Countermeasures to the Bioterrorist Threat of Smallpox

Peter B. Jahrling1,*, Elizabeth A. Fritz2 and Lisa E. Hensley2

1National Institute of Allergy and Infectious Diseases, National Institutes of Health, 6700-B Rockledge Drive, Bethesda, MD 20892-7609, USA
2Virology Division, United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Frederick, MD 21702-5011, USA

Abstract: Variola, the agent of smallpox, is a bioterrorist threat, as is monkeypox virus, which also occurs naturally in Africa. Development of countermeasures, in the form of improved vaccines, antiviral drugs, and other therapeutic strategies are a high priority. Recent advances in molecular biology and in animal model development have provided fresh insight into the virulence determinants for smallpox and the pathophysiology of disease. The complex replication cycle for orthopoxviruses, and the pivotal role for viral-specific immunomodulatory proteins which contribute to escape from immunologic surveillance, provide many unique targets for therapeutic intervention. The “toxemia” of smallpox has been elucidated in part by variola-infected primate studies which revealed the central role of apoptosis and the evolution of a cytokine storm leading to hemorrhagic diathesis, resembling fulminent “black” smallpox. This suggests a potential role for therapeutic strategies developed for septic shock, in treatment of smallpox. Drugs licensed for other viruses which share molecular targets with orthopoxviruses (e.g. Cidofovir) or cancer drugs (e.g. Gleevec and other tyrosine kinase inhibitors) have immediate application for treatment of smallpox and monkeypox and provide leads for second generation drugs with higher therapeutic indices. Recent advances in identification of virulence determinants and immune evasion genes facilitate the design of alternative vaccines to replace live vaccinia strains that are unsuitable for a large proportion of individuals in a mass immunization campaign.

INTRODUCTION

Variola, the virus that causes smallpox, is widely regarded as one of the most significant bioterrorist threat agents. During the 20th century alone, smallpox is estimated to have caused over 500 million human deaths [1]. Yet the disease and the naturally occurring virus itself were eradicated by means of the World Health Organization’s global eradication campaign [2]. This program of intensively vaccinating all humans in a ring surrounding every suspected case of variola infection was successful in part because smallpox is a human-only disease; there are no animal reservoirs to reintroduce the virus into the human population. The impact of a smallpox virus attack in the human population today would be even more catastrophic than during the last century; vaccination programs were abandoned world-wide around 1976, the prevalence of immunosuppressed populations has grown, and mobility, to include intercontinental air travel has accelerated the pace of viral spread world-wide. The plausibility of variola as an agent of bioterrorism is augmented by recent revelations that the former Soviet Union produced ton quantities of smallpox virus as a strategic weapon [3] and even conducted open air testing of aerosolized variola on Voz Island in the Aral Sea [4]. These tests resulted in at least one documented exposure 15 km downwind and the seeding of a small outbreak in Aralsk, Kazakhstan, in 1971. It is for these reasons that considerable investment is being made into development of improved countermeasures against smallpox, including new vaccines and antiviral drugs [5]. A 1999 National Academies report summarized and assessed scientific needs for live variola virus [6]. These countermeasures are also needed to respond to the public health threat of monkeypox virus, a virus that produces a disease in man that closely resembles smallpox and which occurs naturally in western and central Africa. This virus was imported inadvertently into the United States in a shipment of rodents originating in Ghana demonstrating the potential public health impact of this agent as well as the potential bioterrorist threat [7].

BACKGROUND

Poxviruses are large DNA viruses that replicate entirely in the cytoplasm. Among their ~ 200 genes are many which code for gene products that interact with and modulate essential functions of the host cells and immune processes [8]. The limited host range of variola is thought to reside in part to the unique association of viral gene products with
the myriad of host signaling pathways. Therefore, strategies which block such key pathways in the replication and maturation of poxviruses provide potential targets for therapeutic intervention.

Poxviruses infect most vertebrates and invertebrates, causing a variety of diseases of veterinary and medical importance. The Poxvirus family is divided into two main subfamilies, the chordopoxvirinae, which infect vertebrates, and the entomopoxvirinae, which infect insects. Chordopoxvirinae are divided into eight genera, one of which is Orthopoxvirus, which includes the human pathogens variola, monkeypox virus, and other species which infect humans including cowpox and vaccinia viruses.

