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Identification and preliminary characterization of vaccinia virus (Dryvax) antigens recognized by vaccinia immune globulin

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Abstract

Using vaccinia immune globulin (VIG), a high-titer antibody preparation from immunized subjects, we demonstrate that the humoral immune response in humans is directed against numerous antigens in the Dryvax vaccine strain. Western blot and immunoprecipitation analyses revealed highly antigenic proteins associated with both the extracellular enveloped virus and intracellular mature virus forms. The modified vaccinia virus Ankara (MVA), a new generation smallpox vaccine that is attenuated for replication in humans, expresses most, but not all, of the major vaccinia antigens recognized by antibodies in VIG, lacking the highly antigenic protein corresponding to the A-type inclusion body protein. Since new-generation smallpox vaccines such as MVA will require extensive comparison to traditional smallpox vaccines in animal models of immunogenicity and protection, we compared the vaccinia virus antigens recognized by VIG to those recognized by sera from Dryvax and MVA immunized mice. The humoral immune response in immunized mice is qualitatively similar to that in humans.

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Keywords: VIG; Dryvax; MVA; Humoral response; Vaccinia; Inclusion body protein

Introduction

The elimination of smallpox disease from the human population was one of the greatest public health accomplishments of the 20th Century. A primary reason for this accomplishment was the widespread use of live viral vaccines based on vaccinia. Immunization by scarification using vaccinia-based vaccines evoked a potent humoral and cellular immune response (Belshe et al., 2004; Ennis et al., 2002) almost universally effective against smallpox.

Although current licensed vaccines were extremely effective against smallpox, a number of adverse events, especially in the immune-compromised population, were observed (Bray, 2003; Fulginiti et al., 2003). Since it was believed that some of the more severe adverse events arose from defects in antibody production, the immunotherapeutic drug, vaccinia immune globulin (VIG), was developed to treat adverse events arising from vaccination. The VIG was prepared from the collected plasma of recently vaccinated individuals. Plasma samples were pooled and the IgG fraction was concentrated 5- to 8-fold through a series of ethanol precipitation steps. Therefore, VIG represents the humoral arm of the human immune response elicited by Dryvax, a licensed vaccinia-based vaccine protective against smallpox.

During the latter stages of smallpox vaccination in the United States, VIG was used to treat almost all patients with vaccination complications. In uncontrolled trials, an intramuscular injection of 0.6 ml of VIG per kg of body weight was effective in ameliorating cases of generalized vaccinia and eczema vaccinatum (Goldstein et al., 1975; Kempe et al., 1956; Sharp and Fletcher, 1973; Sussman and Gross-

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man, 1965). Some cases of progressive vaccinia were successfully resolved after multiple injections of VIG over the course of weeks. VIG was also recommended for prophylactic use at a dose of 0.3 ml/kg during vaccination of contraindicated individuals who had been exposed to smallpox. This treatment did not prevent the occurrence of a vaccination take (Kempe et al., 1956). However, there is no evidence that VIG was useful in the treatment of post-vaccinal encephalitis. Therefore, patients with eczema vaccinatum, some patients with severe generalized vaccinia, many patients with severe reactions to inadvertent inoculation, and some with milder forms of progressive vaccinia benefit from treatment with VIG. In addition to its activity against vaccinia, VIG activity against smallpox was demonstrated in one small, uncontrolled trial in which administration of VIG in conjunction with simultaneous vaccination reduced the incidence of smallpox in exposed individuals (Kempe et al., 1961).

In recent years, the consequences of an accidental or deliberate release of smallpox (Henderson, 1999) have led to a concerted effort by the public health community to meet this potential threat by the development of diagnostic tools, the pursuit of antiviral drugs for therapeutic options, and the manufacture and stockpile of vaccines against smallpox. The spectrum of adverse events associated with vaccinia-based smallpox vaccines, especially in the immunocompromised population, has spurred the development of new generation smallpox vaccines with superior safety profiles to augment, or possibly replace the current licensed smallpox vaccines. The identification of the important components of the elicited immune response and the comparison of that response in new vaccines to known effective vaccines are critical in the evaluation of the new generation vaccines.

In the present report, we present a series of experiments identifying viral protein targets recognized by VIG, the humoral component of the protective immunological response to smallpox. As demonstrated by the Western blot analysis, the antigens to VIG are numerous and include proteins from both the extracellular enveloped virus (EEV) and intracellular mature virus (IMV). The prominent antigens are not restricted to abundant viral structural proteins but include members of the early gene class and core proteins.

Attenuated smallpox vaccines were developed at the end of the program to eradicate smallpox by multiple passages of vaccinia-based vaccines in tissue culture substrates (Fenner et al., 1988). The most widely used attenuated vaccine, Modified Vaccinia Ankara (MVA), was derived from over 500 passages in primary chicken embryo fibroblasts (Mayr et al., 1975). The efficacy evaluation of this vaccine will rely on a comparison of the MVA elicited immune response to Dryvax and the ability of MVA to protect against lethal poxvirus challenge in animal models. The suitability of the mouse model will be reflected in its ability to mimic the immune response observed in humans. This report also describes the humoral response in mice vaccinated with Dryvax or MVA via a characterization of the protein targets recognized by Western blot using antisera from mice vaccinated with Dryvax or MVA. The pattern of proteins recognized by mouse sera derived from animals vaccinated with Dryvax or MVA was qualitatively similar to the pattern recognized by VIG.

Results

Human VIG recognizes many proteins in Dryvax and MVA virions and virus-infected cells

A preliminary identification and characterization of the targets of the humoral response against the smallpox vaccine Dryvax were undertaken using Western blot analysis. A collection of human and monkey cell lines were infected with either Dryvax or MVA and proteins analyzed by Western blot using VIG (Fig. 1). Human anti-vaccinia virus antibodies in VIG were primarily reactive against 13 vaccinia virus proteins with molecular mass of approximately 95, 77, 62, 59, 42, 40, 37, 35, 33, 28, 25, 18, and 14 kDa. The pattern of viral proteins recognized by VIG was similar in both human and monkey cells infected with Dryvax or MVA. There was, however, one prominent band with a molecular mass greater than 80 kDa in Dryvax-infected cell lysates and purified virus that was not present in MVA-infected cell lysates or purified virus (Fig. 1, arrow). There were a few reproducible differences in the Western blot pattern between viral particles and infected cells for both Dryvax and MVA. A band at 62 kDa was more intense in the Dryvax and MVA virion samples, but weaker in the infected cell lysates. Conversely, a band at 59

