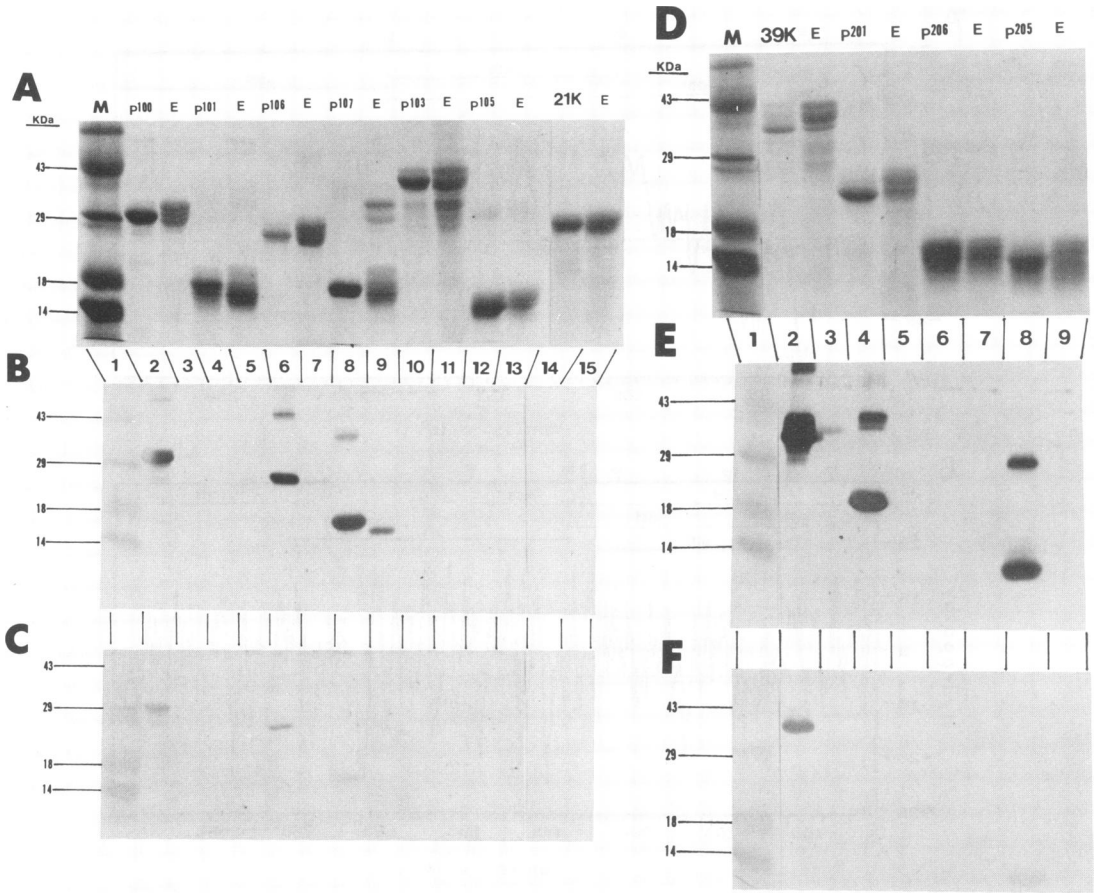

FIG. 8. Mapping of the antigenic domains of the VV p4a and 39-kDa proteins. Different-size fragments of the VV p4a and 39-kDa proteins expressed in *E. coli* as in Fig. 7 were cut from the gels, electroeluted, and concentrated, and 2 μg of each fragment was run on 12% SDS-PAGE. The gel was either stained with Coomassie blue (panel A for p4a; panel D for the 39-kDa protein) or blotted and reacted with rabbit anti-VV serum (panel B for p4a; panel E for the 39-kDa protein) or human immune serum (panel C for p4a; panel F for the 39-kDa protein). Lane M, molecular weight markers; lane E, *E. coli* protein electroeluted from uninduced cultures that was similar in size to the VV protein observed in gels from induced cultures. Plasmid origins are described in Materials and Methods.

Other groups of mice were mock treated or immunized with the *E. coli*-expressed 14-kDa envelope protein. Blood was collected from each mouse 10 days after the booster, and serum was tested by ELISA for the presence of antibodies reactive against VV proteins as well as for the presence of neutralizing antibodies to VV by plaque reduction assay. It was found that all mice have antibodies to these proteins, but the levels were in the order 39 kDa > 14 kDa > 62 kDa. Neutralizing antibodies were detected only in mice immunized against the 14-kDa protein (data not shown). Following 14 days after the booster, the animals were challenged with 40 LD_{50} of wild-type VV and survival was scored over a 3-week period. As shown in Fig. 10, control animals died by day 4 after virus challenge, while animals immunized with the 14-kDa protein demonstrated no ill effects of the virus burden and were all alive after 3 weeks. Fifty percent of the 39-kDa protein-immunized animals and 75% of the 62-kDa protein-immunized animals remained alive after 3 weeks. The findings of Fig. 10 provided evidence that VV proteins of 62, 39, and 14 kDa stimulate, to different degrees, immune responses capable of conferring protection against VV disease.