The evolutionary relationships among the poxviruses have been facilitated by the recent availability of complete sequences for over 30 species. Variola major carried a 30% fatality rate and was associated with nearly 100% of the hemorrhagic manifestations of smallpox. In contrast, variola minor carried a fatality rate < 1%, yet at the genome level, variola major and variola minor are > 98% identical over the length of the 185,000 kb genome. At the genome level, poxvirus strains differ markedly in virulence, (e.g. variola major versus variola minor are > 98% identical over the length of the 185,000 kb genome). This suggests that virulence or attenuation may hinge on a relatively few number of genetic determinants. Phylogenetic relationships among the orthopoxviruses; camelpox and variola are more closely related to each other than to any other virus [9, 10]. Concern has been raised that minor modifications to the camelpox virus genome might conceivably result in a virus with the attributes of variola.

Poxviruses have complex structures and replication strategies which have been described in detail elsewhere [8]. Orthopoxviruses are oval, brick-shaped particles 220 nm to 450 nm in length and 140 nm to 260 nm in width. The outer envelop consists of a lipoprotein layer embedding surface tubules and enclosing a bi-concave core. The core contains the viral DNA and core fibrils, and is surrounded by the core envelop and a tightly arranged layer of rod-shaped structures known as the palisade layer. Two oval masses known as the lateral bodies are usually present between the palisade layer and the outer envelop. Two infectious forms of orthopoxviruses (described below) result from the replication cycle.

Viral replication begins with attachment of viral particles to host cell surface, most likely through cell receptors, followed by fusion of the viral and cellular membranes, uncoating, and release of the viral core into the cytoplasm where all subsequent steps take place. The core synthesizes early mRNA which is translated by cellular processes. The synthesis of early proteins results in release of the nucleoprotein complex from the core and initiation of translation of intermediate and late genes, resulting in synthesis of essential viral proteins which are then assembled into progeny virus particles. The form of the initial viral progeny is termed the immature virion which then matures into the brick-shaped intracellular mature virion (IMV) which is infectious only when it is released by cell lysis. IMV particles can acquire a second membrane from an early endosomal component to form the intracellular enveloped virion (IEV). IEVs migrate to the cell surface via microtubules and fuse with the cell membrane to form cell-associated virions (CEV). Finally, CEV induce polymerization of actin to form filaments which affect the direct transfer of CEV to adjacent cells. If CEV become dissociated from the cell membranes they are termed extracellular enveloped virions (EEV). While IMVs are produced in greatest abundance in cell culture, and are the most stable to environmental degradation, CEV and EEV are suspected to play a more critical role in cell-to-cell spread in the intact animal.

**IMMUNE MODULATION BY VIRAL PROTEINS**

One of the unique characteristics of these viruses is their ability to modulate the host's immune response. Poxviruses encode proteins that target or interrupt the natural inflammatory response, interfere with apoptosis, synthesis of steroids, and initiation of the complement system. Many of these viral defenses can be broken in to two categories: 1) those proteins that block extracellular immune signals, e.g., proteins that mimic or interfere with cytokine/chemokine proteins and/or receptors and 2) intracellular proteins which interfere with apoptosis, targeting by the immune system, or intracellular immune cell signaling. Both of these mechanisms are inextricably entwined and together, allow the virus to overcome immunologic surveillance and establish clinical disease in the host.

One of the main targets of these viral proteins is the interferon (IFN) system. Orthopoxviruses encode for an IFN-γ receptor homolog, B9R, as well as an interleukin (IL)-18 binding protein protein [IL-18BP: B6L/D5L in variola virus; D6L in monkeypox virus]. The IL-18 binding protein is believed to be a homologue of the protein thought to be responsible for regulating IL-18 activation of natural killer (NK) cells and T lymphocytes (T-cells) and production of IFN-γ [11]. NK cells and CTLs are mediators of the cell-mediated immune response and cell-mediated immunity is critical in the clearance of infected cells. Similar to IL-1, IL-18 is requires cleavage by caspase-1 to become active. Many poxviruses encode serine or cysteine protease inhibitors (SERPIN), which prevent IL-18 cleavage from the pro-form to the active form. A summary of the immunomodulatory genes discussed in this paper is presented in Table 1. Animal studies conducted using viruses with deletions in the IL-18BP gene showed increased NK cell activity, increased cytokine production and decreased viral replication [12]. Poxviruses also produce a secreted protein(s) that
Table 1. Summary of select immunomodulatory genes.