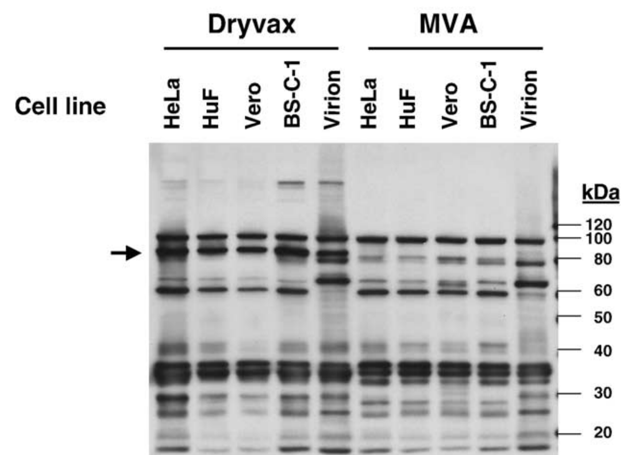

Fig. 1. Western blot analysis of proteins expressed in cell lines infected with Dryvax or MVA. Lysates from HeLa, human fibroblast (HuF), Vero and BS-C-1 cells infected at an MOI of 10 with Dryvax or MVA (leftmost four lanes in each group), and 500 ng purified Dryvax and MVA virions (Virion) were transferred to nitrocellulose and used for Western blot analysis using VIG as the primary antibody. The protein detected only in Dryvax virions or infected cell lysates is marked with an arrow.

kDa was prominent in cells infected with either vaccinia strain, but only faintly visible in purified virus preparations. Since the virion samples are composed predominantly of the IMV form, bands prominent only in cell lysates may correspond to poorly packaged proteins, proteins specific for EEV, or a modification of the 59 kDA protein after packaging. VIG did not cross-react with cellular proteins from the human or monkey cell lines and human vaccinia naïve serum did not react with infected lysates or purified virions (data not shown).

The data from Fig. 1 suggest that most of the proteins that react with VIG fall into two regions, with apparent molecular mass between 25 and 40 kDa or 60 and 100 kDa. The relationship between the relative abundance of viral proteins and their reactivity with VIG was investigated by electrophoresing purified Dryvax and MVA virions through 4–12% polyacrylamide gels and transferring the proteins to nitrocellulose. Duplicate filters were stained with colloidal gold (Berggren et al., 1999) to detect proteins (Fig. 2A) or analyzed by Western blot using VIG (Fig. 2B). The most abundant viral proteins, as determined by staining with colloidal gold, did not always coincide with the most intensely stained bands after Western blot analysis using VIG. The viral proteins that elicited a strong antibody response after vaccination were those associated with the range of 30–38 kDa proteins and the protein associated with the greater than 80 kDa band found only in Dryvax. The intense band at 62 kDa (Fig. 2B) did correlate with an abundant viral protein. The abundant viral proteins at approximate molecular mass of 42 kDa and 59 kDa were recognized poorly by VIG. The phenomenon of immunodominance of vaccinia virus proteins has been observed in mice, rabbits, and humans (Demkowicz et al., 1992; Maa and Esteban, 1987; Wilton et al., 1986).

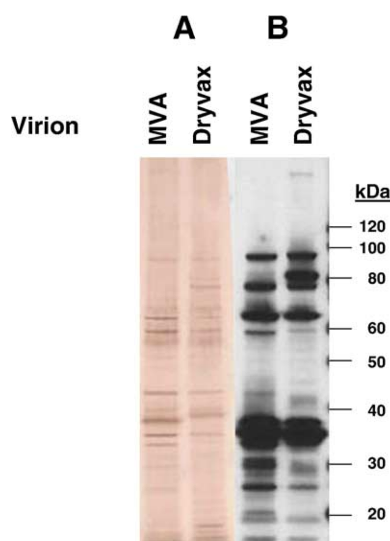

Fig. 2. Viral protein concentration does not correlate with VIG reactivity. Proteins from purified Dryvax and MVA virus were electrophoretically resolved on a 4–12% bis-Tris gel, transferred to nitrocellulose membrane and (A) stained with colloidal gold; or (B) used for Western blot analysis using VIG as the primary antibody (chemiluminescent detection).

VIG recognizes both early and late viral proteins

Further characterization of the many vaccinia proteins recognized by VIG using Western blotting was accomplished by investigating the temporal class of the reactive viral proteins. To determine whether vaccinia virus early proteins elicit a humoral response during vaccination, Western blot analysis using VIG was performed on lysates from cells infected with Dryvax in the presence or absence of cytosine arabinonucleoside (AraC). A number of bands with molecular mass between 20 and 40 kDa increased in intensity from 2 to 18 h post-infection, most prominent of which were the bands observed at 37, 33, and 25 kDa. A band at about 62 kDa was relatively unchanged over time and probably corresponds to a protein from the input virus (Fig. 3A). Since many more viral proteins were observed in lysates from cells infected in the absence of AraC (Figs. 3A and B), the majority of viral proteins recognized by VIG are members of the late class of viral proteins. The pattern of recognized early viral proteins was similar in lysates from monkey (BS-C-1) or human fibroblast cell lysates (HuF) infected with Dryvax or MVA (Fig. 3B).

VIG recognizes soluble and core viral proteins

Next, a physical fractionation of virion proteins was performed prior to Western blot analysis with VIG. Dryvax and MVA virions were separated into soluble membrane and insoluble core protein fractions and analyzed by Western blot using VIG. The prominent 62 kDa and 37 kDa proteins were found in the virus core, while the 28 kDa protein fractionated in the soluble, envelope fraction (Fig. 4). The 32–35 kDa band was found in all fractions, and may represent different proteins with similar migration patterns or a partially soluble protein. The overall distribution of the bands among fractions, except for the band missing in MVA, was the same for MVA and Dryvax virions.

VIG recognizes EEV-specific viral proteins

The biologically important EEV form of vaccinia virus contains a set of proteins absent in the IMV form. Since the viral particles used for Western blot analysis with VIG were prepared from infected cells and were predominantly IMV, a different approach was required to determine if VIG recognized the EEV-specific viral proteins. Recombinant Semliki Forest Virus constructs were prepared expressing the EEV A33R, A34R, A56R, or B5R proteins. The EEV proteins were purified from infected cells and analyzed by Western blot analysis with VIG (Fig. 5). Three EEV proteins were strongly reactive with the antibodies in VIG. In contrast, the protein isolated from a Semliki Forest virus recombinant expressing the EEV-specific A34R protein was

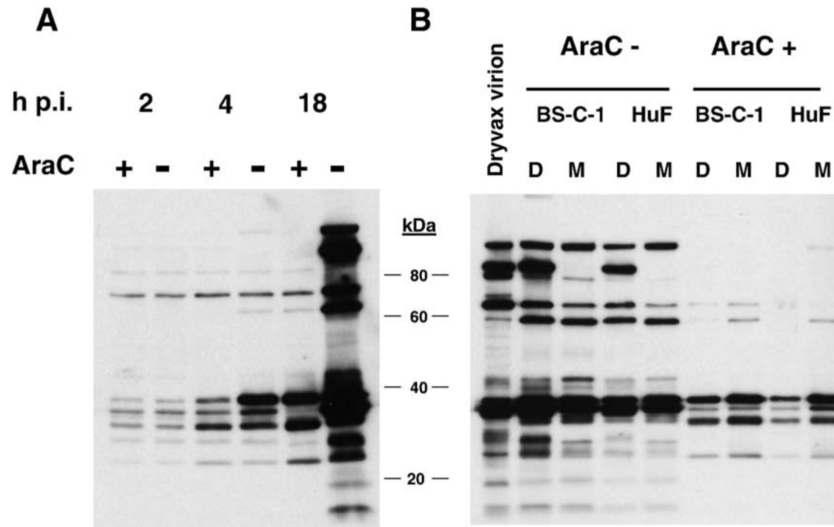

Fig. 3. VIG recognizes vaccinia early and late proteins. (A) Lysates were prepared from BS-C-1 cells infected with Dryvax at an MOI of 5 in the presence (+) or absence (−) of 40 μg/ml of cytosine arabinoside (AraC) at 2, 4, and 18 h post-infection. Proteins were electrophoretically separated, transferred to nitrocellulose, and used for Western blot analysis using VIG as the primary antibody. (B) Lysates were prepared from BS-C-1 or human fibroblasts (HuF) infected with Dryvax (D) or MVA (M) at an MOI of 5, in the presence or absence of AraC at 18 h post-infection. Proteins were electrophoretically separated, transferred to nitrocellulose, and used for Western blot analysis using VIG as the primary antibody.

not detected by Western blot analysis with VIG (data not shown). Control sera from humans did not react with any of the EEV proteins (data not shown).

