The Purified 14-Kilodalton Envelope Protein of Vaccinia Virus Produced in *Escherichia coli* Induces Virus Immunity in Animals

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Vaccinia virus (VV) was successfully used as a live vaccine to eradicate smallpox, but the nature of viral proteins involved in eliciting viral immunity has not yet been identified. A potential candidate is a 14-kDa VV envelope protein that is involved in virus penetration at the level of virus-cell fusion, in cell-cell fusion late in infection, and in virus dissemination. The 14-kDa envelope protein has been produced in *Escherichia coli*, with properties similar to those of the native protein found in the virus particle and in infected cells (C. Lai, S. Gong, and M. Esteban, *J. Biol. Chem.* 256:22174-22180, 1990). In this investigation, we showed that mice immunized with purified VV 14-kDa protein synthesized in *E. coli* in the form of a monomer or a trimer develop high-titer neutralizing antibodies and are protected when challenged with lethal doses of wild-type VV. Our findings demonstrate that it is possible to confer protection against VV through immunization with the 14-kDa envelope protein.

One of the major successes in modern health care is the eradication of smallpox, because vaccinia virus (VV) was proven to be a potent immunogen (1, 2). Live recombinants of VV are now being considered as candidate vaccines against human and animal pathogens (15, 20). A concern with the use of VV recombinants is that VV is a pathogen and has a wide host range. Thus, a priori vaccination of humans may result in a potential hazard due to spread of the virus to different tissues and to the brain, particularly in immunosuppressed individuals; if inoculated into wild animals, it might lead to generation of new variants because of homologous recombination with closely related poxviruses (2, 16). A major effort has been invested in the identification of regions in the viral genome which, when deleted or mutated, lead to virus attenuation. Many genes determining virulence (28) as well as those viral genes that confer tropism to the virus (19) have been identified. Thus, it is now possible to generate VV recombinants that are highly attenuated in animals and have restricted tropism, minimizing the risks of vaccination with live virus.

Despite an increased understanding of the structure, transcription, and DNA replication of VV (14), little is known about the details of the interaction between VV and the host immune system (15). Indeed, vaccination with live VV is known to induce a potent activation of humoral and cell-mediated immune responses, both of which are thought to play a major role in immunity to the virus (3, 9, 15). Because the complete sequence of VV DNA has recently been deduced (10), it should now be possible to define which of the viral gene products are involved in active humoral and cellular immunity leading to protection against virus infection. As yet, only a few structural proteins of VV that induced neutralizing antibodies in animals have been identified. These are a 58-kDa surface tubule protein that induces cell-cell fusion (27), a 14-kDa protein that induces virus-cell fusion (12, 24), and several other uncharacterized proteins with molecular masses of 54, 34, 32, 29, 25, and 17 kDa (17) and of 30, 16, and 14 kDa (4). Of the VV genes encoding

antigenic proteins with neutralizing ability, only the gene encoding the 14-kDa fusion protein has been identified (21). Previous studies have shown that the 14-kDa protein plays a major role in virus-host cell interaction. This protein is localized on the virus envelope in the form of a trimer and is involved in virus penetration, acting on fusion of the virus envelope with the cell membrane (12). The 14-kDa protein is also involved in cell-cell fusion, which occurs late in virus infections (12, 24), and in spreading of the virus to uninfected cells (5, 6, 26). The 14-kDa envelope protein of VV could, therefore, be a potential component vaccine. Recently, we have shown that large amounts of 14-kDa protein could be produced in soluble form in *Escherichia coli* by using a high-level prokaryotic expression system. The *E. coli*-produced 14-kDa protein was shown by several criteria to exhibit structural and functional properties similar to those of the protein found in the virus particle (13). In this article, we report a procedure for the purification of the 14-kDa envelope protein of VV from *E. coli* cells harboring the expression plasmid pT7Nd14K and show that mice primed and boosted with the 14-kDa protein induced high-titer neutralizing antibodies and were fully protected against challenge with wild-type VV.