Cellular proliferative responses induced by VV antigenic proteins. Since immune protection was observed with VV proteins that do not induce neutralizing antibodies, it was of interest to determine whether protection correlated with activation of cellular immune responses. Thus, as an index of cellular immune responses, we measured the ability of spleen cells from mice primed with live VV to be stimulated in response to VV proteins. Each of the BALB/c mice was primed i.p. with 10^7 PFU of VV; splenocytes were prepared 5 to 8 weeks after virus inoculation and cultured in the presence of different amounts of *E. coli*-expressed VV proteins of 62, 39, 32, 25, and 14 kDa, and [^{3}H]thymidine incorporation was measured as described in Materials and Methods. After three independent experiments, we could obtain significant proliferative responses only when spleen cells were cultured with VV proteins of 32 and 14 kDa (Fig. 11). Clearly, spleen cells from animals immunized with VV elicit higher proliferative response to the 14-kDa protein than do spleen cells from nonimmune animals (Fig. 11A). The proliferative response was specific, since spleen cells from immune animals showed no significant stimulation with bovine serum albumin (Fig. 11B). Similar findings were observed with spleen cells from immune and nonimmune animals in response to the 32-kDa protein (Fig. 11C and D). We conclude that VV proteins of 32 and 14 kDa elicit cellular immune responses.

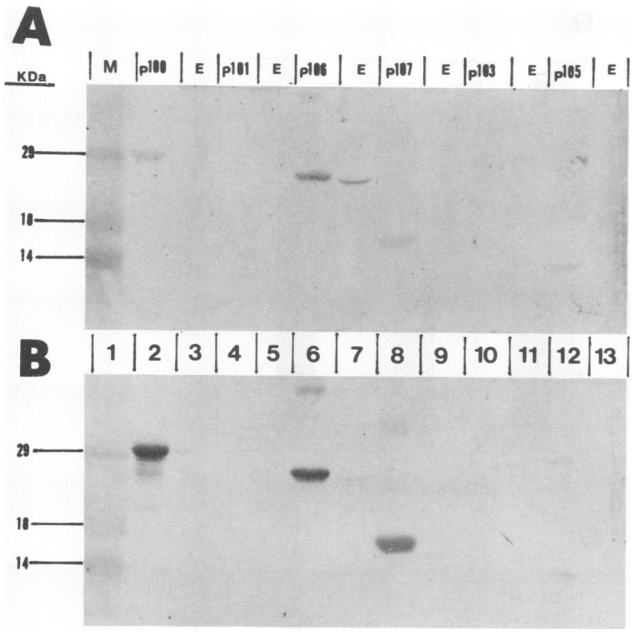

FIG. 9. Extent of antibody reactivity to VV p4a from human immune serum before and after revaccination. Serum was collected from an individual who was vaccinated 23 years ago and then again 3 weeks after revaccination. The serum (1:50), before (A) and after (B) revaccination, was reacted against different fragments of VV p4a expressed in *E. coli*, and the protein profile was visualized by immunoperoxidase staining. Lane M, molecular weight markers; lane E, *E. coli* proteins similar in size to the VV proteins. Plasmid origins are described in Materials and Methods.

DISCUSSION

In this investigation, we have identified VV proteins that play a major role in the immune response elicited after humans are vaccinated against smallpox and after animals are immunized with VV. The genes encoding VV antigenic proteins were cloned, sequenced, and assigned to specific ORFs. We characterized the mode of expression of these viral proteins during virus infection, defined their compartmentalization in the virion, and identified the most antigenic domains within some of these proteins. Finally, the ability of VV proteins to induce immunity and to activate cellular immune responses was also characterized. In light of the unique properties exhibited by each of the VV antigenic proteins, we will discuss the immunological characteristics and the structure-function relationships of these proteins separately.