VIG contains antibodies to the IMV proteins H3L and A27L

We were also interested in determining if VIG recognized specific protein targets present in the IMV form of vaccinia, particularly those known to elicit neutralizing antibody response. The core protein H3L elicits a strong antibody response in mice, sheep, rabbits, and humans (Lin et al., 2000; Zinoviev et al., 1994) and encodes a major epitope for T-cell recognition in humans (Drexler et al., 2003). The plasmid pH3L containing the H3L open reading frame linked to an HA epitope tag proximal to a CMV promoter was transfected into Vero cells. Cellular extracts were resolved by electrophoresis, transferred to nitrocellulose, and analyzed by Western blotting using VIG as the primary antibody. A band with molecular mass consistent

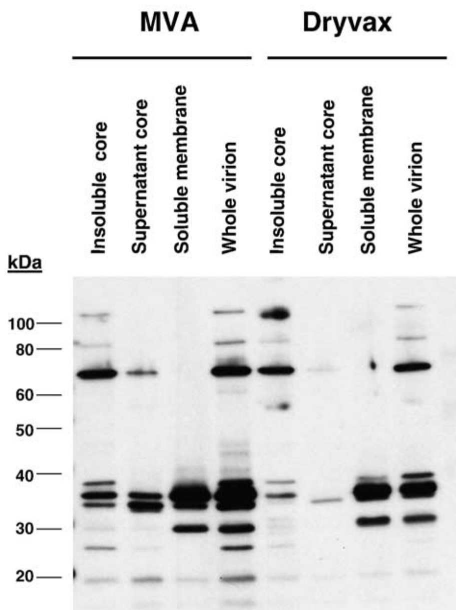

Fig. 4. VIG contains antibodies to core and membrane vaccinia virus proteins. Approximately 500 ng of MVA and Dryvax virions was separated into the viral insoluble core, supernatant core, and soluble membrane fractions. These fractions and purified virions (Whole virion) were electrophoretically separated, transferred to nitrocellulose, and analyzed by Western blot using VIG as the primary antibody.

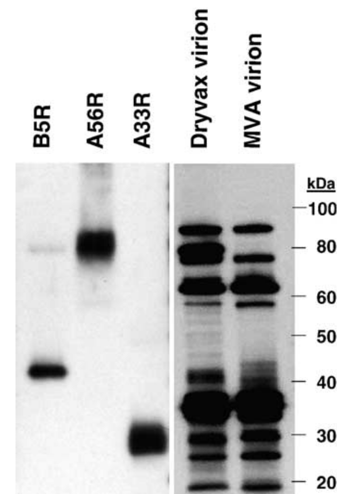

Fig. 5. VIG contains antibodies to vaccinia EEV proteins. Affinity purified preparations of the vaccinia virus proteins A33R, B5R, and A56R were electrophoretically separated, transferred to nitrocellulose, and used for Western blot analysis using VIG as the primary antibody (left panel) and compared to proteins from purified Dryvax or MVA virions electrophoretically separated, transferred to nitrocellulose, and used for Western blot analysis using VIG as the primary antibody (right panel).

with the H3L protein was observed only in cells transfected with pH3L (Fig. 6A).

Antibodies directed against the IMV protein A27L also has been shown to be neutralizing for vaccinia virus infection (Ichihashi et al., 1994; Rodriguez and Esteban, 1987; Wolffe et al., 1995). To determine whether VIG contains antibodies to the IMV A27L protein, lysates from uninfected cells or lysates from cells infected with Dryvax were immunoprecipitated with VIG. The samples were resolved by electrophoresis through polyacrylamide gels and Western blotted with polyclonal rabbit antisera made to A27L protein. A band of approximately 14 kDa was only observed in lysates immunoprecipitated with VIG or lanes containing Dryvax-infected cell lysates or virions (Fig. 6B).

Mouse antisera to Dryvax recognizes a similar set of viral proteins as VIG

The viral proteins recognized by Dryvax or MVA hyperimmune sera from mice were also characterized by Western blot analysis using pooled sera. The sera were used as the primary antibody in Western blotting against virion particles or lysates from cells infected with Dryvax or MVA (Fig. 7). One cellular protein with a mass of approximately 62 kDa was detected when uninfected cell lysates were probed with sera from mice vaccinated with Dryvax (leftmost lane); no cross-reactivity was observed when sera raised against MVA were used (data not shown). No bands were observed when infected or uninfected cell lysates were incubated with naive mouse sera (data not shown). As observed for VIG, many bands were present when the Dryvax or MVA hyperimmune serum from mice was used as the primary antibody in Western blot analysis. The pattern of bands observed after using Dryvax hyperimmune serum as the primary antibody against Dryvax and MVA viral particles was almost identical to the pattern observed for VIG, including the presence of an approximately 80 kDa protein only in samples derived from Dryvax. Some of the low molecular weight bands in the virion and cell lysate samples were more intensely reactive than their cognate bands in samples screened with VIG. This may reflect differences in the binding among the antibody panoply elicited in mice and humans.

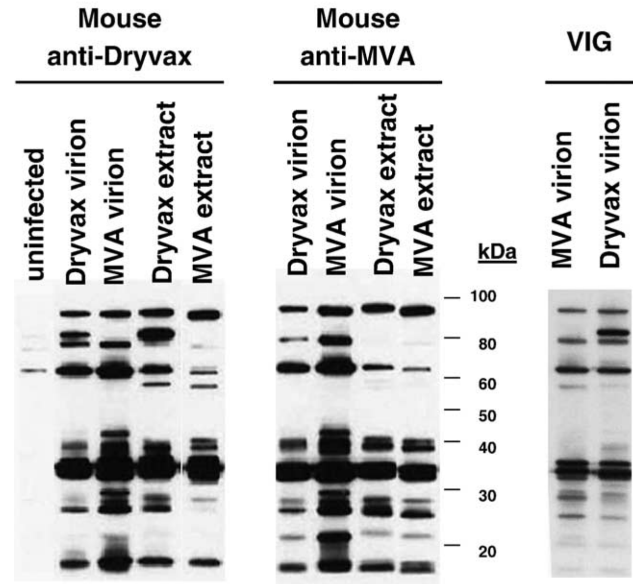

Fig. 7. The murine humoral response to Dryvax or MVA vaccination is qualitatively identical to the human humoral response to Dryvax vaccination. Lysates from BS-C-1 cells infected with Dryvax or MVA, and purified MVA or Dryvax virions were electrophoretically separated, transferred to nitrocellulose, and analyzed by Western blot using sera from mice vaccinated with Dryvax (leftmost panel) or MVA (center panel) as the primary antibody. Lysates from uninfected BS-C-1 cells probed with sera from mice vaccinated with Dryvax appear in the leftmost lane. The rightmost panel contains purified MVA or Dryvax virions electrophoretically separated, transferred to nitrocellulose, and analyzed by Western blot using VIG as the primary antibody.