<table>
<thead>
<tr>
<th>Viral Protein</th>
<th>Function in Immune-Modulation</th>
<th>Vaccinia</th>
<th>Variola-Major</th>
<th>Variola-Minor</th>
<th>MPV</th>
<th>CPV</th>
<th>MCV</th>
<th>MYX</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18 binding protein (IL-18BP)</td>
<td>Inhibition of IL-18</td>
<td></td>
<td>D5L</td>
<td>D6L</td>
<td>C8L</td>
<td></td>
<td>MC054</td>
<td></td>
</tr>
<tr>
<td>IFN-γ receptor</td>
<td>Binds IFN-γ</td>
<td>B9R</td>
<td>B9R</td>
<td>H9R</td>
<td>B9R</td>
<td>B7R</td>
<td></td>
<td>M007R/L</td>
</tr>
<tr>
<td>IFN-α/β binding protein</td>
<td>Binds IFN-α/β</td>
<td>B20R</td>
<td>D9R</td>
<td>M16R</td>
<td>B17R</td>
<td></td>
<td>M135R</td>
<td></td>
</tr>
<tr>
<td>dsRNA binding protein</td>
<td>Inhibition PKR and 2',5'-OAS</td>
<td>E3L</td>
<td>E3L</td>
<td>C3L</td>
<td>F3L</td>
<td>F3L</td>
<td></td>
<td>M029L</td>
</tr>
<tr>
<td>eIF-2 alpha homolog</td>
<td>Inhibition of cellular translation;</td>
<td>K3L</td>
<td>C3L</td>
<td>F3L</td>
<td></td>
<td></td>
<td>M3L</td>
<td>M156R</td>
</tr>
<tr>
<td>Chemokine binding protein (CBP-II)</td>
<td>CC chemokine binding and inhibition of CC receptor</td>
<td>C23L</td>
<td>G3R</td>
<td>G3R</td>
<td>J1L</td>
<td>D1L</td>
<td></td>
<td>M001R/L,M007R/L</td>
</tr>
<tr>
<td>SPI-2/Crm A, Serpin homolog</td>
<td>Inhibition of apoptosis, inhibition of IL-1β and IL-18 processing</td>
<td>B13R/B14R</td>
<td>B13R</td>
<td>D2R</td>
<td>B12R</td>
<td>B12R</td>
<td></td>
<td>M151R</td>
</tr>
<tr>
<td>TNF receptor homolog, Crm B</td>
<td>Inhibition of TNF and lympho toxicin-α</td>
<td>C22L</td>
<td>G2R</td>
<td>G2R</td>
<td>J2R</td>
<td>D2L</td>
<td></td>
<td>M002RL</td>
</tr>
<tr>
<td>TNF receptor homolog, Crm C</td>
<td>Inhibition of TNF, lympho toxicin-α and TNF-induced apoptosis</td>
<td>A53R</td>
<td></td>
<td>A56R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF receptor homolog, Crm D</td>
<td>Inhibition of TNF and TNF-induced apoptosis</td>
<td>K3R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF receptor homolog, Crm E</td>
<td>Inhibition of TNF</td>
<td>K1R</td>
<td>K2R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β receptor homolog</td>
<td>Inhibition of IL-1β</td>
<td>B16R</td>
<td>B15R, B16R</td>
<td>D4R</td>
<td>B14R</td>
<td>B14R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibitor of Toll-like receptor (TLR)</td>
<td>Inhibition of TLR, IL-1, IL-18 receptor signaling</td>
<td>A46R, A52R</td>
<td>A52R</td>
<td>A56R</td>
<td>A47R</td>
<td>A49R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Death domain homolog (v-FLIP)</td>
<td>Inhibition of apoptosis</td>
<td>N1L, K1L, A52R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MC159L, MC160R</td>
</tr>
<tr>
<td>Glutathione peroxidase homolog</td>
<td>Inhibition of oxidant-induced apoptosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MC066L (M66)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: monkeypox virus (MPV), cowpox virus (CPV), molluscum contagiosum virus (MCV), myxoma virus (MYX).
Figure 1. All images are from tissues obtained at necropsy from variola virus-infected cynomolgus monkeys [22].

Panel A. Tingible body macrophage. Note the red apoptotic cell or body and the blue, unstained nuclei of the cell. Apoptosis staining was performed using a stain for single strand DNA breaks.

Panel B. Migratory infected monocytes / macrophages are observed in non-human primates. The presence of viral antigen is indicated in red. Identification of cell populations was performed using a polyclonal antibody for macrophages shown in green.

Panel C. Infection of the capsule of axillary lymph node. Viral antigen is indicated in red.

Panel D. Pox lesions on the arm of a non-human primate.

