The strategic use of novel smallpox vaccines in the post-eradication world

We still face a threat of orthopoxviruses in the form of biological weapons and emerging zoonoses. Therefore, there is a need to maintain a comprehensive defense strategy to counter the low-probability, high-impact threat of smallpox, as well as the ongoing threat of naturally occurring orthopoxvirus disease. The currently licensed live-virus smallpox vaccine ACAM2000 is effective, but associated with serious and even life-threatening adverse events. The health threat posed by this vaccine, and other previously licensed vaccines, has prevented many first responders, and even many in the military, from receiving a vaccine against smallpox. At the same time, global immunity produced during the smallpox eradication campaign is waning. Here, we review novel subunit/component vaccines and how they might play roles in unconventional strategies to defend against emerging orthopoxvirus diseases throughout the world and against smallpox used as a weapon of mass destruction.

Keywords: biological warfare • disease eradication • DNA vaccines • humoral immunity • orthopoxvirus • subunit vaccines • vaccination • variola virus • viral vectors • zoonoses

Pre-eradication world

Variola virus (VARV), a member of the Orthopoxvirus family, is the causative agent of smallpox [1,2]. It is believed to have emerged early in humanity, around 10,000 BC when people began congregating in small settlements [3,4]. Countless numbers of people, regardless of age, sex or vocation, were killed or maimed by smallpox; and even whole civilizations were destroyed [4,5]. The history of smallpox and the havoc it wreaked on humanity has been reviewed extensively elsewhere [3–6].

While smallpox was devastating, it did spur the development of technologies aimed at combating infectious disease, including the first vaccine. The process of variolation was an attempt to control smallpox by choosing a specific time, dose, and route of infection. Variolation consisted of the delivery of VARV material from infected persons to the uninfected person via nasal mucosa or subcutaneously [7]. The technique evolved in the east, in places such as China and India [7] and spread to Europe in the 18th Century [8]. Variolation was extremely dangerous (i.e., 2–5% fatal) and there was a high risk of inadvertently starting a smallpox epidemic [7]. Nevertheless, the risk of variolation was deemed lower than the risk of uncontrolled smallpox, which was 30% fatal. Edward Jenner’s 1796 experiment using cowpox virus (CPXV) delivered to the skin by scarification was a monumental technological breakthrough [9]. This process, from which the word ‘vaccine’ was derived (Latin vacca for cow), provided the means to eliminate naturally occurring smallpox.

Eradication

Jenner’s vaccine, coupled with the fact that VARV has no nonhuman reservoir, made smallpox eradication possible. If person-to-person transmission could be prevented in the absence of a nonhuman reservoir, then the disease would die-out after infecting its last victims. After approximately 200 years of sporadic vaccine use, the cycle of person-to-person transmission was finally broken. Endemic smallpox was eradicated during the WHO-sponsored Smallpox Eradication Program, which involved a sophisticated worldwide monitoring effort and ring vaccination [4]. Use of Jenner’s vaccine was not without risks. In the beginning, the vaccine was distributed arm-to-arm, spreading other diseases such as tetanus and syphilis [4]. Eventually, immunization with CPXV became less common and vaccinia virus (VACV) became the vaccine of choice. The historical context of
this transition is interesting, since the specific origin of VACV remains unknown. To produce the vaccine, VACV was grown on the skin of calves [10]. Lymph collected from the lesions was used as the vaccine inoculum until the end of the eradication era [11].

During the mid-20th Century, as endemic smallpox become more sporadic, a shift in vaccination strategies emerged. Mass vaccination was replaced with ring-vaccination [4], which involves vaccinating those with contact to a smallpox-infected person (first ring) and vaccination in a second ring of those who have contact with the first ring. The vaccine used in this strategy was live-VACV smallpox vaccine derived from calf lymph. Ring-vaccination had the advantage of sparing vaccine and eliminated the costs associated with coordinating mass vaccination efforts.

As the eradication campaign matured, and country after country declared their population smallpox-free, it became clear that the vaccine itself posed a significant risk to not only the vaccinated person, but also to their close contacts. In fact, vaccination with the live-VACV smallpox vaccine became a greater risk than the risk of acquiring smallpox. Owing to the shifting risk–benefit ratio posed by the approaching eradication of VARV, an ongoing effort to develop safer smallpox vaccines was initiated. A safer vaccine was especially needed for children and persons with compromised immune systems. It was reasoned that, if the virus used in the vaccine could be inactivated or attenuated, then perhaps the deleterious effects of live-virus vaccination could be eliminated without reducing efficacy. Inactivation by various means rendered VACV incapable of eliciting protective immunity [12–15].

In the 1930s, Rivers et al. were the first to report the generation of an attenuated vaccine virus derived from serial passage of the VACV New York Board of Health strain in chick embryo tissue [16,17]. Rivers et al. found that vaccination with the attenuated VACV was not as efficacious as parental VACV and therefore would likely not function as a stand-alone vaccine. This led to the testing of a prime/boost strategy, whereby persons were vaccinated with the attenuated strain and 6–12 months postvaccination were boosted with the standard live-VACV vaccine [16,17]. This technique successfully reduced reactogenicity of the vaccine [17]. In 1968, Rivers’ vaccine was modified by Kempe et al. by additional passaging in chicken embryos and termed the CVI-78 vaccine [18]. CVI-78 proved to have an improved safety profile for use in children with eczema. Wesley et al. tested this vaccine in the 1970s and reported that, while it eliminated the dangers associated with live-virus vaccination, it seemed to have been ‘over attenuated’ (i.e., there were fewer ‘takes’ and reduced prevalence of neutralizing antibody) [19]. In a parallel study, Speers et al. investigated the quality of the prime/boost strategy [20]. They found that revaccination with parental live-VACV vaccine after a prime with CVI-78 did not necessarily eliminate complications. Around the same time, there were other efforts to attenuate VACV. For example, in the 1960s modified vaccinia Ankara (MVA) was developed by serially passaging VACV strain Ankara in chick embryo explants [21,22]. The result was a highly attenuated virus that lost approximately 15% of its genome. MVA was tested in over 120,000 human subjects in the 1970s [22]. MVA requires two vaccinations to achieve levels of humoral immunity that are similar to the levels achieved following a single live-virus vaccination [23]. Because of the low level of humoral immunity generated by MVA and CVI-78, and the fact that MVA required more than one dose to reach acceptable titers, Japan started to develop other highly attenuated VACV strains [24,25]. This led to the isolation of the LC16 attenuated strain, derived from VACV strain Lister [24]. This strain was created by serial passage in rabbit kidney cells at low temperature. Ultimately, strain LC16m8 was found to elicit an immune response similar to strain Lister, with the exception that the attenuated strain significantly reduced fever and local induration [24]. While not licensed by the US FDA for use in the USA, LC16m8 is licensed for human use in Japan by the Japanese government [25]. With the eradication of naturally occurring smallpox, the continued need for further development of attenuated smallpox vaccines appeared to vanish. Furthermore, none of the attenuated live-virus vaccines were tested against human smallpox and consequently it remains unknown how effective these vaccines would be at preventing VARV infection, or ameliorating disease.

On October 26 1977, the last naturally occurring smallpox case was reported [4–6,26]. Approximately 1 year later, on September 11 1978, Janet Parker became the last person to perish from a smallpox infection [27]. She acquired it in a laboratory setting by unknown means despite that fact that she had been vaccinated with the live-VACV smallpox vaccine a decade earlier [27]. The disease spread to Janet Parker’s mother, who received emergency smallpox vaccination as part of a ring-vaccination effort to contain the disease. She survived infection, exhibiting only minor disease and is the last person history records as having been infected with VARV [4]. In the spring of 1980, the WHO declared smallpox officially eradicated [28]. This event is a hallmark of the power of vaccination.

Post-eradication world

Naturally occurring smallpox is no longer a threat. Unfortunately, the properties of VARV that made it such a scourge to humanity also make it a daunting biological weapons threat. For example, the virus is relatively stable in the environment; easy to replicate; aerosolizable; contagious before, during and after disease onset; and produces an often lethal disease [29]. Moreover, the grotesque manifestations of the disease (i.e., characteristic pox lesions) achieve demoralizing and debilitating terror in an unprotected population. Indeed, the use of smallpox as a biological weapon is not novel. An invoice for replacement of blankets and a handkerchief dated June 1763 reads, “…Sundries got to replace in kind those which were taken from people in the Hospital to convey the Smallpox to the Indians” [30]. This now infamous event led to widespread infection in the Native American population around Fort Pitt (Pittsburgh, PA, USA), where the sundries, blankets and a handkerchief were distributed [30–32]. The concept of utilizing orthopoxviruses as biological weapons continued into the 20th Century. For example, the former USSR included VARV as part of its offensive biological weapons program [31–33]. There is evidence that this program continued after the 1972 Biological Weapons treaty and involved reactor-based culturing methods.
and field-testing the use of smallpox as a weapon [31–33]. The disposition of the VARV stocks used in this program is unclear. Furthermore, it is unclear if, and to what extent, terrorist or other state-sponsored groups are interested in using orthopoxviruses as weapons.