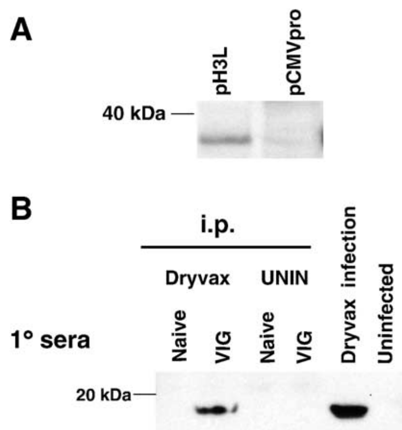

Fig. 6. VIG contains antibodies to the vaccinia H3L and A27L IMV proteins. (A) Lysates prepared from Vero cells transfected with 2.5 μg of pH3L, a plasmid containing the H3L open reading frame under the control of the strong CMV promoter, or 1.7 μg of pCDNA/tks/RfA (pCMVpro), a plasmid containing the CMV promoter without an insert, were electrophoretically separated, transferred to nitrocellulose, and analyzed by Western blot using VIG as the primary antibody. (B) Lysates from human fibroblasts infected with Dryvax (two leftmost lanes) or uninfected controls (two middle lanes) were immunoprecipitated with naïve human serum (first and third leftmost lanes) or VIG (second and fourth leftmost lanes). These samples were electrophoretically separated, transferred to nitrocellulose, and analyzed by Western blot using purified rabbit anti-A27L as the primary antibody. The gel also included lysates from human fibroblasts and fibroblasts infected with Dryvax (two rightmost lanes).

When samples were analyzed by Western blot using the MVA hyperimmune serum, numerous proteins were detected, including an intense cluster of bands around 35 kDa. The pattern of bands was identical for both MVA- and Dryvax-infected cells. The total number of bands observed in the samples screened with the MVA-derived serum was almost the same as the total observed for those screened with Dryvax. The Dryvax-specific 80 kDa protein detected by VIG was not observed in the Western blot analysis using MVA hyperimmune serum in either Dryvax or MVA samples. Since the protein is present in Dryvax and inoculation of mice with MVA did not elicit antibodies

against this protein, these results suggest that the inability of VIG to recognize this band in MVA is due to the absence of the protein epitope, and not to aberrant electrophoretic migration.

The strongly antigenic protein missing in MVA is the A-type inclusion body protein

Serum raised in mice vaccinated with MVA did not recognize the strongly antigenic, approximately 80 kDa band in Dryvax, implying the protein may be missing from the MVA genome. A comparison of the vaccinia WR and MVA genomes using the web site www.poxvirus.org provided four candidate genes present in vaccinia WR and missing in MVA with protein molecular masses greater than 65 kDa: C9L (VACWR019), O1L (VACWR068), the A-type inclusion body protein (VACRW148), and B4R (VACWR186). A set of four recombinant vaccinia WR genomes was constructed, each of which was missing one of the candidate genes. Western blot analysis using VIG as the primary antibody against protein extracts from BS-C-1 cells infected with these recombinants revealed that the recombinant missing the A-type inclusion body, v644 (-ATI), no longer had a strongly reactive 80 kDa protein recognized by VIG (Fig. 8). To confirm the identity of the 80 kDa protein, the open reading frame for the A-type inclusion body under the transcriptional control of the bacteriophage T7 promoter and linked to an HA epitope tag was inserted into the v644 (-ATI) genome by homologous recombination. The resultant virus, vATI-REST, was used to infect BOS cells and protein extracts were analyzed by Western blot analysis using VIG as the primary antibody (Fig. 9). The approximately 80 kDA Dryvax specific protein was detected by VIG in the ATI-restored virus, but not in the ATI-deleted virus. Western blot analysis of these extracts using an HA antibody as the primary antibody showed a strongly reactive band at 80 kDa that co-migrated with the VIG reactive protein only in vATI-REST samples (data not shown). Taken together, the data indicate that the strongly immunogenic protein recognized by VIG in Dryvax, but not MVA corresponds to the A-type inclusion body protein.

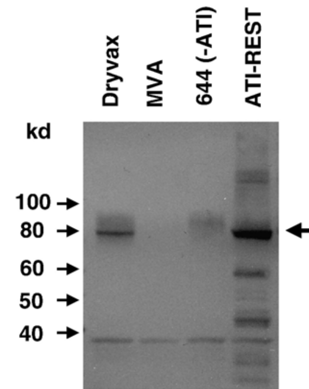

Fig. 9. The antigenic VIG target missing in MVA is encoded by the A-type inclusion body protein. Lysates from BOS cells infected with Vaccinia WR, MVA, the recombinant viruses missing the open reading frame encoding the A-type inclusion body (ATI), or the recombinant virus containing the ATI open reading frame inserted into the thymidine kinase locus of v644 (-ATI) were electrophoretically separated, transferred to nitrocellulose, and analyzed by Western blot using VIG as the primary antibody. The arrow refers to the position of the protein recognized by VIG in cells infected with Dryvax but missing in MVA.

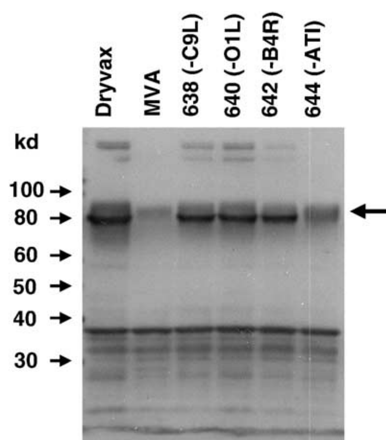

Fig. 8. The antigenic VIG target missing in MVA requires expression of the A-type inclusion body protein. Lysates from BS-C-1 cells infected with Dryvax, MVA, or recombinant viruses missing the open reading frame encoding C9L, O1L, B4R, or the A-type inclusion body (ATI) were electrophoretically separated, transferred to PVDF (Invitrogen), and analyzed by Western blot using VIG as the primary antibody. The arrow refers to the position of the protein recognized by VIG in cells infected with Dryvax but missing in MVA.