Panel E. cDNA microarray performed on total peripheral blood mononuclear cells collected from non-human primates infected with variola virus. Each triangle represents time course in individual animals. The pink triangles represent animals challenged by the i.v. and aerosol route; the purple by the i.v. route. The orange triangles were from animals in a dose response study with the animals receiving the lowest dose on the right. All animals were normalized to bleeds prior to infection, indicated in black; down regulation of genes is visualized as green and upregulation of genes is visualized as red. Of particular note are the strong interferon response, cell proliferation, and immunoglobulin expression. Interestingly, changes in the IFN-related/induced genes were seen at the very earliest time points. Also of interest was a virtual absence of a TNF-α/NF-κB response (Microarray photo kindly provided by Kathleen Rubins and David Relman, Stanford University).
smallpox-infected NHP [22, 24]. A marked upregulation of numerous IFN-regulated genes such as MX1, MX2, 2'-5'oligoadenylate synthetase (OAS), and STAT 1/2 was, however, demonstrated at the transcriptional level with the peak mRNA levels at days 3 to 5 postinfection [24]. These IFN-stimulated genes are induced by both type I and type II IFNs. These data suggest that some but potentially not all of the above mentioned immunomodulatory proteins may be affecting the ability of the host to mount an effective anti-viral IFN response. Further studies will need to be performed to address whether these genes are being expressed, at what levels, and if these genes are fully functional in NHPs.

Another facet of orthopoxvirus immune evasion is the encoding of chemokine binding proteins and cytokine/chemokine receptor homologues. Modulation of the chemokine response is common to all poxviruses. Chemokines are important regulators of immune cell trafficking to the site of viral infection. Although chemokines are a common target, only the Orthopoxvirus and Leporipoxvirus genera encode for CC-chemokine binding proteins which are classified as type II CBP (CBP-II) [17]. The CBP-II proteins are demonstrated to bind monocyte chemoattractant protein (MCP)-1, as well as other CC chemokines with high affinity [17, 25]. MCP-1 is a chemoattractant for monocytes/macrophages as well as antigen presenting cells and is important in immune cell recognition of infected cells. Surprisingly, deletion of the CBP-II protein in myxoma virus or rabbitpox virus did not affect virulence, but did result in increased leukocyte trafficking to virus-infected tissues [26, 27].

Orthopoxviruses are also reported to have one to three intact tumor necrosis factor (TNF) receptor homologues (cytokine response modifier (crm)B, crmC, and crmD). CrmB has also been shown to bind lymphotoxin-α, and crmC and crmD were shown to inhibit TNF-α promoted cell death [28]. Variola virus encodes only for crmB while monkeypox virus encodes for crmB and crmE [17]. Orthopoxviruses also have sequences to encode for secreted homologues of the IL-1β receptor [29]. In addition, IL-1β activity is targeted by poxviruses through some of the same protease inhibitor mechanisms described for IL-18 [30]. Similar to other studies, deletion of these gene may alter the virulence in vivo [29-31]. The TNF receptor and IL-1β receptor homologs are a unique set of immunomodulatory genes found only within the Orthopoxvirus genera.

In addition to the production of cytokine/chemokine binding proteins, and decoy cell proteins, recent work suggests that pox viruses may modulate the immune response through the host cell transcription factor, nuclear factor-kappa B (NF-κB). NF-κB is a modulator of numerous proinflammatory, apoptosis, and immune response genes; control of this host cell pathway would be critical for the survival of the virus. Studies with cowpox virus suggest that regulation of NF-κB activity is targeted at the degradation of the inhibitor of NF-κB protein, I-κB [32]. Treatment of cowpox virus-infected cells with TNF-α can induce phosphorylation of I-κBα; however, further processing is blocked [32]. molluscum contagiosum virus (MCV) encodes for a viral protein, MC159L, which blocks NF-κB activation downstream of PKR activation [33]. Vaccinia virus also encodes a number of genes to target this pathway. KIL blocks NF-κB activation at the level of I-κBα degradation [34] while another vaccinia virus gene A52R, inhibits NF-κB activation by multiple toll-like receptors (TLR) most likely through the interaction of this protein with downstream signaling molecules involved in TLR signaling and NF-κB activation [35]. Vaccinia virus N1L gene, which is similar to the A52R gene [36] was found to inhibit NF-κB signaling through toll-like-interleukin-1 resistance domain (TIR) of the TLR and TNF-R superfamily [36, 37]. NIL disrupts NF-κB signaling by targeting the IKK complex, the upstream signaling regulator of I-κBα phosphorlyation. NIL was also recognized to inhibit IFN regulatory factor (IRF)-3 signaling which links this immunomodulatory protein to the IFN response [37] and NIL was shown to be an important virulence factor in vivo. TLR inhibitors are unique to the Orthopoxvirus and Leporipoxvirus genera. Both variola virus and monkeypox virus have genes which potentially encode for TLR inhibitors (A56R/A52R and A47R, respectively) [17]. Further support for the importance of NF-κB modulation can be gained by examining modified vaccinia virus Ankara (MVA), an attenuated vaccinia virus. Unlike the viruses just described, this attenuated virus induces NF-κB activation [32]. Sequence analysis of MVA has demonstrated that this virus lacks many immunomodulatory genes present in its wild-type vaccinia virus Ankara parent; insertion of the KIL gene from wild-type Ankara back into MVA conferred the ability of this recombinant MVA virus to inhibit NF-κB activation similar to vaccinia virus.