A recent report by the Commission on the Prevention of Weapons of Mass Destruction highlighted biological weapons as the gravest weapons of mass destruction (WMD) threat to the world [34]. It was argued that the technical knowledge needed to develop biological weapons was widespread and publicly available and the financial hurdle relatively low [34]. Orthopoxviruses are DNA viruses that are highly malleable and relatively easy to engineer using very basic molecular biology techniques. In fact, both VACV and MVA have been used as vaccine platforms to deliver immunogens from other pathogens including rabies and HIV. Furthermore, advances in molecular biology have made it possible to clone entire orthopoxvirus genomes into plasmids allowing more rapid manipulation of the genome [35]. More importantly, advances in synthetic biology and reverse genetics have made it possible to synthesize entire viral genomes de novo and to produce an infectious virus in the laboratory [35,36]. The rapid advances in synthetic biology capabilities have raised serious concerns in the biodefense community [37]. Elimination of the last remaining stocks of VARV would be moot because the published VARV genome could, theoretically, be synthesized and the virus reanimated. As disturbing as that prospect is, an even more disturbing prospect is the intentional development of genetically modified poxviruses (i.e., enhanced agents or advanced agents) that are not only as virulent or more virulent than smallpox, but also engineered to evade existing medical countermeasures. History has demonstrated that naturally occurring smallpox disease outbreaks are difficult to contain [4]. An intentional attack using smallpox or enhanced/advanced poxvirus on an unprotected population would be a national and international emergency of historic proportions [38].

The smallpox response plan

The US Advisory Committee on Immunization Practices and the US Healthcare Infection Control Practices Advisory Committee issued a comprehensive smallpox response strategy in 2001 and 2003 [39,40]. In these reports, they stated that the risk of a smallpox incidence was low, but existed and necessitated a contingency plan. The plan consists of a pre-release and a post-release phase. In the pre-release stage, the plan asserts each state form at least one smallpox response team (SRT) that consists of hospital staff and support staff trained to deal with a smallpox outbreak and maintain a continuity of care during the outbreak [40]. Optimally, SRT members should be vaccinated with live-virus before an event occurs; although there is no requirement that team members be vaccinated. Because live-virus vaccination can be associated with mild-to-serious adverse effects, both the Advisory Committee on Immunization Practices and US Healthcare Infection Control Practices Advisory Committee recommended that those previously vaccinated during the smallpox eradication program should be revaccinated. The rationale was that previously vaccinated persons would suffer fewer serious adverse events than naive vaccines as their immunity is primed for a recall response by the boosting vaccination. However, members of the SRT were reluctant to get vaccinated, a direct result of fears from the adverse events associated with the vaccine [41–43]. These fears were substantiated in an analysis of those who were vaccinated between 24 January and 31 December 2003. During this period, there were 857 adverse events reported and among these, 12.5% were considered serious and three people died from ‘possible contribution of smallpox vaccination to ischemic cardiac events’ [44,45]. To respond to future vaccine injuries, the US government began the Smallpox Vaccine Injury Compensation Program in April 2003. This program covers the medical expenses of persons who suffered ill effects directly caused by the vaccine [46].

The post-release CDC guidance calls for vaccination of medical staff responding to the outbreak, including medical laboratory personnel in the community where the outbreak is occurring [39]. Team members are also to take part in isolation and treatment of suspected and confirmed cases accompanied by contact tracing and ring vaccination. This initial response is a ring-vaccination strategy. The CDC guidance calls for mass community vaccination only if it becomes warranted by the size of the outbreak and permitted by public health officials [47]. Under this plan, modeling suggests it may take up to a year to extinguish smallpox cases if the event occurs from a single focal point [48].

Several people have criticized the response plan as being inadequate to respond to an actual release of smallpox as a biological weapon where the attack would likely be multifocal and involve exposure doses and routes different from those experienced in a natural outbreak. A smallpox outbreak was modeled in a 2001 exercise entitled Dark Winter [49]. In this hypothetical event, a single outbreak in Oklahoma (USA) rapidly became epidemic after a few months. Multiple factors contributed to the escalation and failure of containment. Among these included a problem in rapid vaccination of healthcare workers, conflicts between governmental entities (local, state and federal) and unpredictable actions of affected citizens [49]. Smith and McFadden wrote in a 2002 review that the Dark Winter scenario suggests any release of smallpox would “probably quickly escalate into a national, and then global, health emergency” [38].

Mass vaccination in a post-eradication world

In a review on smallpox policy, Bicknell suggested that ring-vaccination fails to meet the unique challenge associated with deliberate release of smallpox [50]. He argued that it may take up to 12–17 days before an initial infection will be discovered and in this time, those infected could be spreading virus for up to 5–7 days. A recent case of severe eczema vaccinatum in an infant exposed to VACV from a vaccinated parent reinforced Bicknell’s theory; the child presented with a severe rash that did not resemble chickenpox and was only diagnosed as a poxvirus disease after several days [51]. Historical reports suggest vaccination 4 days postinfection could prevent severe disease [52]. However, according to Bicknell, that first round window would be lost in a covert attack [50,53]. In 2003, Bicknell advocated for a larger voluntary pre-event vaccination program as
the best defense [54]. This recommendation was based on the fact that pre-event vaccination would be logistically less complicated, would reduce accidental infection among the immunocompromised, and it would enhance the overall herd immunity [50,53]. In 2002, other leaders, such as National Institute of Allergy and Infectious Diseases (NIAID)’s Anthony Fauci, supported more widespread voluntary pre-event vaccination if sufficient supply was available [55]. Maurice Hilleman, who was involved in the development of many of the vaccines used today, wrote in 2002 that mass vaccination may be the only means to control smallpox, particularly given the likelihood of being released in multiple locations. This included acknowledging that the vaccine itself would ‘kill’ a certain number of people [56]. However, ethical concerns with pre-event vaccination remain. Just as was observed in the 2003 vaccination program, pre-event vaccination with live virus will lead to more vaccine-caused deaths and many more serious health problems including unintentional spread of VACV to close contacts. The risk from disease may be too low for vaccine risks to be acceptable in a pre-event world. However, even if pre-event mass vaccination is the unacceptable, Kaplan et al. presented evidence that it may be the best strategy, even after an event [47]. These authors simulated a smallpox outbreak in a large city using disease transmission models. They found mass vaccination immediately after viral detection resulted in a decreased number of deaths and faster eradication compared with a ring-vaccination strategy. However, it is not clear if during an event enough vaccine doses could be distributed for a mass vaccination campaign, particularly if there was worldwide demand. In addition, a mass vaccination campaign using a vaccine with a history of serve adverse events could be politically sensitive if any potential smallpox outbreak was limited and rapidly contained. If, on the other hand, safer, cheaper and more widely available vaccines could be produced, then mass vaccination, pre- and post-event could be justified.

**Second- & third-generation smallpox vaccines**

As the demand for vaccine dwindled post-eradication, the smallpox manufacturing capability eroded and regulatory issues in the archaic, calf-lymph-based manufacturing process arose. Then, in the late 1990s, revelations that the USSR had continued a massive biological weapons program after 1972, combined with the emerging threat of the use of WMD by terrorists organizations, led to an effort to develop a more modern smallpox vaccine. The US government contracted Acambis (now Sanofi-Pasteur) to develop a cell culture-produced version of the live-VACV smallpox vaccine [57]. Briefly, Acambis plaque-picked clones from Dryvax, evaluated safety in animals, and chose a clone with minimal reactivogenicity for expansion, eventually naming it ACAM2000 [58]. ACAM2000 is delivered by scarification in a similar manner developed by Jenner more than 200 years ago. The vaccine elicits a virtually identical immune response compared with a calf-lymph-produced vaccine [54]. Unfortunately, this second-generation smallpox vaccine has a virtually identical adverse event profile as Dryvax [59,60]. These events can include autoinoculation of the eye, or spread of the virus to close contacts [60]. They may also be severe and include generalized vaccinia, eczema vaccinatum and progressive vaccinia [60]. In particular, eczema vaccinatum may be more aggressive when acquired from contact with vaccinated individuals, opposed to by direct vaccination [61,62]. A very severe case of ‘contact’ eczema vaccinatum in the child of a military vaccinee who received ACAM2000 was recently reported [63]. ACAM2000 may also cause myocarditis, a potential deadly condition [59]. Moreover, during clinical trials, 18% of subjects had false-positive syphilis tests [64], a known hallmark of the live-VACV vaccine. A request for approximately 200 million doses of this vaccine for the US National Strategic Stockpile was made in 2001. ACAM2000 was licensed by the FDA in 2007 and subsequently replaced all stocks of virus produced from infected calf lymph [57].

Because of the adverse events associated with ACAM2000, there has been a renewed push to develop a third-generation vaccine that can be used in immunocompromised individuals and other persons contraindicated for ACAM2000. A number of MVA-derived viruses (e.g., ACAM3000 and TBC-MVA), other attenuated strains of VACV (e.g., LCl6m8 and NYSVAC), as well as avipoxviruses (e.g., ALVAC fowlpox) have been investigated as alternatives to live VACV. The lead candidate is an MVA-derived vaccine from Bavarian Nordic called IMVAMUNE® [65]. IMVAMUNE is safe and immunogenic in humans and protective in animal models [65]. However, the immune response is notably less potent than that generated by ACAM2000 and requires a second vaccination [51]. Interestingly, IMVAMUNE has been shown to induce more rapid protection from lethal orthopoxvirus challenge in animal models when compared with the live-VACV vaccine, suggesting that it might be useful as an emergency vaccine [66]. Despite these promising data, some evidence suggests the vaccine will not induce long-term immunity, making more periodic revaccination a necessity [67]. However, other evidence suggests the vaccine can indeed induce long-term immunity [68]. More work in this area will be needed to fully address this critical yet controversial issue. Furthermore, there are unresolved issues of the mechanism of attenuation and mechanism of protection of MVA. The MVA genome has six major deletions compared with the VACV genome, in addition to various insertions and mutations [69–71]. Recent attempts by Bavarian Nordic and others to identify the mechanism of attenuation have been unsuccessful [72,73]. This included deletion of all six regions from VACV, which still failed to reproduce the MVA phenotype [72]. Similarly, the mechanism of protection of MVA is unknown. The US FDA has granted ‘fast-track’ status for this vaccine licensure, and the US government is procuring stocks for the US National Strategic Stockpile [74]. However, licensure may remain a daunting challenge because, unlike ACAM2000, MVA differs significantly from the previously licensed VACV vaccines.