Discussion

Recent political events have led to a renewed interest in the evaluation and development of vaccines against smallpox (Henderson, 1999). Historically, live viral vaccines based on vaccinia, such as Dryvax, generated a strong and durable protective response against smallpox. However, these vaccines caused a number of adverse events, especially in immunocompromised people. The immunotherapeutic serum developed for the treatment of complications associated with vaccination, VIG, was derived from the serum of people vaccinated with Dryvax and therefore represents the antibody response to inoculation with a vaccine that protects against smallpox infection. Although the biological activity of VIG has been demonstrated in tissue culture and VIG has been used to treat complications associated with Dryvax vaccination for the last 30 years, no systematic characterization of the viral proteins recognized by VIG has been documented. Here, we describe a preliminary molecular characterization of the viral antigens recognized by VIG.

Another approach to mitigate the complications associated with vaccinia-based smallpox vaccines is the use of new-generation attenuated smallpox vaccines with superior

safety profiles. The efficacy evaluation of attenuated smallpox vaccines will require extensive comparison to traditional smallpox vaccines in animal models for immune response and protection against lethal orthopoxvirus challenge since clinical trials with variola are impossible. The most widely used attenuated vaccine, modified vaccinia virus Ankara (MVA), was derived from over 500 passages in primary chicken embryo fibroblasts (Mayr et al., 1975). MVA is highly attenuated in mammalian cells (Blanchard et al., 1998; Carroll and Moss, 1997; Drexler et al., 1998), but still induces a potent cellular and humoral immune response to vaccinia virus in mice (Belyakov et al., 2003; Drexler et al., 2003). In addition, vaccination with MVA protects mice and monkeys against lethal orthopoxvirus challenge (Earl et al., 2004; Wyatt et al., 2004). Here, we describe a direct comparison of the antibody responses to MVA and Dryvax in mice. The protein targets recognized by VIG in both Dryvax and MVA virions and lysates from infected cells were compared to determine if the highly reactive epitopes present in Dryvax were retained in MVA.

The protein targets of the high-titer antibody serum VIG were characterized by Western blot analysis against viral proteins from purified virions or infected cells. The antibodies present in VIG were directed against numerous antigens in vaccinia. The pattern of reactive bands on Western blots from purified viral particles or cells infected with Dryvax and MVA using VIG was nearly identical. There was, however, one major difference. A band at approximately 80 kDa observed in Dryvax particles and lysates from infected cells was always missing in samples derived from MVA. We concluded that this epitope was missing from MVA, as sera raised in mice treated with MVA did not detect the band corresponding to the 80 kDa protein in MVA or Dryvax whereas sera from mice inoculated with Dryvax did detect the 80 kDa protein. The open reading frame encoding for this protein was determined to be the vaccinia WR gene fragment corresponding to the cowpox A-type inclusion body. This portion of this cowpox gene is highly conserved across poxvirus strains including sharing 92% homology with variola major virus, India strain. Further research is required to determine the importance of the humoral immune response to this gene and its role in virus pathogenicity. Admittedly, characterization of VIG targets by Western blotting reveals only a subset of the total number of antigenic targets of VIG. Binding to denatured proteins on membranes will not reveal antibodies directed against vaccinia virus conformational epitopes or secondary structures. Therefore, the antibodies in VIG almost certainly bind to even more targets than those described in this investigation.

Many bands were observed after Western blot analysis of vaccinia viral proteins with VIG. To more fully characterize the targets of VIG, proteins were fractionated prior to Western blot analysis. Viral transcription in cells infected with vaccinia virus proceeds via a temporal cascade with three discrete classes: early, intermediate, and late (Broyles, 2003). The results presented in this manuscript demonstrate that, in addition to the highly expressed members of the late class of viral genes, VIG recognized members of the early class of gene products. We cannot formally rule out the possibility that these proteins recognized by VIG are expressed both at early and late times after infection.

Detergent fractionation of the viral particle revealed that, in addition to the soluble membrane associated proteins expected to be accessible on the outside of the viral particle, a number of core proteins were recognized by VIG. These results reinforce the conclusion that the human humoral response to Dryvax vaccination is directed against a large number of viral proteins and not restricted to a limited number of virion surface proteins.

The two major infectious forms of vaccinia are the IMV and EEV (Smith et al., 2002). The IMV form is the predominant infectious form in orthopoxvirus infections but the EEV form is important for dissemination within the host. Since the protection provided by vaccinia virus subunit vaccines was most effective when a combination of EEV and IMV antigens was utilized (Fogg et al., 2004; Hooper et al., 2000; Hooper et al., 2003), we predicted that VIG would contain antibodies directed against both IMV and EEV proteins. The three EEV proteins A33R, A56R, and B5R were recognized by VIG. The EEV protein B5R has been shown to be a target for EEV neutralizing antisera in rabbits (Galmiche et al., 1999), and the source of anti-EEV activity in VIG (Bell et al., 2004). In contrast to the first three EEV proteins investigated, the A34R protein was not recognized by VIG. The capacity of VIG to recognize three of the four EEV proteins by Western blot was replicated in EEV single protein ELISAs for each of the four proteins purified from the Semliki Forest Virus expression system (Garcia et al., in preparation). This result is also consistent with the observation that A34R protein did not elicit an EEV neutralizing serum when used to inoculate rabbits (Galmiche et al., 1999). Recently, it has been demonstrated that mice inoculated with MVA also generated a humoral response against EEV specific proteins (Earl et al., 2004; Wyatt et al., 2004).

Prior experiments using Western blot analysis with sera from humans, rabbits, or mice (Demkowicz et al., 1992; Wilton et al., 1986) revealed a similar pattern of reactive protein bands as described in this report for VIG. The protein bands recognized by all sera included an intense 62 kDa band and a cluster of bands around 32–40 kDa. A number of the prominent reactive bands were identified by Western blot analysis of the sera with a bacteriophage λ-based expression library of rabbitpox DNA (Demkowicz et al., 1992). The highly reactive 62 kDa band corresponded to the major proteolytic product of p4a, the A10L gene product of vaccinia virus strain Copenhagen. The prominent reactive bands at approximately 40 kDa, 32 kDa, 25 kDa, and 14 kDa were assigned to the A4L (A5L in vaccinia WR), D8L, L4R, and A27L genes in Copenhagen strain, respectively. In

these experiments, we have demonstrated that VIG reacts strongly with viral proteins with molecular mass of 95, 77, 62, 59, 42, 40, 37, 35, 33, 28, 25, 18, and 14 kDa. The bands at 62 kDa and 32 kDa may correspond to the large fragment products of the A10L and the D8L gene, respectively. The reactive 14 kDa probably corresponds to the A27L protein as the data illustrated in Fig. 7 confirmed that VIG contained antibodies to immunoprecipitate A27L. It is more difficult to discern if VIG contained antibodies to A4L and L4R, as reactive bands corresponding to those molecular masses were not impressive. However, sera from mice inoculated with Dryvax or MVA did clearly detect bands with molecular masses consistent with L4R and A4L (Fig. 7). A stronger humoral immune response to L4R in rabbits than humans has been observed (Demkowicz et al., 1992).