A procedure for the purification of the VV 14-kDa protein from the *E. coli* expression system was developed (Table 1). Typically, 16 liters of *E. coli* BL21(DE3) cells transformed with the expression plasmid pT7Nd14K (13) were induced with 400 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) for 2 h, cells were centrifuged at 5,000 rpm for 10 min, and cell pellets were resuspended in 450 ml of a low-salt harvesting buffer (5 mM Tris-HCl, pH 8.0, 2 mM NaCl, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF]). Cells were disrupted by sonication and clarified by centrifugation at 15,000 rpm for 60 min at 4°C, and the supernatant was brought to 30% saturation by ammonium sulfate. After 4 h at 4°C with stirring, followed by centrifugation at 6,000 rpm for 30 min at 4°C, the pellet was resuspended in the harvesting buffer, and the supernatant was brought to 70% saturation by addition of ammonium sulfate. After 2.5 h at 4°C, followed by centrifugation at 10,000 rpm for 30 min, the pellet was

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TABLE 1. Summary for purification of the VV 14-kDa protein produced in *E. coli*^a

Treatment ^b	Vol (ml)	Protein concn (mg/ml)	Total protein (mg)	14-kDa protein total (%) ^c	Yield (%)	Purification (fold)
Crude extract	450	1.29	581.4	ND	100	ND
15,000 sup.	435	1.24	539.4	21	92	1
15,000 ppt.	50	0.84	42.0	ND	ND	ND
30% ammonium sulfate sup.	440	0.92	406.6	25	70	1.2
30% ammonium sulfate ppt.	50	0.60	30.0	ND	ND	ND
70% ammonium sulfate sup.	500	0.12	60.0	ND	ND	ND
70% ammonium sulfate ppt.	55	1.60	88.0	46	15	2.2
DEAE-Sepharose	50	1.44	72.0	73	12	3.5
Hydroxylapatite	27	0.44	11.9	88	2	4.2
Sephadex G-75	20	0.02	0.4	95	0.06	4.5

^a ND, not determined.^b sup., supernatant; ppt., pellet.^c Recoveries of 14-kDa protein should be higher than shown because only a few fractions from one column were loaded onto the next column.

resuspended in 50 ml of deionized water with 1 mM PMSF, dialyzed against 20 mM Tris-HCl (pH 8.0)–1 mM PMSF at 4°C, lyophilized, and resuspended in 30 ml of deionized water with 1 mM PMSF. This dialyzed sample was applied to a DEAE-Sepharose CL-6B column (26 by 40 cm; Pharmacia Fine Chemicals, Piscataway, N.J.) preequilibrated with 20 mM Tris-HCl (pH 8.0)–1 mM PMSF (buffer I). Elution of the proteins was performed at a flow rate of 52 ml/h with 480 ml of buffer I containing a linear gradient of 0 to 1.0 M NaCl. Fractions of about 4 ml each were collected, and the 14-kDa protein content of each fraction was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis after staining with Coomassie blue R-250. The percentage of the 14-kDa protein in each fraction was calculated from the total area of the 14-kDa protein versus the total area of the total proteins measured by densitometric scanning of the gel. The peak fractions containing most purified 14-kDa protein (fractions 62 to 67) were pooled, dialyzed against buffer I, lyophilized, and resuspended in deionized water. Pooled peak fractions were applied to a hydroxylapatite Bio-Gel HTP adsorption column (16 by 40 cm) equilibrated in 20 mM Tris-HCl (pH 8.0)–10 mM KH₂PO₄–1 mM PMSF–300 mM NaCl. Elution was performed at a flow rate of 26 ml/h with 480 ml of a linear gradient of 0 to 1.0 M phosphate buffer (NaH₂PO₄–Na₂HPO₄, pH 8.0). Fractions of about 4 ml each were collected and analyzed by SDS-PAGE. Fractions containing the most purified 14-kDa protein (fractions 70 to 76) were pooled, dialyzed, lyophilized, resuspended in deionized water, and applied to a Sephadex G-75 gel filtration column (16 by 70 cm). Elution was performed at the same flow rate of 26 ml/h with 100 ml (about two bed volumes) of 20 mM Tris-HCl, pH 8.0. Fractions of about 4 ml each were collected and analyzed by SDS-PAGE, and peak fractions were pooled, lyophilized, and resuspended in water. Representative results for the last step in purification, which involves Sephadex G-75 gel filtration, are shown in Fig. 1. The VV 14-kDa protein was purified to 95% homogeneity (Table 1). As determined by SDS-PAGE analysis under nonreducing conditions (13), the purified 14-kDa protein was largely in the form of a trimer of about 40 kDa (data not shown).