**Future-generation smallpox vaccines**

The mechanism by which the live-VACV vaccine confers protective immunity against orthopoxviruses, including VARV, remains incompletely understood [75]. Protection against primary exposure to orthopoxviruses, including primary vaccination with VACV, requires both T cells and B cells. The most convincing
The strategic use of novel smallpox vaccines in the post-eradication world

The VACV (MVA and ACAM2000 included) genome consists of hundreds of genes that encode more than 200 structural and nonstructural proteins [1,69]. Many of these proteins have unknown functions, and it is likely that only a small subset actually contribute to protective immunity. Comprehensive investigations have identified poxvirus proteins that are the targets of antibodies [83] or T cells [84]. However, it is important to distinguish between the identification of immune response targets versus protective immune response targets. Investigators have used genome sequence data and recombinant DNA techniques to identify several VACV proteins that contribute to protective immunity in laboratory animals. A list of immunogens that have conferred >60% protection in animal models is provided in Table 1. Most of these proteins are structural components found within the membranes of the virion. Recently, one Orthopoxvirus immune mediator, an interferon-binding protein, was shown to be the target of protective immunity [85], at least under low-dose challenge conditions [86]. In addition, a peptide from at least one target, C7L (also called host range protein 2) of cell-mediated immunity was identified [87]. A comprehensive review of these immunogens can be found elsewhere [88].

Orthopoxviruses exist as two antigenically distinct infectious particles: the MV and the EV. Many of the identified targets are present on the surface of the MV including the neutralizing antibody targets L1, D8, H3 and A27 [89–105]. Galmiche et al. investigated the protective efficacy of EV-specific molecules and identified B5 and A33 as protective antigens [106]. Anti-B5 antibodies are a major component of VIG and have been shown to neutralize EV in cell culture [107]. Anti-B5 and A33 antibodies inhibit the spread of virus in cell culture as measured by ‘comet’ inhibition assays [93]. In 2000, we reported that combining MV (L1) and EV (A33) targets as a single DNA vaccine provides superior protection compared with targeting the individual molecules [96]. The concept of vaccinating with combinations of MV and EV immunogens to elicit improved protection has subsequently been reproduced using several subunit vaccine technologies including DNA vaccines, protein subunit vaccines, Alphavirus replicon-based vaccines, adenovirus viral vector vaccines and vesicular stomatitis virus vaccine vectors [89,91–104]. To date, the vaccinia L1, B5, A33 and A27 proteins and their orthologs have been the most extensively tested orthopoxvirus immunogens (Table 1). Note that we have used the term subunit vaccine to describe all vaccine approaches involving the delivery of orthopoxvirus subunits/components; in contrast to live virus, attenuated virus, or nonreplicating virus.

Vaccines consisting of combinations of individual MV and EV targets might not be able to cross-protect as efficiently as redundant combination vaccines (e.g., multiple MV targets combined with multiple EV targets). Although the protective immunogens discovered to date are highly conserved across species, there are still subtle antigenic differences in the vaccine targets that can affect immunity. For example, by investigating the cross-reactivity of monoclonal antibodies against A33 orthologs from VACV, VARV and monkeypox virus (MPXV), we discovered an important epitope varied among these agents such that protective antibodies that bound the VACV A33 failed to bind the MPXV A33 ortholog [108]. Similarly, Aldaz-Carroll et al. identified monoclonal antibodies that bound the VACV B5 but did not cross-react with the VARV B5 ortholog [109]. In 2003, we used redundant targeting of both the EV and MV (i.e., A33, B5, L1 and A27) in a DNA vaccine delivered by gene gun to provide potent protection in a MPXV, nonhuman primate challenge model [97].

While subunit smallpox vaccines pose a multitude of advantages over the current vaccine, there are disadvantages that warrant mention. For example, it is unlikely that any subunit

---

Table 1. Orthopox proteins that contribute to protective immunity.

| Open reading frame | Location in virion | Molecular vaccine platform | Tested in NHPs | Ref. |
|--------------------|--------------------|----------------------------|---------------|------|
| L1R                | MV membrane        | P/D/V                      | Yes           | [92,93,96–100,102,104,154] |
| D8L                | MV membrane        | D                           | No            | [102,103] |
| A27L               | MV membrane        | P/D/V                      | Yes           | [92,93,96–102,105,154,155] |
| A33R               | EV membrane        | P/D/V                      | Yes           | [92,93,96–102,104,106,154,156] |
| B5R                | EV membrane        | P/D/V                      | Yes           | [92,93,96–104,154] |
| H3L                | MV membrane        | P/D                         | No            | [90] |
| A28L + H2L         | MV membrane        | D                           | No            | [357] |
| B18R               | Not in virion      | P/D                         | No            | [85,86] |
| A10L               | Core               | P                           | No            | [105] |
| C7L                | Not in virion      | Peptide                     | No            | [87] |

1Greater than 60% survival in at least one animal model.
2Based on vaccinia virus strain Copenhagen.
3Confirmed independently.
4All protein and peptide vaccinations involved the use of adjuvant.
5The use of immunostimulatory molecules for the poxvirus DNA vaccines have not yet been published; however, it is likely that these types of adjuvants will be used in the future.
6D: DNA; EV: Enveloped virion; MV: Mature virion; NHP: Nonhuman primate; P: Protein; V: Virus-vectored.

---

www.expert-reviews.com

---

1025
Table 2. Comparison of licensed and novel smallpox vaccine technologies.

| Vaccine                     | Status     | Safety            | Efficacy                              | Regulatory | Mechanism of action | Mechanism of attenuation |
|-----------------------------|------------|-------------------|---------------------------------------|------------|---------------------|--------------------------|
| Live VACV (i.e., ACAM2000)  | Licensed   | Major concern     | Noninferior to historical vaccine; protective in animal models | Unknown    | Unknown             | Not attenuated           |
| Attenuated VACV (e.g., MVA) | Clinical trials in progress§ | Appears safe | Protective in animal models | Unknown    | Unknown             | Unknown*                 |
| DNA (e.g., 4pox plasmid)    | Preclinical | Unknown*          | Protective in animal models | Targets identified | NA                  |
| Protein (e.g., 4pox protein)| Preclinical | Unknown*          | Protective in animal models | Targets identified | NA                  |
| Virus-vectorized (e.g., 4pox VEE replicon) | Preclinical | Unknown* | Protective in animal models | Targets identified | NA                  |

1 IMVAMUNE® has been fast-tracked for approval by the US FDA.
2 Other DNA vaccine human trials have shown no serious safety issues [158–163].
3 Other protein vaccines have been licensed for human use, including hepatitis B [162–163], and show no safety issues.
4 Virus-vectored vaccines have been used in human trials with no safety issues.
5 Recent work by Bavarian Nordic has shown that attenuation does not involve the six major deleted regions of MVA [70].
6 For example gene gun, electroporation and needle-free jet.
7 Two vaccinations for standalone vaccine. One vaccination if used as focused prime or boost.
8 The logistics associated with primary cells and potential association of these cells with endogenous retroviruses have prompted research into the development of safer and easier production cell lines [111].
9 GMP: Good manufacturing practice; MVA: Modified vaccinia Ankara; NA: Not applicable; TCID: Tissue culture infectious dose; VACV: Vaccinia virus; VEE: Venezuelan equine encephalitis virus.

Vaccine could obtain the high degree of potency conferred by the live-VACV vaccine. This is because the current vaccine is an infectious, mildly pathogenic, replicating virus that elicits a robust immune response. By contrast, subunit vaccines are not replicating organisms, and lack such inherent potent immunostimulatory effects associated with an infection. The addition of adjuvants, including novel plasmid-encoded immunostimulatory molecules, will increase the potency of candidate subunit vaccines; however, it will be exceedingly difficult to match the potent response elicited by live VACV. Another potential disadvantage of a subunit approach is that the level of cross-protection against heterologous challenges might be reduced. This may be of particular relevance in biodefense where an engineered virus is a concern. Nevertheless, the use of redundant antigens (e.g., B5 and A33 redundantly target EV) in the subunit vaccine appears to compensate for cross-protection defects.

**Novel strategies to strengthen our defenses against orthopoxviruses**

In the post-eradication world, it is possible to look beyond ‘smallpox vaccine’ per se, and think of molecular vaccines as ways to augment our defense against orthopoxviruses. It is possible to devise strategies where a safe subunit vaccine is used in conjunction with the licensed second- and third-generation vaccines. For example, a subunit vaccine that elicits high-titer MV and/or EV neutralizing antibodies could be used to safely boost persons previously vaccinated with Dryvax or ACAM2000 (e.g., most of the population born before the 1970s and segments of the military). Another strategy could be to use the subunit vaccines to safely prime selected groups of people, such as first responders and the military, or even the entire population. The immunity elicited by the prime could then be rapidly recalled with the live-VACV vaccine (ACAM2000 or IMVAMUNE) if an attack occurred or was imminent. There are several advantages to this strategy. It would safely provide some level of protection to our first responders pre-event. It would reduce possible adverse events in individuals receiving emergency vaccination with ACAM2000. It would also decrease the time required for a vaccinee to attain protective levels of immunity, because it would be recalling established (e.g., memory) immune responses. Finally, a subunit prime could significantly reduce the dose of the ACAM2000 or IMVAMUNE to be used in emergency. For example, it has been reported that even simultaneous administration of the killed rabies vaccine, along with a rabies DNA vaccine, can reduced the amount of killed vaccine 25-fold [110]. This dose-sparing effect would provide cost savings and ease logistical pressures by lowering the amount of doses of live-virus needed to be stored, replaced and maintained.