The evaluation of the efficacy of new generation smallpox vaccines will include a comparison of the immune response to that of an effective vaccine, such as Dryvax, in humans and surrogate animal models. Antibody production induced by inoculation with MVA or Dryvax plays an important role in protecting mice against disease caused by pathogenic vaccinia viruses (Belyakov et al., 2003). As an initial step in the comparison of the humoral response in mice and humans, we measured vaccinal antigen targets of antibodies elicited in response to inoculation of mice with Dryvax or MVA using a Western blot analysis. The pattern of bands observed to react with mouse antiserum to Dryvax in MVA or Dryvax samples was identical except for the previously discussed 80 kDa band missing in MVA. The murine antisera against Dryvax detected the same set of proteins by Western blot analysis as VIG. In addition, the murine antibodies produced proportionally darker bands for some low molecular mass proteins in Dryvax and MVA particles or lysates from infected cells. The murine antibody response was qualitatively identical, albeit with some differences in the relative intensities of bands, to the human antibody response to vaccination with Dryvax measured by Western blot. There is a band corresponding to a low molecular weight protein of approximately 20 kDa that appears more intense for the MVA virion sample than the Dryvax virion sample especially when the MVA hyperimmune mouse sera are used as the primary antibody (Fig. 7). However, the band is not consistently observed when VIG is used as the primary antibody and it is unclear if this band corresponds to a unique protein in MVA, a protein in both MVA and Dryvax that is a better target for VIG in MVA, or an MVA truncated protein corresponding to a higher molecular weight protein in Dryvax.

The data presented here demonstrate that early and late class genes, components of the IMV and EEV, and both membrane and core proteins are recognized by Western blot analysis using VIG. The unambiguous identification of the protein targets and the demonstration of their importance in the biological activity of VIG will provide an inventory of the important targets of the human humoral response protective against smallpox infection. The most straightforward identification of VIG targets can be accomplished by Western blot analysis on specific candidate genes with VIG. We have demonstrated that the EEV proteins encoded by A33R, A56R, B5R, and the IMV protein encoded by H3L and the A-type inclusion body are recognized by VIG using this approach. Additionally, we demonstrated that the protein encoded by A27L is immunoprecipitated by VIG. The identification of the candidate genes as targets of VIG will be facilitated by using the data from the fractionation of the viral extracts presented in this report with the properties of the poxvirus proteins tabulated at the Poxvirus Bioinformatics Resource Center (www.poxvirus.org).

A complementary approach utilizing a peptide expression library expressed in bacteria and immobilized on a filter microarray system has recently been described (Davies et al., 2005). The authors generated a vaccinia expression library from a set of gene-specific PCR primers to one strain of vaccinia virus. The sizes of many of the vaccinia proteins that react with VIG on the microarray filter correspond to prominent bands on Western blots with Dryvax, the licensed vaccine strain that is a mixture of many strains. The majority of the proteins identified using the microarray have masses between 20 and 42 kDa, similar to the pattern of reactive bands in this work. All of the proteins we have identified directly as targets of VIG between 12 and 38 kDa (A27L, A33R, A56R, B5R, and H3L) are detected in the microarray analysis. However, the overall set of proteins identified using the two approaches does not correspond exactly. We observed four prominent high molecular mass bands at approximately 97, 77, 62, and 59 kDa. The microarray analysis using VIG detects only two proteins with molecular masses greater than 42 kDa, the 102 kDa A10L major core protein, which is most likely cleaved to a 62 kDa fragment in our samples and the 62 kDa D13L protein. The prominent 80 kDA protein corresponding to the A-type inclusion body protein is not detected by the microarray analysis. The open reading frame for the vaccinia WR A-type inclusion body does not have a homologue in the Copenhagen genome and therefore the appropriate peptide may not have been part of the array. The comparative analysis of mouse sera to VIG using the microarray approach revealed seven vaccinia proteins exclusively detected with mouse sera, five with molecular masses less than 43 kDa (K2L, H6R, A4L, H7R, and N1L) and two with a molecular mass of 72 kDa (A3L and D11L). Although we do observe an increase in the intensity of some lower molecular mass bands, no new band corresponding to the 72 kDa proteins was observed by Western blot of infected cell extracts or virion particles using mouse MVA or Dryvax hyperimmune sera. The disparity in intensity or number of targets between the two approaches has a number of possible explanations. The entire set of Dryvax epitopes

may not be contained in the bacterial expression array derived solely from one strain, presumably Copenhagen, of vaccinia since Dryvax is a heterogeneous population of vaccinia virus. The set of antigens recognized after immobilization with bacterial proteins on nitrocellulose may differ from those recognized after electrophoresis through SDS gels. The sensitivity of the two approaches may differ since all peptides are expressed at relatively equivalent levels before immobilization in the bacterial expression system whereas the Western blots contain the viral proteins at the disparate levels normally found in infected cells or in the viral particle. The proteins detected by the microarray may not correspond to a band at the expected mobility on a Western blot since post-translation modifications in the infected mammalian cells may alter the apparent molecular mass.

In summary, the experiments presented here provide the first molecular characterization of VIG, the therapeutic antisera derived from vaccination with Dryvax, using a Western blot analysis. The protein targets recognized by the antibodies in VIG were numerous. The breadth of the elicited antibody response probably explains the universal effectiveness of the myriad strains used during the smallpox eradication campaign and underscores the observation that vaccinia-based vaccines are protective against a broad range of orthopoxviruses. The experiments also provided a direct comparison of the scope of the humoral response in mice, as measured by Western blotting, for Dryvax, a vaccine with demonstrated efficacy against smallpox, and MVA, a candidate vaccine. In the absence of efficacy trials using variola, and without direct knowledge of the critical components of the protective immune response, it is vitally important to understand any differences in the elicited immune response between the vaccines known to be effective against smallpox and new generation vaccines. There were few differences noted in the humoral response against Dryvax and MVA in mice, with one notable exception, the response to the A-type inclusion protein missing in MVA. The contribution of this epitope to the protective immune response elicited by Dryvax is not known.

Materials and methods

Cells and viruses

Vero, BS-C-1, and BHK-21 cells were maintained in Dulbecco's modified Eagles's medium (DMEM). HeLa and primary chick embryo fibroblast (CEF) cells were maintained in Eagle's minimal essential medium (MEM). BOS cells, a BS-C-1 derived cell line expressing the bacteriophage T7 RNA polymerase, were obtained from the laboratory of Dr. Bernard Moss, NIAID, NIH, and were maintained in complete MEM, 10% FBS supplemented with 1 mg/ml G418 (Sigma). The thymidine kinase deficient cells, 143B (ATCC CRL8303), were maintained in MEM, 10% FBS supplemented with 50 μg/ml bromodeoxyuridine (BrdU). Both MEM and DMEM were supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 50 μg/ml gentamicin. Human skin fibroblasts Detroit 551 (ATCC CCL-110) were maintained in MEM, 1 mM sodium pyruvate, non-essential amino acids, 10% FBS, and 2 mM glutamine. Cells were incubated in a humidified air-5% CO_2 atmosphere at 37 °C.