p62K. The 62-kDa immunodominant core antigen identified in this study is encoded by the gene corresponding to VV (Copenhagen [CO]) ORF designated A10L (9). This gene encodes the precursor polypeptide of the 62-kDa protein, p4a, that is expressed late in infection and is posttranslationally modified to the mature 62-kDa major core antigen (Fig. 6). This major core antigen has an important role in the host immune response to VV because it induces long-term antibodies and confers in vivo protection against VV disease (Fig. 1 and 10). Through expression of this gene, in addition to gene fragments in a bacterial expression system, we have shown that the most antigenic domain within this protein is localized within its N terminus (Fig. 8). This antigenic domain was also found to markedly boost antibodies in humans after revaccination (Fig. 9), suggesting that this domain contributes to the generation of B-cell memory. When mice were immunized with this protein and challenged with VV, 75% of the animals survived (Fig. 9). Taken together, these results strongly suggest that the 62-kDa major core antigen is an important determinant in the immune response mounted against VV. The reason why this protein induces virus immunity is not known, but it could be related to activation of a cellular immune response. Posttranslational cleavage of p4a must occur at the C terminus, since we found that the gene which encodes the 21-kDa protein mapped to the C terminus of the 891-aa precursor protein p4a. By scanning the predicted p4a amino acid sequence (37), a potential cleavage site, Ala-Gly-Thr, was found that could result in cleavage at position 697, generating a protein equivalent to 21 kDa. The p4a protein could represent the common nucleoprotein antigen (34) found by the precipitation test in most members of the poxviruses (41) and could be the same cross-reactive polypeptide of 64 to 65 kDa previously identified with homologous and heterologous antiserum and with monoclonal antibodies (13, 15).

p39K. The gene for the 39-kDa core protein identified in this study was found to encode a polypeptide of 281 aa that we previously identified as immunodominant (18). The sequence of the VV 39-kDa gene (Western Reserve strain) was deduced (Fig. 3) and corresponds to the VV(CO) ORF designated A4L (9). Comparison of our deduced 39-kDa protein sequence for VV (WR) with A4L of VV(CO) strain revealed 98.2% identity in the 281-aa residues. We found five amino acid differences between the two proteins. The amino acid changes in the 39-kDa protein of WR versus CO occurred at positions Thr-62 for Met, Val-63 for Ala, Val-98 for Ala, Asn-236 for Ser, and Arg-239 for Lys. These substitutions, with the exception of Thr-62 for Met, do not appear to change the secondary structure of the protein. Comparison of the 39-kDa protein sequence with that of other cellular proteins revealed a subregion which shares identity to cytoskeleton keratins at aa 227 to 231, Met-Asp-Leu-Lys-Asp. This result might explain the ability of the

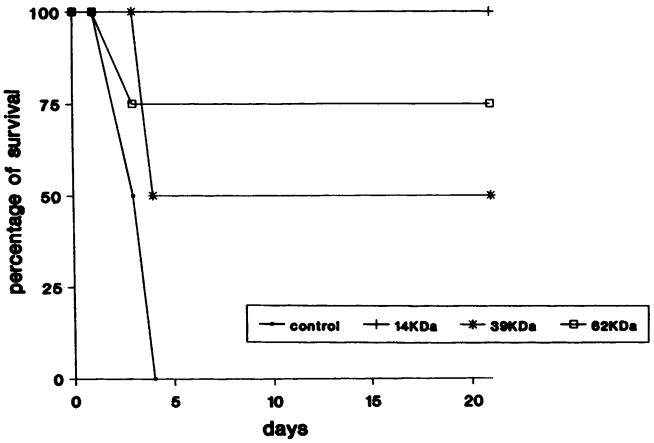

FIG. 10. Survival of mice immunized with antigenic VV proteins after challenge with a lethal dose of wild-type virus. BALB/c mice, four per group, were immunized i.p. with either 62-, 39-, or 14-kDa VV proteins as described in Materials and Methods. These VV proteins were purified by gel electroelution from SDS-PAGE. Control animals received the same immunization protocol but in the absence of VV proteins. Two weeks after the booster, the mice were challenged i.p. with 40 LD_{50} of VV. Survival was scored over a 3-week period.