We next sought to determine the antigenic characteristics of the *E. coli*-produced VV 14-kDa protein. BALB/c mice were immunized intraperitoneally (i.p.) with different amounts of the column-purified 14-kDa protein or with a noninduced *E. coli* extract as a control. Antiserum from each

FIG. 1. Sephadex G-75 gel filtration column chromatography. Pooled peak fractions from the hydroxylapatite Bio-Gel HTP column were loaded onto a Sephadex G-75 column preequilibrated with 400 ml of 20 mM Tris-HCl, pH 8.0, at an adsorption rate of 15 ml/h. Elution was performed with the same buffer, fractions were collected, and proteins in each fraction were analyzed on SDS–12% PAGE under reducing conditions and stained with Coomassie blue. Relative areas of the 14-kDa protein (Δ) and the total proteins ($\circ$) and percentage of the 14-kDa protein in each fraction ($\blacktriangle$) were plotted versus fraction numbers. C1, pooled peak fractions from the hydroxylapatite Bio-Gel HTP column; C2, the 30 to 70% ammonium sulfate-fractionated induced *E. coli* cell extract.

FIG. 2. Western blot analysis of antiserum produced in mice immunized with the *E. coli*-synthesized VV 14-kDa protein. BALB/c mice, 6 weeks old, were immunized i.p. with the purified 14-kDa protein or with a control lysate from *E. coli*. Each mouse received 10 or 50 μ g of 14-kDa protein or an equivalent amount of *E. coli* lysate in PBS mixed with an equal volume of Freund's complete adjuvant. Three weeks later, each mouse received i.p. the same amount of protein but mixed with Freund's incomplete adjuvant. After 2 more weeks, the blood was collected from the tail vein and the ability of antibodies in serum to react with the 14-kDa protein was determined by immunoblot analysis at a 1:100 dilution. The nitrocellulose filter from SDS-12% PAGE contains lysates from noninduced *E. coli* cells (lanes 1), from 4-h-induced *E. coli* cells expressing the 14-kDa protein (lanes 2), and from BSC-40 cells infected for 24 h with 2 PFU of VV per cell (lanes 3). Antigen-antibody reactivity was detected by immunoperoxidase staining, as previously described (13). Each panel contains serum from a different origin: A, serum from a mouse immunized with 50 μ g of a control *E. coli* lysate; B, monoclonal antibody C3 as ascites (monoclonal antibody C3 is an immunoglobulin G2b, and its origin has been described previously [23]); F and H, sera from two individual mice immunized with 50 μ g of purified 14-kDa protein; G and I, sera from two individual mice immunized with 10 μ g of 14-kDa protein; P, hyperimmune anti-VV rabbit serum; N, serum from a nonimmune mouse. Molecular weight markers (lane M) are indicated in kilodaltons.

mouse was tested by Western blot (immunoblot) analysis with nitrocellulose filter strips containing lysates from non-induced *E. coli* cells (Fig. 2, lanes 1), from 4-h-induced *E. coli* cells (Fig. 2, lanes 2), and from cultured cells (BSC-40) infected for 24 h with VV (Fig. 2, lanes 3). Clearly, antiserum from four mice immunized with different concentrations of the 14-kDa protein (Fig. 2F, G, H, and I) does not react with control *E. coli* lysates (Fig. 2, lanes 1), while it reacts strongly with the 14-kDa protein present in induced *E. coli* lysates (lanes 2) and with the 14-kDa protein synthesized in virus-infected cells (lanes 3). Antiserum from mice immunized with control *E. coli* lysates does not react with the 14-kDa protein (Fig. 2A). The mouse antibodies were capable of immunoprecipitating the 14-kDa protein from VV-infected cells and of inhibiting cell-cell fusion induced by VV, strain IHD-W, at late times postinfection (data not shown).

The serum from 14-kDa-protein-immunized animals was tested by enzyme-linked immunosorbent assay (ELISA) for antibody levels and by plaque reduction assay for neutralizing antibodies. The humoral immune response was then compared with the ability of animals to allow virus gene expression in the spleen and to survive subsequent virus challenge.