Another strategy employed by these viruses to circumvent the host’s immune response is targeting of the programmed cell death function. Apoptosis, or programmed cell death, is a distinct morphological process characterized by activation of cellular proteases, or caspases. Apoptosis may be triggered through multiple pathways including Fas, members of the TNF superfamily, and hydrogen peroxide (reactive oxygen species) produced by macrophages or viral proteins. During viral infection apoptosis may aid the immune system by inducing the premature death of an infected cell and may also enhance MHC class I presentation of viral antigens. Not surprisingly, many viruses have developed or evolved mechanisms to prevent their host cells from undergoing apoptosis. The SERPIN crmA, not only prevents cleavage of IL-1β from the pro-form (similar to IL-18) by blocking IL-1β converting enzyme (ICE), but also prevents apoptosis by inhibiting other caspase molecules. Therefore, crmA is a potent inhibitor of apoptosis induced by both the Fas and TNF pathways [30, 38]. In vitro studies have also demonstrated that crmA binds to granzyme B, a
serine protease and another important inducer of cell death [39]. Deletion of crmA from cowpox virus, which was administered intranasally, resulted in a dramatic attenuation of disease [40]. In addition, the viral protein MC066L encoded by molluscum contagiosum virus was demonstrated to protect cells against the cytotoxic (pro-apoptotic) effects of hydrogen peroxide or ultraviolet (UV) irradiation (both sources of intracellular reactive oxygen species) [41]. Molluscum contagiosum virus also encodes a viral protein, MC159, which may inhibit association of Fas associated death domain (FADD) with caspase-8, an important step in caspase activation and induction of apoptosis[42]. The E3L gene of vaccinia virus encodes a double-stranded RNA binding protein that protects against PKR activity. Additional proteins in other poxviruses have been identified that have no cellular homologue, but still prevent induction of host cell apoptosis [38].

**DISEASE PATHOGENESIS**

Due to the limited tools that were available when smallpox was an endemic disease, much of the pathogenesis remains a mystery. Previous studies conducted at the United States Army Medical Research Institute of Infectious Diseases and the Centers for Disease Control and Prevention demonstrated the susceptibility of cynomolgus monkeys to i.v. inoculation of variola virus [22, 24] and monkeypox virus [43, 44]. The disease courses are clinically indistinguishable from each other manifesting elements of both classical pox disease and hemorrhagic pox disease. Comparison with archived tissues from human smallpox infections also suggest that the observed pathologies are consistent, and limited studies indicate a complex disregulation of the immune response involving the production of proinflammatory cytokines, lymphocyte apoptosis and the development of coagulation abnormalities. Briefly, the i.v. administration of virus produced an artificial viremia. Two to three days after infection, cell-associated virus was detectable in the blood and infectious virus was recovered from throat swabs. Changes in hematology and clinical chemistry revealed a profound leukocytosis, thrombocytopenia, and elevated serum creatinine levels. By day 3 post inoculation, the development of mucosal and cutaneous vesicles and pustules was evident. Similar to reports in human cases, the distribution of these lesions was centrifugal. At the time of death, high viral burdens were identified in numerous target tissues. It is likely that these viral burdens were associated with organ dysfunction and multi-system failure. Distribution of viral antigens by immunohistochemistry as well as replicating virus by electron microscopy correlated with pathology in the lymphoid tissues, skin, oral mucosa, gastrointestinal tract, reproductive system, and liver. Apoptosis was a prominent observation in lymphoid tissues with a striking loss of T cells observed. The cause of this widespread apoptosis remains unknown. However, it is likely that strong production of proinflammatory cytokines at least in part contributes to the upregulation of various pro-apoptotic genes. The strong upregulation of cytokines may also have contributed to the development of a hemorrhagic diathesis. The detection of D-dimers, as well as other changes in hematology in monkeys that developed classical or hemorrhagic smallpox suggests that activation of the coagulation cascade is a component of both disease syndromes, although in human populations, the prevalence of black or hemorrhagic smallpox was only ~ 1-3% of the total cases observed [45].