As Rivers et al. first demonstrated in the 1930s [16,17], attenuated vaccine viruses, including MVA, could also be used in prime/boost strategies. However, there are several reasons why subunit vaccine approaches might be better suited for these roles...
in a post-eradication world. By definition, subunit vaccines will be more highly defined, consisting of a few known protective immunogens. This is in sharp contrast to the hundreds of open reading frames expressed by MVA that will be targeted during vaccination, most of which will not be involved in protection. Production of subunit vaccines will also be more straightforward compared with MVA, which requires unique expertise. Currently, MVA is produced in primary chicken embryo fibroblasts (CEFs) that must be collected freshly from special pathogen-free chicken stocks and used immediately. This added layer of complication would undoubtedly add cost to the vaccine and possibly present a major delay in production, particularly in a worldwide emergency when access to various regions may be cut off due to quarantine. Recent studies have been investigating new manufacturing methods that circumvent the requirement for CEF cells and could alleviate this complication [111]. Conversely, subunit vaccines consisting of DNA or protein could be rapidly scaled up and generated to GMP standards anywhere in the world with common equipment and from a cold start. For example, it has been shown that a DNA vaccine can be mass-produced to GMP quality within 2–3 weeks [112,113]. Protein and virus-vectored vaccines may take extra time, as they require more specialized purification steps. In the case of the protein vaccine, formulation with an adjuvant is an additional step that is required [92]. Nevertheless, these subunit vaccines could be manufactured more rapidly than live-virus vaccines, which are subject to time-consuming lot release safety tests. CEFs have also been found to contain endogenous retroviruses that can occasionally be activated during vaccine production runs [114,115]. This may add a previously unappreciated safety concern to an already challenging manufacturing environment. This threat does not exist for recombinant protein or DNA vaccines. A comparison of the advantages and disadvantages of live virus, nonreplicating virus and subunit smallpox vaccines is provided in Table 2.

Unlike live-virus vaccines, which are agent specific, subunit vaccines are more amenable to multivalent formulations. Therefore, it could be envisaged that a subunit vaccine platform based on DNA or protein could be developed that consisted of several immunogens targeting a multitude of biodefense or emerging-disease threats, including orthopoxviruses, alphanaviruses, Ebola virus, arenaviruses and anthrax. This vaccine could exist as a priming vaccine that is boosted, if required, by licensed agent-specific vaccines such as ACAM2000 or the anthrax vaccine Biothrax™ (Emergent BioSolutions). Subunit vaccines are also highly adaptable and could be rapidly tailored to a newly emerging threat. Such rapid adaptability is not possible with live-virus vaccines. Finally, it is highly unlikely that the immune response generated by subunit vaccines would be impacted by the coadministration of antivirals or other therapeutics. It is not yet clear to what extent

---

**Table 2. Comparison of licensed and novel smallpox vaccine technologies (cont.)**

| Site       | Method          | Dose          | Schedule                        | Drug substance      | Production medium | Speed              | Scalability       |
|------------|-----------------|---------------|---------------------------------|---------------------|-------------------|--------------------|-------------------|
|            |                  |               |                                  | Purified virus      | Mammalian cell culture | Moderate           | Highly specialized |
| Skin       | Bifurcated needle | 2.5–12.5 x 10⁵ PFU/dose | Single 15–30 prick vaccination |                     |                   |                    |                   |
| Muscle or | Needle           | 1 x 10¹⁴ TCID | Two vaccinations¹ | Purified virus      | Avian primary cell² | Slow. Depends on availability of primary avian cells from pathogen-free flocks | Highly specialized |
| skin       |                  | 0.002–2 mg    |                                  |                     |                   |                    |                   |
| Muscle or | Needle           | 20–100 µg     | Two vaccinations¹ | Purified plasmid DNA | Escherichia coli | Rapid. GMP lots can be made in weeks | Multiple GMP DNA production facilities exist |
| skin       |                  |               |                                  |                     |                   |                    |                   |
| Muscle or | Needle           | 10⁶–10⁷ particles | Two vaccinations¹ | Purified virus vector | Mammalian cell culture | Slow to moderate. Depends on viral vector | Highly specialized |
| skin       |                  |               |                                  |                     |                   |                    |                   |

¹IMVAMUNE® has been fast-tracked for approval by the US FDA.
²Other DNA vaccine human trials have shown no serious safety issues [158–163].
³Other protein vaccines have been licensed for human use, including hepatitis B [162–163], and show no safety issues.
⁴Virus-vectored vaccines have been used in human trials with no safety issues.
⁵Recent work by Bavarian Nordic has shown that attenuation does not involve the six major deleted regions of MVA [70].
⁶For example gene gun, electroporation and needle-free jet.
⁷Two vaccinations for standalone vaccine. One vaccination if used as focused prime or boost.
⁸The logistics associated with primary cells and potential association of these cells with endogenous retroviruses have prompted research into the development of safer and easier production cell lines [111].

GMP: Good manufacturing practice; MVA: Modified vaccinia Ankara; NA: Not applicable; TCID: Tissue culture infectious dose; VACV: Vaccinia virus; VEE: Venezuelan equine encephalitis virus.
antivirals targeting poxvirus would negatively impact vaccination during an outbreak. Evidence suggests that the ST-246 antiviral administered immediately after tail scarification does not impact the take of Dryvax in mice [116].

DNA vaccines may be especially effective for use in focused-prime and focused-boost strategies. It is well established that DNA priming followed by protein or viral vaccine boost elicits significantly greater immunity compared with either alone [117]. This has been observed with MVA–HIV vaccines [118], Ebola virus vaccines [119] and vaccines against other infectious diseases [120–122]. The mechanism of action may involve the production of low levels of high-quality immunogen produced by the DNA vaccine that is targeted by the immune system [117,123,124]. The low levels of a higher quality antigen allows the immune system to more specifically target protective domains, which upon recall with protein-based or whole-virus vaccines, elicit superior responses. Indeed, we have shown that even inefficient DNA priming of nonhuman primates followed by a protein boost produced potent antibody responses and protective immunity [95]. It is probable that a similar response would be observed if live VACV (ACAM2000 or IMVAMUNE) were used as the booster vaccination.

**Emerging Orthopoxvirus zoonoses & the use of subunit vaccines**

The bioterrorism threat posed by orthopoxviruses is the most immediate concern; however, orthopoxviruses also threaten humans in the form of emerging zoonoses [125]. Today, there are several members of the Orthopoxvirus genus that cause significant disease in people. These infections might arise owing to the waning orthopoxviruses immunity [126]. In each case, a role-played by subunit vaccines in protection of people and animals could be envisaged. The most important of these zoonotic poxviruses is MPXV. Human MPXV infections began to be detected when regions of Western and Central Africa became smallpox free [127,128]. The transmissibility of MPXV between humans is low; however, the symptoms, including the characteristic rash and lesions, resemble smallpox [128]. The disease can be fatal and mortality rates have ranged widely from 0 to 17% [128]. Given that the rodent population serves as a reservoir for MPXV in Africa [128], the disease will probably continue to cause human infections in Africa. Evolution of this virus to a more virulent or transmissible pathogen cannot be ruled out.

And, as was seen in the 2003 MPXV outbreak in the USA, MPXV can spread beyond Africa [129].

Cowpox virus is an emerging zoonosis in Eurasia [125] where it has been found to infect domestic cats [130,131] and zoo animals, including elephants [132], banded mongooses, jaguarundis [133] and exotic felines [134], with rats as the primary reservoir [135]. Humans can acquire CPXV from these animals and the resulting infection can lead to localized pustular skin lesions on the hands, face, neck and feet [125]. In some cases, the lesions can lead to significant damage, as was recently reported in a 19-year-old veterinary student who was infected by an unknown animal type (presumably cats) [136]. The student developed a small red plaque on her cheek that evolved into a larger ulcerated nodule surrounded by inflammation. The nodule demarcated but persisted and eventually required a complete necrectomy and plastic surgery to repair the damage. Given the broad-species range of CPXV, it will continue to be a threat for persons working with animals.

Beside MPXV and CPXV, VACV itself has become a source of infection throughout the world. Ironically, the emergence of VACV in local animal populations may be a direct result of the live virus used during the smallpox vaccination program spreading to animals [137,138]. Buffalopox virus has been observed in a broad geographical location including India, Russia, Indonesia, Egypt, Pakistan and Italy [137] where it infects domesticated buffalos and cattle. During occasional outbreaks, this disease impacts milk production and can even kill the animals [137]. Animal workers can be infected by Buffalopox virus and develop classic poxvirus lesions on their hands, feet and forearms [139]. For over a decade, VACV-related poxviruses have also been found to cause disease in wild and domestic animals, as well as humans, in Brazil [138,140,141]. Data suggest that the disease is endemic, consists of multiple strains of VACV, and impacts a large area of Brazil [125,142].