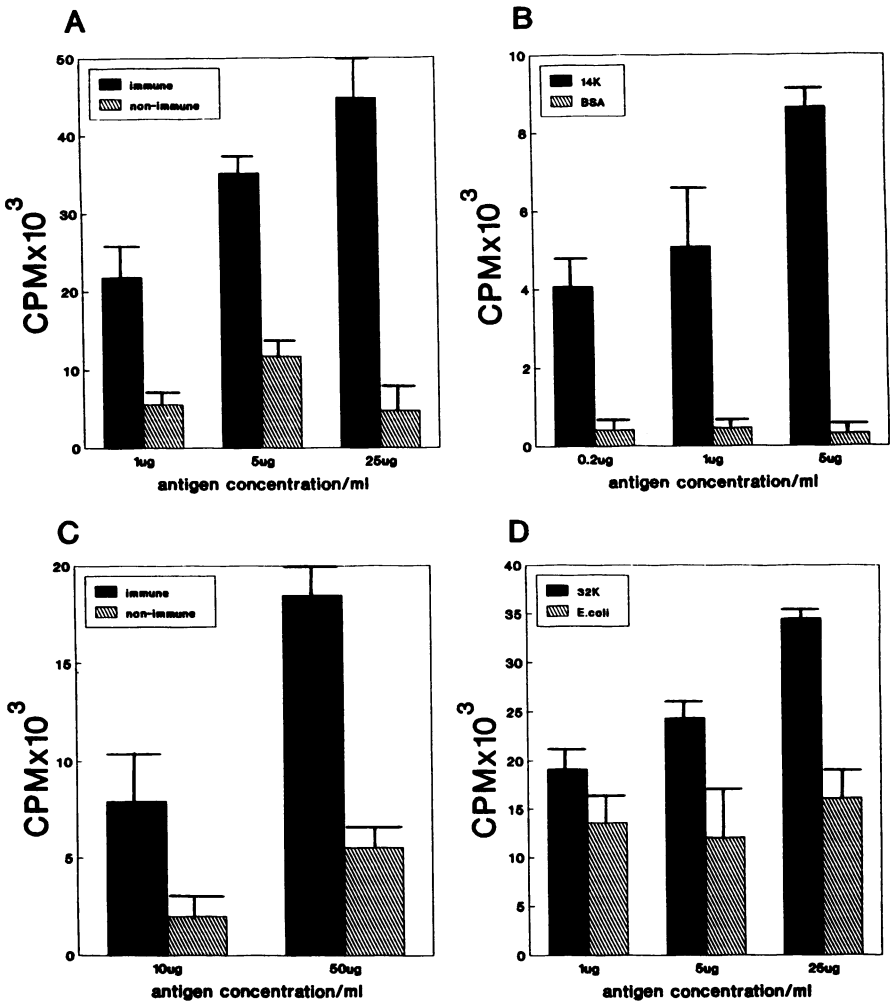

FIG. 11. Proliferative response of spleen cells from primed mice to VV antigenic proteins. Mice were primed i.p. with VV (10^7 PFU per mouse), and 5 to 8 weeks after virus inoculation, spleens were removed and spleen cells (5×10^5) were incubated with various concentrations of VV antigens. [^{3}H]thymidine incorporation was measured as described in Materials and Methods. The 14 kDa protein was purified by column chromatography to 95% homogeneity (17a), and the 32-kDa protein was purified from SDS-PAGE by electroelution. (A) [^{3}H]thymidine incorporation by spleen cells from a mouse immunized with live VV (immune) and incubated with the 14-kDa protein in comparison with spleen cells from a normal mouse (nonimmune) incubated with the 14-kDa protein; (B) [^{3}H]thymidine incorporation by spleen cells from an immune mouse after incubation with either the 14-kDa protein or bovine serum albumin; (C) similar to panel A, but spleen cells were cultured with the 32-kDa protein; (D) similar to panel B, but spleen cells were cultured with either the VV 32-kDa protein or the *E. coli* protein of similar size. The results shown in each panel were derived from different animals. The standard deviation is given. All assays were carried out by using triplicate wells.

39-kDa monoclonal antibody B6 to recognize cytoskeleton-like structures in uninfected cells (18). A recent report has described a 39-kDa immunodominant protein in fowlpox virus that maps immediately upstream from the fowlpox virus 4b gene and contains multiple copies of a 12-aa serine-rich repeated sequence (2). Although the VV 39-kDa gene also maps immediately upstream of 4b, comparison of the VV 39-kDa protein sequence with the fowlpox 39-kDa protein sequence reveals that these two proteins are different (8.2% identity), and only a region spanning aa 156 to 169 of the VV protein was found to have significant homology to aa 92 to 104 of the fowlpox virus protein. It appears that the 39-kDa immunodominant proteins in VV and fowlpox virus have evolved divergently. Our study determined that this protein elicits strong humoral immune responses in humans and animals (Fig. 1) and that the strongest antigenic domain is contained within the C-terminal 103 aa (Fig. 7). Significantly, prior immunization with the VV 39-kDa protein could protect 50% of mice against VV challenge (Fig. 9). While we do not know why the 39-kDa protein induces virus immunity, it is significant that this core antigen can be detected on the surface of virus-infected cells (data not shown). Antibody-dependent cell-mediated cytotoxic mechanisms may contribute to the partial protection seen by immunization with this protein. Whether this protein induces specific cellular immune responses important for clearance of the virus-infected cells remains to be determined.