Fenner and colleagues mentioned the “toxic appearance” of patients with hemorrhagic smallpox, and offers the suggestion that complement activation due to high levels of viral antigen/IgM antibody complexes might contribute to the observed symptoms [46]. Dixon remarked that patients with hemorrhagic smallpox die “peacefully due to increasing toxemia” [47]. Moreover, he noted that, since antibiotics did not improve the prognosis of hemorrhagic disease, death was probably a result of extensive tissue damage [48]. From recent studies of variola and monkeypox virus infection in primates, the “toxemia” described by clinicians for human smallpox, may be fundamentally related to the processes underlying septic shock [49]. Among processes, mediators and pathways thought to be disregulated in monkeypox, smallpox and septic shock are: 1) lymphocyte apoptosis (massive apoptosis of lymphocytes both intravascularly and in lymphoid organs); 2) proinflammatory cytokines (exuberant production of type I IFNs, IL-6, TNF-α, and IFN-γ measurable in plasma); 3) the coagulation cascade (disseminated intravascular coagulation [DIC]); and 4) nitric oxide. Aberrant activation of all of these pathways is a hallmark of pathological activation of the innate immune system. All can contribute to fatal shock and all are likely interrelated.

**VACCINE RELATED ISSUES**

During the WHO Global Eradication Program, most of the human population received vaccinia virus by skin scarification. Although there were multiple manufacturers world-wide, and vaccine lots varied with respect to potency and purity, almost all vaccinia administered was derived from one of two lineages, the New York Board of Health and Lister strains [46]. Live virus was placed as a drop on the skin or drawn up by capillary action between the tines of a bifurcated needle; the nominal dose of live vaccinia was about 10^5 virions. Primary vaccination is usually uneventful; following introduction into the skin, the virus replicates in basal layer keratinocytes, spreading cell-to-cell, and leading to formation of discrete vesicles. Within a week, the vesicle evolves into a pustule surrounded by inflammatory tissue. This lesion scabs over within 10-14 days; eventually, the scab is shed. It was not uncommon for vaccinees
to experience tender axillary lymph nodes, fever, and malaise for brief periods. Occasionally, however, complications arose with varying degrees of severity. Accidental transfer of vaccinia from the inoculation site was fairly common, but of little consequence unless transferred to the eye. Generalized vaccinia, which involved systemic spread of the virus and eruption of multiple pocks at distant sites, was more serious; in individuals with eczema or atopic dermatitis, however, this could lead to extensive inflammation and secondary bacterial infection. More serious, life threatening complications arose in vaccinees with defects in cell mediated immunity; the vaccination site frequently enlarged to form an ulcer, secondary ulcers appeared, and the infection cleared slowly or not at all. The most serious event was post-vaccinal encephalitis. Although rare, this condition was frequently fatal. Historically, death occurred in approximately 1 in one million primary vaccinations [50, 51]. The fear is that these adverse events (AE’s) might be more frequent and severe if mass immunization were to be resumed in a population which now includes transplant recipients on immunosuppressive drugs, HIV-infected persons, and geriatric patients. An effective, but less reactogenic vaccine is urgently needed.

An alternative vaccine must be shown to be “non-inferior” to live vaccinia, and this sets a high standard since the successful immunization or “take rate” was > 95%, both historically and in a more recent series of > 450,000 military vaccines [52]. In this series, one case of encephalitis was documented and 37 cases of myopericarditis in this pre-screened and healthy, young adult population. The incidence of myopericarditis, while below the historical average and mild, was worrisome and may have been a factor in the general reluctance of the civilian health care population to accept vaccination [53]. Recently, a potential replacement vaccinia was prepared in massive quantities (> 300 million doses) by selection of plaque-purified progeny virus from the NYBOH strain and amplification in Vero cell cultures (ACAM 2000 [54]). This vaccine is more purified and free of adventitious agents in comparison with its predecessor, which was prepared on calf skin. Phase I safety and immunogenicity trials for ACAM 2000 indicate > 95% take rates and comparable AE’s [55]. Historically, live vaccinia immunization was also believed to protect recipients if administered therapeutically, within four days of exposure, although controlled clinical trials do not exist to support this claim; it is unlikely that an alternative, non-replicating vaccine will be effective therapeutically. The recent immunization of modest numbers of military and civilian individuals has provided an opportunity to study the nature of AE’s using modern tools of immunology. A strong association was established between AE’s and increased systemic cytokines, in particular, IFN-γ, TNF-α, IL-5, and IL-10 [56]. Although the numbers are small, some have speculated that cardiac events may be related to dramatic alterations in cytokine profiles.