Animal workers are at an increased risk at being infected with many of the emerging orthopoxviruses. During the 2003 USA MPXV outbreak, most of those infected were animal workers or veterinarians [143]. In Europe, CPXV can cause significant disease in veterinarians and animal workers [125]. Dairy workers and farmers throughout the world are also at increased risk from infection by circulating VACV-like viruses [137,138]. Accordingly, there is a potential market for using subunit vaccine in animal workers, farmers and veterinarians. Kuntze suggested that all persons working with elephants get vaccinated using MVA [144]. Certainly MVA could function in this setting. Because MVA preferentially infects avian cells [145], there is a chance that this vaccine may be pathogenic to avian species that are in close proximity to vaccinated animals. A highly defined, inexpensive and efficacious protein or DNA-based subunit vaccine may provide a more attractive product.

There may also be a market in the zoo and dairy industries for a subunit animal vaccine. Such a vaccine could mitigate the impact of orthopoxviruses on animals in regions where livestock and zoo animals are infected. A USDA-licensed DNA vaccine against West Nile virus named West Nile-Innovator DNA vaccine from Fort Dodge Animal Health, is currently used in horses [146]. The advantage of a subunit vaccine in livestock is that it is highly defined and, thus, any impacts on the animals and animal products (i.e., milk) could be well understood. Moreover, it is relatively easy to distinguish animals that seroconvert due to the subunit vaccine from those that seroconvert due to infection. Distinguishing vaccinated versus previously-infected animals has hampered the use vaccines for some infectious diseases of agricultural importance (e.g., foot-and-mouth disease). Future studies aimed at understanding the efficacy of candidate subunit vaccines in various animal species, including zoo animals and livestock, may be of interest.
Novel approaches to licensure

Because smallpox no longer exists as a naturally occurring disease [28], it will not be possible to test any vaccine, including IMVAMUNE or subunit vaccines, for efficacy against VARV in humans. Regulations have been written to allow the FDA to deal with countermeasures for which human efficacy challenge studies are not ethical or possible [147]. Under 21 CFR 601 Subpart H, known as the ‘Animal Rule,’ appropriate surrogate animal challenge models can be used to demonstrate efficacy. These efficacy trials would be in addition to human trials aimed at proving safety [147]. It remains unclear how this rule will be used. However, there may be a way to license certain vaccines without the Animal Rule. For example, subunit vaccines, as well as attenuated vaccines such as IMVAMUNE, might be licensed as a biological product aimed at boosting or priming the immunity to the currently licensed vaccine, ACAM2000, as described in previous sections of this article. It might be less cumbersome to demonstrate that a biological has the desired effect, such as increased MV and EV neutralizing antibody titers, than to prove efficacy under the animal rule. Whether or not the FDA would consider this approach to licensure as viable is unclear.

Expert commentary

Naturally occurring smallpox has been eradicated. A new live-VACV vaccine has been licensed and stockpiled and an attenuated-VACV vaccine is moving towards licensure. An antibody-based product, VIG, to treat adverse events associated with the VACV vaccines has been licensed. There are at least two small-molecule compounds that show promise as drugs to treat Orthopoxvirus disease. Is there any reason to continue to modernize and strengthen our defenses against orthopoxviruses? In our opinion the answer is yes. As Benjamin Franklin, whose young son Franky died of smallpox before he could be inoculated by variolation, said, “an ounce of prevention is worth a pound of cure.” Pretreatment (vaccines) against orthopoxviruses make sense when one considers the ramifications of a multifoci attack. However, the existing live-virus vaccine is not fulfilling a pretreatment role. Safety concerns of the licensed vaccine decrease its de facto efficacy because much of the population, including the military and first responders, cannot receive the vaccine due to contraindications or refuse to receive the live-VACV vaccine due to the well-documented risk factors. MVA should be an excellent addition to our pretreatment capabilities. Nevertheless, the complexity of MVA vis-à-vis production and logistical issues of storage along with more immediate challenges of animal rule licensure, caution that back-up approaches are warranted. The current economic situation impacting the world also serves as a warning that cheaper methods may be needed to defend against biological weapon threats that are potentially catastrophic, but low probability. Safer and less expensive vaccines used as stand-alone or priming/boosting vaccines for ACAM2000 would allow the expansion of pretreatment programs. For example, a focused-boost approach of previously vaccinated persons would reverse the trend of waning herd immunity. A vaccine without the safety concerns associated with ACAM2000 would increase the numbers of SRT members, other first responders, and military personnel to be pretreated and thereby have immune systems primed for an event that could be, and would be, boosted with ACAM2000 or perhaps MVA. In addition, subunit vaccine technology might facilitate the development of multi-agent vaccines designed to confer protection or at the minimum prime the immune system against multiple high-impact biodefense threats. These threats are significant given the world of synthetic biology. For example, a potential ST-246 escape mutant has been described in a publicly accessible journal article [148]. The use of a pre-event vaccine would decrease our reliance on a rapid and complicated postevent response, and would decrease the likelihood that a smallpox attack would have its desired effect.

However, as is true for many of the pathogens considered to be biological weapons threat agents, it is myopic to consider the human orthopoxvirus threat as being solely that of a weapon of terror. Orthopoxviruses currently disrupt commerce and the lives of persons throughout the world [125]. Therefore, in addition to strengthening our defenses against orthopoxviruses as WMD, the continued modernization of smallpox vaccines and vaccine augmenters (i.e., priming and boosting subunit vaccines) will allow us to more effectively prevent and contain naturally occurring and newly emerging Orthopoxvirus outbreaks. Recent zoonotic outbreaks such as SARS, Q fever, hantavirus pulmonary syndrome and Nipah virus have prompted government agencies, academic institutions and scientific professional societies to come together to promote a ‘One Health’ concept [149]. One of the tenants of this concept is acknowledging the critical importance of zoonoses, both known and unknown, on human health. As recent outbreaks of MPXV and CPXV attest, orthopoxviruses in the environment can and cross-over into the human population [150]. It is likely that this is how the ancestral smallpox virus originally entered what would later become its sole host. In the advent of modern antibiotics and vaccines in the 1960s, the US Surgeon General William Stewart was once quoted as having said, “time to close the book on infectious diseases” [151]. More widespread vaccination with a safe vaccine (e.g., MVA or subunit vaccine) in regions with known circulating orthopoxviruses (e.g., central Africa, Brazil) would promote human health and possibly prevent large disease outbreaks and curtail intrahuman virus evolution. A safe, efficacious and cheap subunit vaccine devoid of complex manufacturing idiosyncrasies may offer the best solution towards this goal.

In this article, we have described two unconventional ways in which subunit vaccines could be used to mitigate the orthopoxvirus threat. The first is a focused boost approach, where a subunit vaccine is used to safely increase the anti-Orthopoxvirus immune response in previously vaccinated individuals. The second is a focused prime where a subunit vaccine is used to prime naive individuals. A booster vaccination with live-VACV vaccine or MVA would only be needed if an event occurred or was imminent. Use of subunit vaccines as augmenters could shore-up our smallpox defenses and also reduce the chances that a natural Orthopoxvirus zoonotic might expand in a population increasingly vulnerable to infection with orthopoxviruses. Several of these vaccines, in
particular the L1, A33, B5 and A27 targeting DNA and protein vaccines are ready for clinical trials, pending the support from an appropriate private or governmental champion.

Five-year view
In the upcoming 5 years, we expect a multitude of events to occur pertaining to protection against orthopoxviruses. First and foremost will be the development of animal models that are sufficient for licensure of medical countermeasures in the USA under 21 CFR 601 Subpart H. Among these animal models may be the recently described low-dose aerosol rabbitpox challenge model [252]. Using these models systems, along with clinical data, we predict IMVAMUNE will be licensed for human use in the next 5 years. This vaccine has already been fast tracked for licensure. It is also possible that LC16m8 will emerge on the market [25]. In addition to the momentum sweeping attenuated Orthopoxvirus vaccines, it is likely that in the next 5 years, at least one subunit smallpox vaccine will be tested for safety and immunogenicity in a Phase I trial. Such a trial will probably consist, initially, of a focused boost in individuals previously vaccinated with a live-virus vaccine (ACAM2000 and Dryvax). The capacity of a subunit vaccine to prime immune responses that are then boosted by the licensed live-virus vaccine (ACAM2000 and Dryvax) may also occur given the proper resources and programmatic support.

Over the next 5 years, there will be an increase in zoonotic Orthopoxvirus infections throughout the world. This will be attributed to the waning immunity of the population. Whether this will lead to increased pressure for a vaccine solution cannot be predicted, as it will likely depend on the number of cases, economic impact, and the availability of a safe and inexpensive vaccine. During the next few years, we predict that there will be an increased interest in using a vaccine to immunize persons against the various zoonotic orthopoxviruses. These would include animal workers and veterinarians who are impacted by CPXV and VACV-like viruses, in addition to the MPXV threat [125]. In addition, studies determining the efficacy of the most advanced subunit vaccines in cattle and perhaps exotic zoo animals, including wild cats and elephants, will have begun. This effort would support the One Health concept and ensure that an orthopoxvirus does not naturally emerge as smallpox did so long ago.

From a broader perspective, advances in gene-based subunit vaccine technology and protein-based vaccine technologies will allow the development of candidate vaccines against exotic infectious diseases, including biological threat agents. DNA vaccines may be best poised for this goal as they are highly malleable, rapid, combinable and inexpensively produced [153]. It is still relatively early in the process of DNA vaccine development. The discovery of new practical and efficient delivery technologies, as well as novel adjuvants, including novel plasmid-encoded adjuvants, can be expected as this process evolves. In the next few years, DNA-based vaccines against a handful of WMD threat agents, potentially including smallpox, will be tested in humans. These studies will begin to pave the way for the future DNA vaccines aimed at providing an alternative to the classic processes of developing live virus, attenuated virus and even protein-based vaccines. While this will certainly be beneficial to the development of countermeasures against biological weapons, it may also be extremely important for global health, as many of the infectious agents considered WMD bio-weapons are also emerging infectious disease threats impacting people worldwide. In this regard, defending against bioweapon agents and global health defense may be one and the same, each with a need for cost-effective, malleable, rapidly producible and efficacious vaccines. Championing the devolvement of these technologies over the next few years should become the goal of various funding agencies and organizations intent on dealing with these important infectious disease issues.