BALB/c mice (three or five per group) were immunized i.p. with various amounts of VV 14-kDa protein that was either electroeluted by SDS-PAGE, as previously described (13), or purified by column chromatography, as in Fig. 1. The 40-kDa protein is the trimeric form of the 14-kDa protein, while the 12-kDa protein is the processed form of the 14-kDa protein that lacks the first two amino acids at the N terminus

TABLE 2. Immune responses and viral immunity induced by VV 14-kDa protein in mice

Origin of antiserum ^a	ELISA dilutions ^b	Neutralization titer ^c	Luciferase (U/mg) ^d	Survival ^e
Anti- <i>E. coli</i> (50)	0	0		0/3
Anti-40-kDa ^f protein (50)	1:1,200	1:1,024		3/3
Anti-14-kDa ^f protein (50)	1:600	1:256		3/3
Anti-12-kDa ^f protein (50)	1:300	1:128		3/3
None	0	0		0/3
Anti- <i>E. coli</i> (50)	0	0	77.8	0/5
Anti-40-kDa ^g protein (50)	1:3,200	1:1,024		5/5
Anti-40-kDa ^g protein (10)	1:2,400	1:512		5/5
Anti-40-kDa ^g protein (50)	1:2,800	1:1,024	3.8	ND
Anti-40-kDa ^g protein (10)	1:1,600	1:512	3.7	ND

^a Values in parentheses are the amounts of protein per injection. None, mice were not immunized.

^b Each assay was carried out in triplicate with different dilutions of the pooled sera, and the dilutions giving 50% maximal optical absorbances are presented. Values were determined twice from the same sera, with less than 5% standard error.

^c Neutralization titers determined as previously described (13). Values represent the dilutions of sera giving 80% reduction of VV plaques in BSC-40 cell monolayers. Each assay was performed in triplicate, and absolute values from the pooled sera are given.

^d Values represent averages for five spleens.

^e ND, survival was not determined.

^f Protein electroeluted by SDS-PAGE, as previously described (13).

^g Protein purified by column chromatography, as for Fig. 1.

(13). One group of animals was primed with 50 μ g of protein, while another group was primed with 10 μ g of protein in Freund's complete adjuvant. After 3 weeks, mice were boosted with either 50 or 10 μ g of protein in Freund's incomplete adjuvant. After 10 days, blood was collected from the tail vein and sera from each group of animals were pooled. Two weeks after the booster, mice were challenged i.p. with 1.5×10^8 PFU (10 times the 50% lethal dose) of purified wild-type VV (strain WR) per mouse or with a VV recombinant expressing luciferase (25). Groups of mice were examined for survival during 3 weeks, while other groups of animals were sacrificed 3 days after virus challenge to measure luciferase activity in the spleen, as previously described (25). For ELISA, purified VV diluted in 0.1 M NaHCO₃-Na₂CO₃ (pH 9.6) was fixed overnight in microtiter plates at 2 μ g of viral protein per well. The wells were incubated in 5% BLOTTO (nonfat dry milk) in phosphate-buffered saline (PBS) for 1 h at 37°C and reacted with different antisera diluted in 5% BLOTTO for 1 h at 37°C. Thereafter, wells were washed with PBS, reacted for 1 h at 37°C with a second antibody, goat anti-mouse immunoglobulin G coupled to horseradish peroxidase (Cappel Laboratories), at 1:250 dilution in 5% BLOTTO, and washed with PBS. The antibody reactivity was developed at room temperature for 3 min with 50 μ l of 25 mM sodium citrate (pH 4.5) containing 0.02% 3,3',5,5'-tetramethyl-benzidine dihydrochloride and 0.003% hydrogen peroxide (5 ml of 25 mM sodium citrate, 100 μ l of 1.0% 3,3',5,5'-tetramethyl-benzidine dihydrochloride in H₂O, 5 μ l of 3% hydrogen peroxide). The reaction was stopped with 50 μ l of 1 M H₂SO₄, and optical densities at 450 nm were measured in a Bio-Rad model 3550 microplate reader (Bio-Rad Laboratories, Richmond, Calif.). As shown in Table 2, the 14-kDa protein induced high neutralizing-antibody titers that correlated well with the extent of immunogenicity of the protein. The neutralizing titers in serum from animals immunized with the 14-kDa protein purified by column chromatography were