Protective immunity elicited by live vaccinia is thought to depend on a combination of humoral and cellular immune responses. Using a monkey model where animals were immunized with vaccinia and challenged with monkeypox, Edghill-Smith has shown that vaccinia-specific B cells are critical for protection [57]. Antibody depletion of B cells, but not CD4+ or CD8+ T cells abrogated vaccinia-induced protection. This author also showed that simian immunodeficiency virus (SIV)-compromised monkeys could withstand vaccinia if it was preceded by a dose of non-replicating, MVA strain vaccinia, but they were not protected against monkeypox challenge when their CD4+ T cell counts were < 300/mm³ [58]. Lack of protection appeared to be associated with a defect in vaccinia-specific Ig switching from IgM to IgG. The conclusion was that vaccination strategies for severely immunosuppressed individuals would have to bypass CD4+ help to elicit high affinity antibodies to effect protection. In human vaccinees, longitudinal studies have demonstrated that vaccinia raised robust CD4+ responses with a Th1-dominant profile, in addition to CD8+ [59]. A significant proportion of vaccinated individuals lost detectable CD8+ memory while retaining CD4+ memory. It was concluded that vaccinia provides long-lasting CD4+ help that may be critical to long-lived B-cell memory. Protective immunity may last longer than previously imagined; following the recent monkeypox outbreak in the United States, three individuals whose vaccinia immunizations were administered 13, 29, and 48 years previously were identified to have sustained inapparent infections [60, 61]. Antibodies from 104 vaccinees were found to neutralize both infectious forms of vaccinia, IMV and EEV. All individuals retained some ability to neutralize IMV more than three decades after immunization; however, some drop-off in EEV neutralizing activity was noted after three decades. It is unknown whether this loss of anti-EEV correlates with waning protection.

MVA is an alternative vaccine that has promise as a non-replicating immunogen. MVA was utilized in Germany in the later stages of global eradication; it was shown to be safe and immunogenic, but its protective efficacy was not formally established in humans. Recently, MVA was demonstrated to protect monkeys against a monkeypox virus challenge, after one or two doses of MVA or MVA followed by Dryvax [44]. Surprisingly, a single dose of MVA also protected when challenge followed immunization by as little as 10 days, although protection was not absolute; a modest number of poxcs and a low level viremia occurred in the MVA recipients following challenge. Recently, rhesus monkeys were used in a similar i.v. challenge model to evaluate a DNA vaccine strategy; a combination of four genes (L1R, A27L, A33R, and B5R) with promising results [62].
MVA was generated by more than 500 passages in chick embryo fibroblasts, which resulted in multiple deletions and mutations and an inability to replicate efficiently in human and most other mammalian cells [63]. Ultrastructural examination of purified MVA reveals that most of the particles are enveloped; the host restriction occurs at a late stage of maturation. MVA particles label poorly with antibodies against IMV but label strongly with anti-envelope [64]. There is some evidence that MVA may be able to resist humoral antibody displayed by previously vaccinated individuals. MVA, unlike standard vaccinia, activates monocyte-derived human dendritic cells (DC), as measured by increases in surface co-stimulatory molecules and the secretion of pro-inflammatory cytokines [65]. In contrast, vaccinia virus does not activate DC unless it is UV-inactivated, suggesting that a vaccinia gene product prevents DC activation. DC’s are also activated by MVA-infected HeLa cells, and under these conditions can induce IFN-γ more efficiently than when replication competent vaccinia is used. Activation of DC may help explain the remarkable immune stimulating capacity of MVA in the absence of viral multiplication.

**ANTIVIRAL DRUG DISCOVERY**

The elaborate replication strategy of poxviruses offers a number of potential targets for therapeutic intervention [66]. While inhibition of viral replication may be necessary to reverse that pathogenic disease course, it may not be sufficient. It may also be necessary to reverse the effects of the cytokine storm which account for the “toxicity” of systemic orthopoxvirus infection. In this regard, cytokine antagonists developed to treat bacterial sepsis and other conditions may play a role in effective management of smallpox- and monkeypox-infected patients.

Initial studies to identify effective antiviral agents for orthopoxviruses tested drugs developed for other viruses which share molecular targets with poxviruses [67]. The most promising candidate using this approach was cidofovir, which is a dCMP analog [68]. Cidofovir is licensed for treatment of cytomegalovirus-associated retinitis, and is thought to act by inhibiting the cytomegalovirus DNA polymerase, a target shared with the poxviruses. Cidofovir is also thought to inhibit the activity of the proofreading exonuclease, leading to error-prone DNA synthesis during poxvirus replication. Cidofovir has been demonstrated to protect monkeys against severe disease in both the monkeypox and authentic smallpox primate models, provided the drug is administered within 48 hours of i.v. exposure to the virus [23]. Although the drug formulation used in these studies has been criticized because it requires i.v. administration and has been associated with nephrotoxicity, oral formulations with better bioavailability and lower toxicity are under development [69].