Disclaimer
Opinions, interpretations, conclusions and recommendations are those of the author and not necessarily endorsed by the US Army or the Department of Defense.

Financial & competing interests disclosure
The authors are co-inventors on smallpox vaccine-related patents, issued and pending. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues
- The licensed live-vaccinia virus smallpox vaccine is contraindicated for several populations owing to adverse events in vaccinees and their close contacts. The safety issues associated with this vaccine limits its usefulness as a pretreatment product.
- There is a need for a scalable, safe, efficacious and affordable vaccine to defend against the biological weapons threat potentially imposed by smallpox.
- Novel protein, DNA and viral vector-based subunit vaccines have been developed that are safe, highly-defined and effective in a multitude of relevant animal models.
- Subunit vaccines could be used to prime immune responses in select groups (e.g., first responders, governmental officials and military) whereby boosting with the live-virus vaccine (ACAM2000 or IMVAMUNE®) would only occur in the event of an attack.
- Subunit vaccines could be used to boost persons historically vaccinated with a licensed live-vaccinia virus vaccine.
- Orthopoxviruses are also emerging zoonoses, and subunit vaccines may provide a means of safely protecting animal workers and animals themselves from these diseases.
The strategic use of novel smallpox vaccines in the post-eradication world

References

Papers of special note have been highlighted as:

• of interest
  of considerable interest

1. Moss B. Poxviruses and their replication. In: Fields Virology. Knipe DM, Howley PM (Eds). Lippincott, Williams and Wilkins, Philadelphia, PA, USA, 2905–2946 (2007).

2. Damon I. Poxviruses In: Fields Virology. Knipe DM, Howley PM (Eds). Lippincott, Williams and Wilkins, Philadelphia, PA, USA, 2947–2976 (2007).

3. Fenner F, Henderson D, Arita I, Jezek Z, Ladnyi ID. Smallpox and Its Eradication. World Health Organization, Geneva, Switzerland (1988).

4. Hopkins DR. The Greatest Killer: Smallpox in History, With a New Introduction. University of Chicago Press, Chicago, IL, USA (2002).

5. Barquet N, Domingo P. Smallpox: the triumph over the most terrible of the ministers of death. Am. Intern. Med. 127(8 Pt 1), 635–642 (1997).

6. Eyler JM. Smallpox in history: the birth, death, and impact of a dread disease. J. Lab. Clin. Med. 142(4), 216–220 (2003).

7. Klebs AC, Lausanne MD. The historic evolution of variolation. The Johns Hopkins Hospital Bulletin 24(265), 1–66 (1913).

8. Dinc G, Ulman YI. The introduction of variolation ‘A La Turca’ to the West by Lady Mary Montagu and Turkey’s contribution to this. Vaccine 25(21), 4261–4265 (2007).

9. Jenner E. Inquiry into the Causes and Effects of the VariOLate Vaccine. Murray and Highley, London, UK (1798).

• The first scientific report on the use of a vaccine in human history.

10. Dudgeon JA. Development of smallpox vaccine in England in the eighteenth and nineteenth Centuries. BMJ 1(5342), 1367–1372 (1963).

11. Nalca A, Hatchin JM, Garza NL et al. Evaluation of orally delivered ST-246 as postexposure prophylactic and antiviral therapeutic in an aerosolized rabbiPoxPox model. Antiviral Res. 79(2), 121–127 (2008).

12. Appleyard G, Hapel AJ, Boulter EA. An antigenic difference between intracellular and extracellular rabbiPoxPox virus. J. Gen. Virol. 13(1), 9–17 (1971).

• This paper is among the initial studies demonstrating that mature virion and enveloped virion particles are immunologically distinct.

13. Boulter EA. Protection against poxviruses. Proc. R. Soc. Med. 62(3), 295–297 (1969).

14. Turner GS, Squires EJ. Inactivated smallpox vaccine: immunogenicity of inactivated intracellular and extracellular vaccinia virus. J. Gen Virol. 13(1), 19–25 (1971).

15. Turner GS, Squires EJ, Murray HG. Inactivated smallpox vaccine. A comparison of inactivation methods. J. Hyg. (Lond.) 68(2), 197–210 (1970).

16. Rivers TM, Ward SM. Jennerian prophylaxis by means of intradermal injections of culture vaccine virus. J. Exp. Med. 62(4), 549–560 (1935).

17. Rivers TM, Ward SM, Baird RD. Amount and duration of immunity induced by intradermal inoculation of cultured vaccine virus. J. Exp. Med. 69(6), 857–866 (1939).

• The Rivers group was the first to develop and use an attenuated version of the wild-type vaccine to mitigate the adverse effects of the wild-type vaccine. They were also the first to use an attenuated version as a priming vaccine, followed by a boost with the conventional wild-type vaccine.

18. Kempe CH, Fulginiti V, Minamitani M, Shinefeld H. Smallpox vaccination of eczema patients with a strain of attenuated live vaccinia (CVI-78). Pediatrics 42(6), 980–985 (1968).

19. Wesley RB, Speers WC, Neff JM, Ruben FL, Lourie B. Evaluation of two kinds of smallpox vaccine: CVI-78 and calf lymph vaccine. I. Clinical and serologic response to primary vaccination. Pediatr. Res. 9(8), 624–628 (1975).

20. Speers WC, Wesley RB, Neff JM, Goldstein J, Lourie B. Evaluation of two kinds of smallpox vaccine: CVI-78 and calf lymph virus. II. Clinical and serologic observations of response to revaccination with calf lymph vaccine. Pediatr. Res. 9(8), 628–632 (1975).

21. Hochstein-Mintzel V, Hanichen T, Huber HC, Stickl H. An attenuated strain of vaccinia virus (MVA). Successful intramuscular immunization against vaccinia and variola. Zentralbl. Bakteriol. Orig. A 230(3), 283–297 (1975).

22. McCurdy LH, Larkin BD, Martin JE, Graham BS. Modified vaccinia Ankarana: potential as an alternative smallpox vaccine. Clin. Infect. Dis. 38(12), 1749–1753 (2004).

23. Slifka MK. The future of smallpox vaccination: is MVA the key? Med. Immunol. (Lond.) 4(1), 2 (2005).

24. Hashizume S, Yoshizawa H, Morita M, Suzuki Kp-IGVQe. Vaccinia viruses as vectors for vaccine antigens. Properties of attenuated mutant of vaccinia virus, LC16m8, derived from Lister strain. In: Vaccinia Viruses as Vectors for Vaccine Antigens. Quinann GV (Ed.). Elsevier Science Publishing Co, Amsterdam, The Netherlands, 421–428 (1985).

25. Kenner J, Cameron F, Empig C, Jobes DV, Gurswich M. LC16m8: an attenuated smallpox vaccine. Vaccine 24(47–48), 7099–7022 (2006).

26. Center for Disease Control and Prevention. Epidemiology and Prevention of Vaccine-Preventable Diseases. Atkinson W, Wolfe S, Hamborsky J, McIntyre L (Eds). Public Health Foundation, Washington, DC, USA (2006).

27. Shooter RA. Report on the Investigation into the Cause of the 1978 Birmingham Smallpox Occurrence. Her Majesty’s Stationery Office, London, UK (1980).

28. World Health Organization. No smallpox. Wkly Epidemiol. Rec. 54, 329–336 (1979).

29. Kortepeter MG, Parker GW. Potential biological weapons threats. Emerg. Infect. Dis. 5(4), 523–527 (1999).

30. Fenn EA. Biological warfare in eighteenth–Century North America: beyond Jeffery Amherst. J. Am. Hist. 86(4), 1552–1580 (2000).

31. Alibek K. Smallpox: a disease and a weapon. Int. J. Infect. Dis. 8(Suppl. 2), S3–S8 (2004).

32. Frischknecht F. The history of biological warfare. Human experimentation, modern nightmares and lone madmen in the twentieth Century. EMBO Rep. 4(Spec No.), S47–S52 (2003).

33. Shoham D, Wolfson Z. The Russian biological weapons program: vanished or disappeared? Crit. Rev. Microbiol. 30(4), 241–261 (2004).

34. Graham B, Talent J. Bioterrorism: redefining prevention. Biosc. Bioterror. 7(2), 125–126 (2009).

35. Domi A, Moss B. Cloning the vaccinia virus genome as a bacterial artificial chromosome in Escherichia coli and recovery of infectious virus in mammalian cells. Proc. Natl Acad. Sci. USA 99(19), 12415–12420 (2002).

36. Cello J, Paul AY, Wimmer E. Chemical synthesis of poliovirus cDNA: generation of infectious virus in the absence of natural template. Science (New York) 297(5583), 1016–1018 (2002).
A sobering review of the potential hazards associated with a smallpox outbreak and provides useful and scholarly thoughts on our need to be prepared and vigilant.

Center for Disease Control and Prevention. Vaccinia (smallpox) vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Morb. Mortal. Wkly Rep. 50(RR-10), 1–25 (2001).

Center for Disease Control and Prevention. Recommendations for using smallpox vaccine in a prevent vaccinatino program: supplemental recommendation of the Advisory Committee on Immunization Practices (ACIP) and the Healthcare Infection Control Practices Advisory Committee (HICPAC). MMWR Morb. Mortal. Wkly Rep. 52(RR-7), 1–16 (2003).