The Dryvax virus seed stock was prepared by infecting two 162 cm^2 flasks of Vero cell monolayers with a reconstituted vial of Dryvax, the licensed smallpox vaccine manufactured by Wyeth (Lot number 321401). Infected cells were harvested 3 days after infection, concentrated by centrifugation, resuspended in 2 ml of MEM, and virus released by three freeze-thaw cycles. The seed stock was stored in aliquots at −80 °C. Crude stocks of Dryvax were prepared by infecting BS-C-1 monolayers with the seed stock at a multiplicity of infection (MOI) of 1 PFU/cell and incubating at 37 °C for 72 h. The infected cells were harvested, concentrated by centrifugation, suspended in a small volume of MEM, and lysed by three freeze-thaw cycles. Viral titers were determined by standard plaque assay on BS-C-1 cells (Earl et al., 1998). Modified vaccinia virus Ankara (MVA) was a gift of Linda Wyatt and Bernard Moss (Laboratory of Viral Diseases, NIAID, NIH). The MVA seed stock was prepared in CEF cells. Purified virus was prepared by centrifugation of infected cytoplasm through a 36% sucrose cushion and recovery of the pellet fraction (Earl et al., 1998). The viral titer was determined on CEF or BHK-21 cells (Earl et al., 1998).

The recombinant viruses missing specific open reading frames were constructed by recombining vaccinia WR with a linear DNA fragment generated by PCR containing the proximal nucleotide sequences to each reading frame flanking the bacterial GFP protein under the transcriptional control of a vaccinia virus promoter. A linear DNA fragment containing the GFP open reading frame was generated by the product of the PCR with oligonucleotides MM646 (CGGTGGCTGGGTACCAGGCGCGCCTTTCATTTT-GTTTTTTTCTATGCTATA) and MM647 (GGTAC-CAGCGGCCGCTTACTTGTACAGCTCGTGCATGCC-GAGAGTGATCCC) with pLW44 DNA, a plasmid containing the GFP gene under the transcriptional control of the modified H5 early/late vaccinia promoter (gift of Linda Wyatt). Site-specific excision fragments were constructed to selectively remove four open reading frames; C9L (VACWR019), O1L (VACWR068), the A-type inclusion body protein (VACRW148), and B4R (VACWR186). The fragment for deletion of each gene was constructed in two steps. First, a pair of PCRs generated the DNA fragments containing the nucleotide sequences corresponding to the left and right flanking regions of the target gene. Each of these fragments contains a 20 nucleotide overlap with the PCR product of MM646/647. The DNA fragments containing the GFP gene and the two fragments containing

the nucleotide sequences flanking each open reading frame were pooled and a PCR with the outermost oligonucleotides was undertaken to synthesize the DNA fragment comprising the flanking sequences from each open reading frame interspaced with the GFP gene. The appropriate DNA fragment was separated from the reaction products by electrophoresis through an agarose gel and purified using a Geneclean kit. The purified DNA fragment was used directly to generate recombinant vaccinia virus WR (Earl and Moss, 1991). Plaques expressing GFP protein were purified through three passages on B-SC-1 cells. The genomic structure of each recombinant virus was confirmed by PCR. The fragment for deletion of the C9L gene was constructed using the product of PCR with MM648 (GAATGAGCTACTGTGAACTCTATA) and MM649 (GCGCCTGGTACCCAGCCACCGAAAAATAGAAAC-TATAATCATATA) and the product of PCR with MM650 (AAGTAAGCGGCCGCTGGTACCGATTGGTTGTT-GATGCTATCG) and MM651 (CAACCATTTAATAAAT-TAAACATT). The PCR fragments from MM646/647, MM648/649, and MM650/651 were combined and used in a PCR with oligonucleotides MM648 and MM651. The resulting fragment was used to produce v638 (-C9L). The fragment for deletion of the O1L gene was constructed using the product of PCR with MM652 (GTTTAGACGG-CAACATAGAAGCGT) and MM671 (GCGCCTGGTACC-CAGCCACCGTACCGTACCTTCTTATTGTATCA) and the product of PCR with MM654 (AAGTAAGCG-GCCGCTGGTACCCGACATGATTAAGTATTGTTTTTT-CAT) and MM655 (ATGCTCGTCGTAATTATGTTTTTT-ATA). The PCR fragments from MM646/647, MM652/671, and MM654/655 were combined and used in a PCR with oligonucleotides MM652 and MM655. The resulting fragment was used to produce v640 (-O1L). The fragment for deletion of the B4R gene was constructed using the product of PCR with MM656 (CTTCCTTTATACTCT-CATCTTTTA) and MM657 (GCGCCTGGTACCCAGC-CACCGTTTATATTCTGTAACATGTTATCC) and the product of PCR with MM658 (AAGTAAGCGGCCG-CTGGTACCCACTATTAAAATATAAAATCTAAG) and MM659 (TCCGTGTTCTAATTGAAGAGGTTG). The PCR fragments from MM646/647, MM656/657, and MM658/659 were combined and used in a PCR with oligonucleotides MM656 and MM659. The resulting fragment was used to produce v642 (-O1L). The fragment for deletion of the A-type inclusion body was constructed using the product of PCR with MM660 (CCGT-TTGTATTGGTCCATGTTTCC) and MM661 (GC-GCCTGGTACCCAGCCACCGTAGAGATAGAA-TCTCAGATATTGA) and the product of MM662 (AAGTAAGCGGCCGCTGGTACCCAAAACTTAACTA-CAATCGGCTAA) and MM663 (ACCCGATAACGA-TAAGCGAATTCA). The PCR fragments from MM646/647, MM660/661, and MM662/663 were combined and used in a PCR with oligonucleotides MM660 and MM663. The resulting fragment was used to produce v644 (-ATI).

The recombinant virus containing the ATI gene inserted at the thymidine kinase locus of v644 (-ATI) was isolated from the recombination products of v644 (-ATI) and pT7ATI, a plasmid containing the ATI gene under the transcriptional control of the bacteriophage T7 promoter. The recombinant viruses were purified by three passages on thymidine kinase deficient cells in the presence of BrdU (Earl and Moss, 1991). The genomic structure of the recombinant virus vATI-REST was confirmed by PCR.

Recombinant plasmids and proteins

The open reading frames corresponding to the vaccinia EEV proteins A33R, A34R, A56R, and B5R were cloned as PCR fragments from Dryvax into a Semliki Forest virus expression plasmid. The proteins were purified from BHK-21 cells infected with recombinant Semliki Forest viruses (Garcia et al., in preparation).

The plasmid encoding the Dryvax open reading frame corresponding to the vaccinia virus strain Copenhagen H3L core protein, pH3L, contains the open reading frame for H3L linked to an influenza hemagglutinin (HA) epitope tag proximal to a CMV promoter. The DNA fragment generated by PCR with primers MM614 TTATTATTAGGCATAGT-CAGGCACGTCATAAGGATAGATAAATGCGGTAAC-GAATGTTCC and MM615 CACCATGGCGGCGGC-GAAAACTCCTGTT and Dryvax DNA was cloned into the pENTR-D/TOPO (Invitrogen) vector. The N-terminal and C-terminal junction regions were confirmed by determining the nucleotide sequence (Applied Biosystems). The region corresponding to H3L was recombined into a plasmid pCDNA/tks/RfA, a Gateway Destination vector (Invitrogen) modified to contain the intermediate early CMV promoter (A. Woerner, unpublished).