The first drug used to empirically treat progressive vaccinia and smallpox was Marboran, a compound of the class of N-aminomethyl-isatin-beta-thiosemicarbazones. As with most early treatment strategies, controlled clinical trials were not reported. Through combinatorial chemistry, potent and much more selective compounds have now been discovered and are in preliminary testing [70]. A number of essential viral enzymes have been targeted using a homology-based bioinformatics approach, such as that used to develop a structural model of vaccinia virus 17L proteinase. A unique chemical library of 51,000 compounds was computationally queried to identify potential active site inhibitors [71]. A subset of compounds was assayed for toxicity and ability to inhibit vaccinia replication, and through this process, a family was identified with 50% minimal inhibitory concentrations of 3-12 microM.

Alternative approaches are many, and include peptide mimetics of IFN-γ that play a direct role in the activation of STAT 1 alpha transcription factor [72]. These mimetics do not act through recognition of the extracellular domain of the IFN-γ receptor, but rather bind to the cytoplasmic domain of the receptor chain and thereby initiate the cellular signaling. Thus, the authors hypothesize that mimetics would bypass the poxvirus virulence factor B8R protein that binds the intact IFN-γ and would prevent its interaction with its receptor. Experimentally, these mimetics, but not intact IFN-γ, inhibited replication of vaccinia in BSC-40 cells. Thus these mimetics can avoid the B8R virulence factor and have potential activity against poxviruses in vivo.

Recently, a drug (Gleevec), licensed for use in chronic myeloid leukemia, was shown to block the egress of vaccinia virus from infected cells [73]. Smallpox virus includes an epidermal growth factor (EGF)-like domain that targets human Erb-1, inducing tyrosine phosphorylation of certain host cell substrates, thereby facilitating viral replication. Poxviruses migrate to the cell membrane via the polymerization of actin tails to produce EEV, which facilitates viral dissemination. The authors reasoned that low molecular weight inhibitors of Erb-1 kinases might function as antiviral agents. One such inhibitor, CI-1033 blocked variola replication in BSC-40 and Vero cells, primarily at the level of secondary viral spreading. CI-1033 protected mice exposed to a lethal vaccinia challenge via the aerosol route. In conjunction with a monoclonal antibody directed against L1R, CI-1033 cleared the lungs of virus within eight days. Gleevec is also a small molecule which inhibits the Abl-1 family of tyrosine kinases, thereby inhibiting the release of EEV from infected cells. Gleevec inhibited the spread of vaccinia virus from the mouse peritoneum to the ovaries and protected the mice from al lethal intranasal challenge. The advantage of Gleevec over other tyrosine kinase inhibitors such as CI-1033 is that Gleevec is already approved for human use. The potential success of Gleevec suggests that strategies which block key host signaling pathways
have merit and augment the approaches which target classical viral replication enzymes. An alternative approach to inhibiting the polymerization of actin, which in turn inhibits the propulsion of viral particles along actin filaments towards the cell membrane, is small interfering RNA (siRNA) directed against the Arp2/3 [74] complex.

Another strategy is to target the viral virulence factors responsible for viral immune evasion. Host preference is profoundly influenced by host modulation of host signaling pathways. The strict human tropism of variola suggests that its proteins are better adapted than vaccinia to overcome the human immune response. One such factor is A46R of vaccinia, which inhibits intracellular signaling by a range of TLRs by targeting TIR of the receptors and adaptor molecules. A46R targets MyD88, TRIF, and NF-κB. Vaccinia virus lacking A46R is attenuated in the murine model [75]. Another potential target may be the smallpox inhibitor of complement enzymes (SPICE). This homologue is almost 100-fold more potent than its vaccinia homologue, vaccinia complement control protein, (VCP), at inactivating C3b and 6-fold more potent at inactivating C4b [76]. By inactivating complement components, SPICE serves to inhibit the formation of C3/C5 convertases necessary for complement-mediated viral clearance. Thus, disabling SPICE using such technologies such as antisense or siRNAs might be therapeutically useful.

Lastly, treatment strategies may be developed to target the toxemia or clinical manifestations of the disease. In particular, modulation of the systemic immune response to orthopox infection, with particular attention to the prevention of organ damage due to vascular leakage and fibrin deposition, may provide a useful therapeutic target. Uncontrolled or inappropriate immune responses can contribute to multiple organ failure and death; in this aspect the "toxemia" associated with fatal orthopox infections resembles severe sepsis. Currently, several treatment strategies for targeting the manifestations of septic shock [77], such as activated protein C and inhibitors of the tissue factor pathway [78] are under consideration for testing in the NHP model for smallpox.

REFERENCES