Kwon N, Raven MC, Chiang WK et al. Emergency physicians’ perspectives on smallpox vaccination. Acad. Emerg. Med. 10(6), 599–605 (2003).

Schraeder TL, Campion EW. Smallpox vaccination – the call to arms. N. Engl. J. Med. 348(9), 381–382 (2003).

Yih WK, Lieu TA, Rego VH et al. Attitudes of healthcare workers in U.S. hospitals regarding smallpox vaccination. BMC Public Health 3, 20 (2003).

Strikas RA, Neff LJ, Rotz L et al. US civilian smallpox preparedness and response program, 2003. Clin. Infect. Dis. 46(Suppl. 3), S157–S167 (2008).

Casey CG, Iskander JK, Roper MH et al. Adverse events associated with smallpox vaccination in the United States, January–October 2003. JAMA 294(21), 2734–2743 (2005).

Clark PT, Levin S. The Smallpox vaccine injury compensation program. Clin. Infect. Dis. 46(Suppl. 3), S179–S181 (2008).

Kaplan EH, Craft DL, Wein LM. Emergency response to a smallpox attack: the case for mass vaccination. Proc. Natl Acad. Sci. USA 99(16), 10935–10940 (2002).

Meltzer MI, Damon I, LeDuc JW, Millar JD. Modeling potential responses to smallpox as a bioterrorist weapon. Emerg. Infect. Dis. 7(6), 959–969 (2001).

O’Toole T, Mair J, Inglesby TV. Shining light on “Dark Winter”. Clin. Infect. Dis. 34(7), 972–983 (2002).

Bicknell WJ. The case for voluntary smallpox vaccination. N. Engl. J. Med. 346(17), 1323–1325 (2002).

Handley L, Buller RM, Frey SE, Bellone C, Parker S. The new ACAM2000 vaccine and other therapies to control orthopoxvirus outbreaks and bioterror attacks. Expert Rev. Vaccines 8(7), 841–850 (2009).

Mortimer PP. Can postexposure vaccination against smallpox succeed? Clin. Infect. Dis. 36(5), 622–629 (2003).

Bicknell W, James K. Smallpox vaccination after a bioterrorism-based exposure. Clin. Infect. Dis. 37(3), 467 (2003).

Bicknell W, James K. The new cell culture smallpox vaccine should be offered to the general population. Rev. Med. Virol. 13(1), 5–15 (2003).

Fauci AS. Smallpox vaccination policy – the need for dialogue. N. Engl. J. Med. 346(17), 1319–1320 (2002).

Hilleman MR. Overview: cause and need for dialogue. N. Engl. J. Med. 346(17), 1301–1306 (2002).

Nalca A, Zumbrun EE. ACAM2000: the new smallpox vaccine for United States Strategic National Stockpile. Drug Des. Dev. Ther. 4, 71–79 (2010).

Weltzin R, Liu J, Pugachev KV et al. Clonal vaccinia virus grown in cell culture as a new smallpox vaccine. Nat. Med. 9(9), 1125–1130 (2003).

ACAM2000*, package insert. Acambis, Cambridge, UK.

Lane JM, Goldstein J. Adverse events occurring after smallpox vaccination. Semin. Pediatr. Infect. Dis. 14(3), 189–195 (2003).

Copeman PW, Wallace HJ. Eczema vaccinatum. BMJ 2(5414), 906–908 (1994).

Lane JM, Ruben FL, Neff JM, Millar JD. Complications of smallpox vaccination, 1968. N. Engl. J. Med. 281(22), 1201–1208 (1969).

Centers for Disease Control and Prevention. Household transmission of vaccinia virus from contact with a military smallpox vaccinee – Illinois and Indiana, 2007. MMWR Morb. Mortal. Wkly Rep. 56(19), 478–481 (2007).

Monath TP, Frey SE. Possible autoimmune reactions following smallpox vaccination: the biologic false positive test for syphilis. Vaccine 27(10), 1645–1650 (2009).

Kennedy JS, Greenberg RN. IMVAMUNE: modified vaccinia Ankara strain as an attenuated smallpox vaccine. Expert Rev. Vaccines 8(1), 13–24 (2009).

Earl PL, Americo JL, Wyatt LS et al. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. Nature 428(6979), 182–185 (2004).

Ferrier-Rembert A, Drillen R, Tournier JN, Garin D, Crance JM. Short- and long-term immunogenicity and protection induced by non-replicating smallpox vaccine candidates in mice and comparison with the traditional 1st generation vaccine. Vaccine 26(14), 1794–1804 (2008).

Earl PL, Americo JL, Wyatt LS et al. Recombinant modified vaccinia virus Ankara provides durable protection against disease caused by an immunodeficiency virus as well as long-term immunity to an orthopoxvirus in a non-human primate. Virology 366(1), 84–97 (2007).

Antoine G, Scheifflinger F, Dorner F, Falkner FG. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. Virology 244(2), 365–396 (1998).
The first vaccine study to use the antigens L1, A33, A27 and B5 in combination to provide protection against a viral challenge in mice and nonhuman primates. It is also the first to use the intravenous monkeypox virus/nonhuman primate challenge model for subunit vaccine testing.

Hooper JW, Ferro AM, Golden JW et al. Molecular smallpox vaccine delivered by alphavirus replicons elicits protective immunity in mice and non-human primates. Vaccine 28(2), 494–511 (2009).

Hooper JW, Golden JW, Ferro AM, King AD. Smallpox DNA vaccine delivered by novel skin electroporation device protects mice against intranasal poxvirus challenge. Vaccine 25(10), 1814–1823 (2007).

Kaufman DR, Goudsmi J, Holterman L et al. Differential antigen requirements for protection against systemic and intranasal vaccinia virus challenges in mice. J. Virol. 82(14), 6829–6837 (2008).

Pulford DJ, Gates A, Bridge SH, Robinson JH, Ulaeto D. Differential efficacy of vaccinia virus envelope proteins administered by DNA immunisation in protection of BALB/c mice from a lethal intranasal poxvirus challenge. Vaccine 22(25–26), 3358–3366 (2004).

Sakhatksry P, Wang S, Chou TH, Lu S. Immunogenicity and protection efficacy of monovalent and polyvalent poxvirus vaccines that include the D8 antigen. Virology 355(2), 164–174 (2006).

Sakhatksry P, Wang S, Zhang C, Chou TH, Kishko M, Lu S. Immunogenicity and protection efficacy of subunit-based smallpox vaccines using variola major antigens. Virology 371(1), 98–107 (2007).

Xiao Y, Aldaz-Carroll L, Ortiz AM et al. A protein-based smallpox vaccine protects mice from vaccinia and ectromelia virus challenges when given as a prime and single boost. Vaccine 25(7), 1214–1224 (2007).

Demkowicz WE, Maa JS, Esteban M. Identification and characterization of vaccinia virus genes encoding proteins that are highly antigenic in animals and are immunodominant in vaccinated humans. J. Virol. 66(1), 386–398 (1992).
• Demonstrates the importance of redundant targeting in the development of subunit vaccines against orthopoxviruses.

107 Bell E, Shamim M, Whitbeck JC, Syfoegra G, Lambris JD, Issacs SN. Antibodies against the extracellular enveloped virus BSR protein are mainly responsible for the EEV neutralizing capacity of vaccinia immune globulin. *Virology* 325(2), 425–431 (2004).

108 Golden JW, Hooper JW. Heterogeneity in the A33 protein impacts the cross-protective efficacy of a candidate smallpox DNA vaccine. *Virology* 377(1), 19–29 (2008).

**Demonstrates the importance of redundant targeting in the development of subunit vaccines against orthopoxviruses.**

109 Aldaz-Carroll L, Xiao Y, Whitbeck JC et al. Major neutralizing sites on vaccinia virus glycoprotein B5 are exposed differently on variola virus ortholog B6. *J. Virol.* 81(15), 8131–8139 (2007).

• Demonstrates the importance of redundant targeting in the development of subunit vaccines against orthopoxviruses.

110 Biswas S, Kalanidhi AP, Ashok MS, Reddy GS, Sririnivasan VA, Rangarajan PN. Evaluation of rabies virus neutralizing antibody titres induced by intramuscular inoculation of rabies DNA vaccine in mice and Bonnet monkeys (*Macaca radiata*). *Indian J. Exp. Biol.* 39(6), 533–536 (2001).

111 Jordan I, Vos A, Beilfuss S, Neubert A, Breul S, Sandig V. An avian cell line designed for production of highly attenuated viruses. *Vaccine* 27(5), 748–756 (2009).

112 Carnes AE, Williams JA. Plasmid DNA manufacturing technology. *Recent Pat. Biotechnol.* 1(2), 151–166 (2007).

113 Forde GM. Rapid-response vaccines – does DNA offer a solution? *Nat. Biotechnol.* 23(9), 1059–1062 (2005).

114 Boni J, Stalder J, Reigel F, Schupbach J. Detection of reverse transcriptase activity in live attenuated virus vaccines. *Clin. Diagn. Virol.* 5(1), 43–53 (1996).

115 Tsang SX, Switzer WM, Shanmugam V et al. Evidence of avian leukosis virus subgroup E and endogenous avian virus in measles and mumps vaccines derived from chicken cells: investigation of transmission to vaccine recipients. *J. Virol.* 73(7), 5843–5851 (1999).

116 Grosenbach DW, Jordan R, King DS et al. Immune responses to the smallpox vaccine given in combination with ST-246, a small-molecule inhibitor of poxvirus dissemination. *Vaccine* 26(7), 933–946 (2008).