comparable to those induced by the 14-kDa protein that was electroeluted by SDS-PAGE (13) (Table 2). The antibody titer was, however, lower in serum from animals immunized with 14-kDa monomers than from animals immunized with trimers as determined by SDS-PAGE (Table 2). To establish whether the immunogenicity and ability to induce a neutralizing immune response by the purified 14-kDa protein correlated with protection of animals from VV disease, two sets of experiments were carried out. In the first set, the ability of a VV recombinant expressing luciferase to replicate in the spleen was evaluated 3 days after virus challenge. In the second set, survival was monitored for 3 weeks after virus challenge. As shown in Table 2, luciferase expression was severely inhibited in the spleens of 14-kDa-protein-immunized animals. Significantly, a 100% survival rate was observed with animals immunized with the 14-kDa protein in the form of a monomer or a trimer. The protected animals were considered cured, since their survival was not altered even 6 months after virus challenge.

We have shown here that immunization of mice with the VV 14-kDa envelope protein produced in *E. coli* elicits an immune response that fully protects animals against a VV disease. Virus immunity could be achieved when the VV 14-kDa protein was purified by column chromatography or electroeluted by SDS-PAGE (Table 2). The advantages of the column-purified 14-kDa protein over the protein purified from virions is that animals are protected when immunized with a protein that resembles the native protein present in the virus envelope, as previously documented (13), and that large amounts of the protein can easily be prepared from *E. coli*.

Protective immunity against poxviruses is thought to be mediated by humoral and cellular immune responses (3, 9), although the nature of the viral proteins involved in virus immunity is not yet known (9). Considering that VV contains about 100 structural proteins (8), how does a single protein induce virus immunity? This ability must be related to the 14-kDa protein playing an important biological role during virus infection. Indeed, previous studies have shown that VV 14-kDa protein is involved in virus penetration by fusion of the virus envelope with the cell membrane (7, 12, 23), can induce cell-cell fusion either at neutral pH in cells infected with some fusogenic VV variants (24) or at acid pH in cells infected with wild-type virus (12), and is involved in virus dissemination (5, 6, 11, 12). Genetic and biochemical studies revealed that mutations in the 14-kDa protein were responsible for the small plaque size phenotype of variants (5, 6, 11, 22). Other studies provided evidence that the 14-kDa protein is essential for the envelopment of intracellular virions by Golgi membrane and for the release of extracellular virus (26). Since extracellular virus is not neutralized in cells in culture by anti-14-kDa protein monoclonal antibody C3, while intracellular virus is severely neutralized by the same antibody (7; our unpublished observations), how does the 14-kDa protein induce protection to VV in animals? The simplest interpretation is that anti-14-kDa protein neutralizing antibodies block virus penetration, since previous studies have shown that anti-14-kDa protein monoclonal antibody C3 does not block virus attachment to cells but blocks virus penetration (23). Input virus particles which escape neutralization will replicate within cells. The progeny virus will then be inhibited by anti-14-kDa protein neutralizing antibodies, if the virus is released before it has acquired the second envelope. This, in turn, will reduce the load of infection. That virus gene expression is markedly reduced 3 days after virus challenge (Table 2) suggests that under the

experimental conditions used, most of the input virus is eliminated by neutralizing antibodies during the first stage of virus infection. Extracellular virus with a second envelope will then escape virus neutralization because antibodies against intracellular virus do not block virus infection mediated by extracellular virus (18). While immunization with purified 14-kDa protein clearly activates a strong humoral immune response, the levels of neutralizing antibodies induced in animals might be sufficient to reduce the spread of virus infection to tissues through elimination of intracellular virus released upon lysis of infected cells. Activation of a cellular immune response by the 14-kDa protein and of other nonspecific host defense mechanisms, such as activation of interferon and natural killer cells as a result of virus infection, might also contribute to virus clearance.

In conclusion, our findings provide the first demonstration that VV disease can be prevented by immunization with the 14-kDa virus envelope protein. This protein could be a candidate component vaccine. Whether 14-kDa protein protects against different strains of poxviruses with conserved 14-kDa protein (23) remains to be determined.

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