The nucleotide sequence encoding A27L was amplified by PCR from vaccinia virus strain WR DNA. The oligonucleotide primer homologous to the 3′ terminus of the A27L gene included nucleotides encoding the HA epitope and histidine (His) tag. The amplification product was then cloned into pAED4 (Doering and Matsudaira, 1996) generating pA27-HA-His. A site-directed mutagenesis kit (Promega) was used to make pAED4 A27L-AA-HA-His, a mutant containing alanine residues at the Cys71 and Cys72 positions. The reaction mix included pAED4 A27L-HA-His with primers GTAACAACAAAGTTTGA-ACAAATAGAAAAGgcagcaAAACGCAACGAT GAAGTTCTATTTAGGTTGG and CCAACCTAAATA-GAACTTCATCGTTGCGTTTtgctgcCTTTTCTATTTGTT-CAAACTTTGTTGTTAC. The lower-case letters designate the nucleotides corresponding to the replacement alanine residues. The identity of each construct was verified by determining its nucleotide sequence (Applied Biosystems).

E. coli strain BL21(DE3)/pLysS bacteria (Novagen) was transformed with pAED4-A27L-AA-HA-His, grown at 37 °C in LB medium to an optical density of 0.8 at 600 nm, and induced with isopropylthio-D-galactoside (IPTG). After

incubation at 37 °C for 3–4 h, cells were concentrated by centrifugation and lysed with glacial acetic acid at 0 °C. The bacterial lysate was centrifuged at 35,000 × *g* for 30 min. The soluble fraction, containing A27L-AA-HA-His, was dialyzed into 5% acetic acid overnight at room temperature. Protein was purified by reverse-phase high performance liquid chromatography on a Delta Pak C-18 preparative column (Waters), using a water-acetonitrile gradient, 0.1% trifluoroacetic acid and lyophilized. The molecular weight of A27L-AA-HA-His was verified using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. Purified A27L-AA-HA-His was dissolved in 6 M guanidinium hydrochloride and refolded by dialysis against phosphate-buffered saline (PBS) at 4 °C.

The plasmid encoding the vaccinia WR open reading frame corresponding to the vaccinia virus A-type inclusion body protein, pT7ATI, contains the open reading frame for the A-type inclusion body linked at the C-terminus to an influenza hemagglutinin (HA) epitope tag under the transcriptional control of the bacteriophage T7 promoter. The DNA fragment generated by PCR with primers MM644 (CACCATGGAGGTCACGAACCTTATT) and MM643 (TTATTACTTGTCGTCATCGTCTTTGTAGTCAGACGTCGCATCTCTCTCTGTTTC) and vaccinia WR DNA was cloned into the pENTR-D/TOPO (Invitrogen) vector generating pENTRY-ATI. The N-terminal and C-terminal junction regions of the insert (including the HA epitope tag) were confirmed by determining the nucleotide sequence (Applied Biosystems). The PCR product of pENTRY-ATI with MM666 (GGGGGGGCCATGGAGGTCACGAACCTTATT) and MM668 (GGGGGGCTCGAGGGGTCGGCGCGCCCACCCTTG) was cloned into the pCR2.1 vector (Invitrogen) generating pCR-ATI. The identity of the ATI gene and HA epitope tag was confirmed by determining the complete nucleotide sequence of the insert. The DNA fragment containing the ATI gene derived from digestion with the restriction enzymes *Nco*I and *Xho*I was ligated to pPG174 digested with the same enzymes, generating pT7ATI. The plasmid pPG174 was a gift from Paul Gershon (TAMHSC-IBT) and was derived from pTM-1 (Elroy-Stein and Moss, 1998). The pPG174 contains the same nucleotide sequence elements as pTM-1 but the T7 RNA polymerase promoter, EMCUTR, and polylinker are inverted with regard to the flanking vaccinia DNA sequences.

Antisera

Massachusetts Biological Laboratories (MBL) vaccinia immune globulin for intravenous administration, 5% (VIGIV), Lot 2 was manufactured in 2001 from the pooled plasma obtained from military personnel vaccinated with the Dryvax vaccine. The VIG was prepared from pooled human sera using a method similar to the Cohn–Oncley cold-ethanol precipitation method for purification of immune globulins at MBL and provided to the FDA as a VIG standard. The VIG was a gift from Dorothy Scott (CBER/FDA).

Rabbit polyclonal antiserum #1087 to A27L was produced by immunization of rabbits with purified protein (A27L-AA-HA) as previously described (Demkowicz et al., 1992). Mouse polyclonal sera were produced from male BALB/cByJ mice (The Jackson Laboratory, Bar Harbor, ME). Groups of 12-week-old mice received two Biojector-2000 delivered (Biojector, Inc., Portland, OR) intradermal immunizations of 10^7 PFU of sucrose-cushion purified Dryvax or 10^8 PFU of sucrose-cushion purified MVA at 3 weeks interval. Seven months later, an additional injection of Dryvax (10^7 PFU) or MVA (10^8 PFU), respectively, was administered intraperitoneally (i.p.). Immune sera were obtained from blood samples collected 3 weeks after the i.p. boost. All work with mice was carried out according to guidelines provided by the Animal Research Advisory Committee of the National Institutes of Health.

Immunoprecipitation and Western blots

Monolayers of tissue culture cells were infected with Dryvax or MVA and incubated at 37 °C, 5% CO_2 for 24 h. Virus-infected or control cells from 6-well plates were washed and scraped into PBS, concentrated by centrifugation, and resuspended in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% TritonX-100, and protease inhibitors. After 30 min on ice, the cell lysates were clarified by centrifugation for 2 min at 13,000 × *g* in a refrigerated bench-top centrifuge. Virus particle fractionation into soluble and core protein samples was performed as previously described (Garcia and Moss, 2001). Immunoprecipitation analysis was performed by adding rabbit polyclonal serum to a 1:10 dilution of the cell lysates in RIPA buffer containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, and 50 mM Tris–HCl, pH 8.0. The samples were rotated for 1 h at 4 °C prior to and after the addition of Protein G Sepharose (Amersham Biosciences). The sepharose beads were concentrated by centrifugation at 2500 × *g* in a refrigerated bench-top centrifuge, washed twice in RIPA buffer, and resuspended in SDS-PAGE loading buffer containing denaturing agent. Proteins were resolved by electrophoresis through 4%–12% bis-Tris acrylamide gels (Invitrogen), and transferred to nitrocellulose membranes (Invitrogen). The membranes were incubated overnight at 4 °C in 5% non-fat dry milk in phosphate-buffered saline containing 0.5% Tween-20 (PBS-T). Membranes were incubated for 1 h at room temperature in a dilution of primary antibody in PBS-T and washed three times in PBS-T. The membranes were then incubated with a secondary antibody, either goat anti-mouse or goat anti-human immunoglobulin G conjugated to horseradish peroxidase (Pierce) and washed three times with PBS-T. Protein–antibody interactions were visualized by treatment with a chemiluminescent reagent (Amersham Biosciences), followed by exposure to film. Membragold (Diversified Biotech) colloidal gold solution was used to stain vaccinia proteins transferred to nitrocellulose membrane.

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