117 Lu S. Heterologous prime-boost vaccination. *Curr. Opin. Immunol.* 21(3), 346–351 (2009).

118 Wang S, Pal R, Mascola JR et al. Polyclonal HIV-1 Env vaccine formulations delivered by the DNA priming plus protein boosting approach are effective in generating neutralizing antibodies against primary human immunodeficiency virus type 1 isolates from subtypes A, B, C, D and E. *Virology* 350(1), 34–47 (2006).

119 Hensley LE, Mulangu S, Asiedu C et al. Demonstration of cross-protective vaccine immunity against an emerging pathogenic Ebolavirus species. *PLoS Pathog.* 6(5), e1000904 (2010).

120 Wei CJ, Boyington JC, McTamney PM et al. Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. *Science* 329(5995), 1060–1064 (2010).

121 Vaine M, Wang S, Hackett A, Arthos J, Lu S. Antibody responses elicited through homologous or heterologous prime-boost DNA and protein vaccinations differ in functional activity and avidity. *Vaccine* 28(17), 2999–3007 (2010).

122 Wang S, Parker C, Taaffe J, Solorzano A, Garcia-Sastre A, Lu S. Heterologous HA DNA vaccine prime–inactivated influenza vaccine boost is more effective than using DNA or inactivated vaccine alone in eliciting antibody responses against H1 or H3 serotype influenza viruses. *Vaccine* 26(29–30), 3626–3633 (2008).

123 Richmond JF, Lu S, Santoro JC et al. Studies of the neutralizing activity and avidity of anti-human immunodeficiency virus type 1 Env antibody elicited by DNA priming and protein boosting. *J. Virol.* 72(11), p9992–p10010 (1998).

124 Wang S, Arthos J, Lawrence JM et al. Enhanced immunogenicity of gp120 protein when combined with recombinant DNA priming to generate antibodies that neutralize the JR-FL primary isolate of human immunodeficiency virus type 1. *J. Virol.* 79(12), 7933–7937 (2005).

125 Essbauer S, Pfeffer M, Meyer H. Zoonotic transmission of cowpox virus infection from domestic cat to man. *Lancet* 1(8444), 1515 (1985).

126 Cho CT, Wenner HA. Monkeypox virus. *Bacteriol. Rev.* 37(1), 1–18 (1973).

127 Di Giulio DB, Eckburg PB. Human monkeypox: an emerging zoonosis. *Lancet Infect. Dis.* 4(1), 15–25 (2004).

128 Reed KD, Melski JW, Graham MB et al. The detection of monkeypox in humans in the Western Hemisphere. *N. Engl. J. Med.* 350(4), 342–350 (2004).

129 Egerberin HF, Willemse A, Horzinek MC. Isolation and identification of a poxvirus from a domestic cat and a human contact case. *Zentralbl. Veterinarmed B* 33(3), 237–240 (1986).

130 Willemse A, Egerberin HF. Transmission of cowpox virus infection from domestic cat to man. *Lancet* 1(8444), 1515 (1985).

131 Kurth A, Wibbelt G, Gerber HP, Petschaelis A, Pauli G, Nitsche A. Rat-to-elephant-to-human transmission of cowpox virus. *Emerg. Infect. Dis.* 14(4), 670–671 (2008).

132 Kurth A, Straube M, Kuczka A, Dunsche AJ, Meyer H, Nitsche A. Cowpox virus outbreak in banded mongooses (*Mungos mungo*) and jaguarundis (*Herpailurus yagouaroundi*) with a time-delayed infection to humans. *PLoS ONE* 4(9), e6883 (2009).

133 Mareniniкова SS, Maltseva NN, Korneeva VI, Garanina VM. Pox infection in *Carnivora* of the family *Felidae. Acta Virol.* 19(3), 260 (1975).

134 Mareniniкова SS, Shelukhina EM, Fimina VA. Pox infection in white rats. *Lab. Anim.* 12(1), 33–36 (1978).

135 Glatz M, Richter S, Ginter-Hanselmayer G, Aberer W, Mullegger RR. Human cowpox in a veterinary student. *Lancet Infect. Dis.* 10(4), 288 (2010).

136 Bhanuprakash V, Venkatesan G, Balamarugan V et al. Zoonotic Infections of Buffalopox in India. *Zoonoses and Public Health* 57(7–8), e149–e155 (2009).

137 Triandide GS, Emerson GL, Carroll DS, Kroon EG, Damon IK. Brazilian vaccinia viruses and their origins. *Emerg. Infect. Dis.* 13(7), 965–972 (2007).

138 Singh RK, Hosamani M, Balamarugan V, Bhanuprakash V, Rasool TJ, Yadav MP. Buffalopox: an emerging and re-emerging zoonosis. *Anim. Health Res. Rev.* 8(1), 105–114 (2007).

139 Abrahao JS, Silva-Fernandes AT, Lima LS et al. Vaccinia virus infection in monkeys, Brazilian Amazon. *Emerg. Infect. Dis.* 16(6), 976–979 (2010).
The strategic use of novel smallpox vaccines in the post-eradication world

141 Medaglia ML, Pessoa LC, Sales ER, Freitas TR, Damaso CR. Spread of cantagalo virus to northern Brazil. Emerg. Infect. Dis. 15(7), 1142–1143 (2009).

142 Silva-Fernandes AT, Travassos CE, Ferreira JM et al. Natural human infections with Vaccinia virus during bovine vaccinia outbreaks. J. Clin. Virol. 44(4), 308–313 (2009).

143 Croft DR, Sotir MJ, Williams CJ et al. Occupational risks during a monkeypox outbreak, Wisconsin, 2003. Emerg. Infect. Dis. 13(8), 1150–1157 (2007).

144 Kuntze A. Elephant pox and microsporum infection-two zoonoses of relevance to the zoo veterinarian. Presented at: European Association of Zoo- and Wildlife Veterinarians, Second scientific meeting. Chester, UK, 21–24 May 1998.

145 Drexler I, Heller K, Wahren B, Erfle V, Sutter G. Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells. J. Gen. Virol. 79 (Pt 2), 347–352 (1998).

146 Redding L, Weiner DB. DNA vaccines in veterinary use. Expert Rev. Vaccines 8(9), 1251–1276 (2009).

147 Gronvall GK, Trent D, Borio L, Brey R, Nagao L. The FDA animal efficacy rule and biodefense. Nat. Biotechnol. 25(10), 1084–1087 (2007).

148 Yang G, Pevear DC, Davies MH et al. An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects mice from lethal orthopoxvirus challenge. J. Virol. 79(20), 13139–13149 (2005).

149 Atlas R, Rubin C, Maloy S, Daszak P, Colwell R, Hyde B. One health-attaining optimal health for people, animals and the environment. Microbe 5(9), 383–389 (2010).

150 Orent W. Will Monkeypox be the Next Smallpox? In: Los Angeles Times. Russ Stanton (Ed.). Los Angeles, CA, USA (2010).

151 Sassetti CM, Rubin EJ. The open book of infectious diseases. Nat. Med. 13(3), 279–280 (2007).

152 Garza NL, Harkin JM, Livingston V et al. Evaluation of the efficacy of modified vaccinia Ankara (MVA)/IMVAMUNE against aerosolized rabbitpox virus in a rabbit model. Vaccine 27(40), 5496–5504 (2009).

153 Dupuy LC, Schmaljohn CS. DNA vaccines for biodefense. Expert Rev. Vaccines 8(12), 1739–1754 (2009).

154 Golden JW, Joselyn MD, Hooper JW. Targeting the vaccinia virus L1 protein to the cell surface enhances production of neutralizing antibodies. Vaccine 26(27–28), 3507–3515 (2008).

155 Rudraraju R, Ramsay AJ. Single-shot immunization with recombinant adenovirus encoding vaccinia virus glycoprotein A27L is protective against a virulent respiratory poxvirus infection. Vaccine 28(31), 4997–5004 (2010).

156 Fang M, Cheng H, Dai Z, Bu Z, Sigal LJ. Immunization with a single extracellular enveloped virus protein produced in bacteria provides partial protection from a lethal orthopoxvirus infection in a natural host. Virology 345(1), 231–243 (2006).

157 Shinoda K, Wyatt LS, Moss B. The neutralizing antibody response to the vaccinia virus A28 protein is specifically enhanced by its association with the H2 protein. Virology 405(1), 41–49 (2010).

158 Graham BS, Koup RA, Roederer M et al. Phase I safety and immunogenicity evaluation of a multiclade HIV-1 DNA candidate vaccine. J. Infect. Dis. 194(12), 1650–1660 (2006).

159 Geier MR, Geier DA, Zahalsky AC. A review of hepatitis B vaccination. Expert Opin. Drug Safety 2(2), 113–122 (2003).

160 Abraham P, Mistry FP, Bapat MR et al. Evaluation of a new recombinant DNA hepatitis B vaccine (Shanvac-B). Vaccine 17(9–10), 1125–1129 (1999).

161 Martin JE, Sullivan NJ, Enama ME et al. A DNA vaccine for Ebola virus is safe and immunogenic in a Phase I clinical trial. Clin. Vaccine Immunol. 13(11), 1267–1277 (2006).

162 Martin JE, Pierson TC, Hubka S et al. A West Nile virus DNA vaccine induces neutralizing antibody in healthy adults during a Phase I clinical trial. J. Infect. Dis. 196(12), 1732–1740 (2007).

163 Jones S, Evans K, McElwaine-Johnn H et al. DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled Phase Ib clinical trial. Vaccine 27(18), 2506–2512 (2009).