p32K. The gene for the 32-kDa protein identified in this study corresponds to the VV(CO) ORF designated D8L (9) and encodes an envelope protein of 304 aa which we have previously identified as a cell surface-binding protein (19). This protein is expressed at late times during virus infection,

contains a domain with strong homology with carbonic anhydrase, and has been found to be nonessential for viral replication in cells in culture (23), but it is important in viral pathogenesis (29a). This protein can elicit strong humoral immune responses in humans and animals (Fig. 1 and 5). Proliferative responses were observed when this antigen was incubated with immune spleen cells, suggesting that this protein is also involved in generating cellular immune responses (Fig. 11). Our identification of the 32-kDa envelope protein as a major determinant involved in the generation of humoral and cellular immune responses against VV underscores its importance in the immune response during VV infection.

p25K. The gene for the 25-kDa protein identified in this study corresponds to the VV(CO) ORF L4R (9) and has previously been shown to encode a 251-aa protein with DNA-binding properties (39). The 25-kDa protein was shown to share amino acid homologies with the v-*erb-B* protein (aa 350 to 604) and with the epidermal growth factor receptor precursor (aa 920 to 1170) (4). Other previous studies have determined that posttranslational cleavage between the glycine and alanine residues at aa 32 and 33 in the 28-kDa precursor polypeptide is responsible for the generation of the mature 25-kDa protein (42). Our studies showed that this core protein, synthesized at late times postinfection, does not possess immunodominant characteristics in vaccinated humans but induces a strong immune response in vaccinated animals (Fig. 5).

p14K. The gene for the 14-kDa envelope protein was found to correspond to the VV(CO) ORF designated A27L (9). It has previously been shown that the protein forms dimers and trimers (28) and is involved in the fusion event between the virus and the cell membrane (27, 28). Antibodies produced against this protein are neutralizing in vitro (27). Our results showed that immunization of mice with this protein confers full protection against VV infection (Fig. 10) (17a) and that this protein elicits cellular immune responses (Fig. 11). These observations strongly suggest that the 14-kDa envelope protein plays an important role in the host immune response to VV.

It should be noted that most vaccinated individuals also contain circulating antibodies reactive against a 36-kDa protein (Fig. 1). Although the VV gene encoding this protein was not identified in this study, despite repeated attempts, it is likely that the 36-kDa protein is encoded by the VV gene previously identified as encoding a 35-kDa envelope protein (11).

Our inability to provide evidence of whether 62-, 39-, and 25-kDa proteins activate a proliferative response of spleen cells from vaccinated animals is probably related to the methodology used. We consistently found high background levels of [^{3}H]thymidine incorporation when spleen cells were cultured in the presence of *E. coli* proteins similar in size to the VV 62-, 39-, and 25-kDa proteins that were electroeluted from SDS-PAGE. Future studies with purified proteins should establish the role of these proteins in cellular immune responses.

The nature of the stimulus required for the continued production of anti-VV antibodies in vaccinated individuals years after vaccination is unknown. Possible stimuli capable of B-cell memory stimulation, and therefore long-lasting antibody production, albeit low levels, are (i) persistent viral antigens within the tissues of vaccinated individuals, (ii) the ubiquity of vaccinia viral antigens, (iii) non-VV antigens capable of cross reactivity, (iv) anti-idiotype antibodies with specificity for the variable domains of VV-specific antibodies capable of acting as or mimicking a foreign antigen, and (v) nonspecific interaction with stimulatory cytokines. Because circulating anti-VV antibodies were found only in immune individuals and have never been found in nonimmune individuals, the second possibility should be excluded. Which of the other possibilities actually plays a major role in the maintenance of anti-VV-specific memory B-cell populations and contributes to the continuous production of circulating antibodies long after the initial vaccination remains unknown.

Elucidation of the immune mechanisms associated with successful vaccination with VV may give us a better understanding of why this virus is such a potent immunogen and may help in the conception and construction of future VV recombinant vaccines. Once the immunological significance of each of the VV components has been established, the genes encoding these proteins could be targets for potential manipulations which alter the virus immunogenicity in a beneficial manner. A genetic manipulation could include introduction of mutations within highly immunogenic epitopes leading to a decrease in the immune response to VV. This, in turn, might allow a stronger interaction of the foreign antigen expressed by a VV recombinant with the host immune system. Another manipulation could involve the fusion of a foreign protein which displays weak immunogenic character to a highly antigenic epitope of a VV protein to augment the immune response directed against the foreign antigen. This strategy has been used for human immunodeficiency virus *env* protein fused to VV 14-kDa protein (29).

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