In the context of history, current smallpox vaccine development brings to mind the phrase, ‘déjà vu all over again’. The textbook by Fenner et al. reveals that scientists of 40 years ago, while successful in the eradication of smallpox, left unanswered the same questions we struggle with today: ‘what is protective immunity?’; ‘how long does immunity last?’; and ‘can we develop a safer smallpox vaccine?’ [1].

Despite our ability to understand human immune responses to viruses at an increasingly advanced cellular level, the elements of effective protective immune memory to viruses are often not well defined. For infectious diseases such as variola, where endemic disease is nonexistent, or others where field study is impractical, translation of immunological biomarkers to vaccine development remains a less than certain art. In this article, we review the current clinical development program by Bavarian Nordic A/S to develop a new strain of modified vaccinia Ankara (MVA) as a safer alternative to first-generation smallpox vaccines such as Dryvax® and the second-generation vaccine ACAM2000™.

Several vaccinia strains were widely used prior to the end of the compulsory smallpox vaccination program. In 1967, the WHO smallpox eradication unit surveyed 59 laboratories producing vaccinia for inoculation of humans: 51 harvested from the skin of calves, six from other bovines, three from the chorioallantoic membrane of chick embryo and three from tissue culture using bovine embryonic fibroblasts [1]. The strains included the Lister-Elstree strain (Lister Institute, UK; 39% of laboratories), New York City Board of Health (NYCBOH) (12%) and the Paris strain (12%), with the remaining laboratories using a variety of other strains (37%), including poorly characterized strains or a mixture of vaccinia and cowpox viruses [1]. Biological properties of a given vaccinia strain were known to be influenced by passage methodology but most of these vaccines had clinical efficacy against variola major. In 1963, the WHO established criteria and standards for the manufacture of vaccinia-derived smallpox vaccines, essentially reducing the derivatives of vaccinia used to three strains: Lister-Elstree, EM63 (Moscow Research Institute of Viral Preparation, Russia) and NYCBOH [2]. The WHO standards required a vaccine to produce major skin reactions in 95% of primary vaccinees and in 90% of those vaccinated 10 or more years previously, using an inoculum virus titer of $10^8$ plaque-forming units per milliliter [1]. The three aforementioned strains of vaccinia virus were considered equivalent in protecting against smallpox during outbreaks,

**Keywords:** adverse event • clinical trial • immunity • modified vaccinia Ankara • smallpox • vaccine • vaccinia

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and provided some protection when administered within 3–4 days of exposure [4]. Although highly effective immunizing agents, each of these vaccines produced significant adverse events [4–8]. The smallpox vaccines in current global stockpiles are derived from these historical vaccinia strains. The strains differ in their pathogenicity in animal models [9,10], and although never shown to be statistically correlated with adverse events, differences were noted in the incidence of adverse events in humans across strains [11,12]. These differences in safety profiles may also reflect changes in vaccination policy. Earlier vaccination of newborns was replaced in the late 1950s with vaccination after 1 year of age. This change significantly reduced serious adverse complications. Therefore, the exact comparator safety profile expected for a modern vaccine remains uncertain based on a number of population concerns, including prevalence of diseases that predispose risk (atopic dermatitis [AD] and immunosuppression), vaccinia strain, method used to inoculate, environmental risk factors and underlying health for those who are currently immunologically naïve to vaccinia. These concerns may alter predicted frequencies of certain adverse events based on historical data [12–15]. Recent experience during the smallpox vaccination campaign demonstrated that the frequency of most adverse events that were anticipated based on historical data were lower than expected [14,16]. By contrast, myopericarditis appeared more frequently than anticipated based on historical age-based data [14,17–20]. Whether differences in the age of those typically receiving their first inoculation with vaccinia, children (<5 years historically) versus the current vaccination programs (aged >18 years), have a role in the risk for myopericarditis is not known [15,19,21–23]. Clinical trials to date with ACAM2000 vaccine have not demonstrated definitive conclusions as to whether this serious adverse event (SAE) is more problematic with this second-generation smallpox vaccine.

Vaccinia strains may differ in the frequency of adverse events elicited following inoculation [12]. NYOBOH-derived strains (as compared with other replicating strains) have historically been associated with less pathogenicity and a safety profile marked by fewer adverse events, such as postvaccinal encephalitis (PVE) deaths or resulting permanent neurological disability [24]. PVE is a complication that is not associated with excessive viral replication and, therefore, could occur as a result of immunization with IMVA-MUNE®. A review of experience in humans comparing several different strains revealed that moderate-to-high pathogenic strains (Lister and Ikeda) produced more neurological events (including non-PVE) than low pathogenic strains (LC16m8, CVI and EM63), despite eliciting equivalent skin-take rates and seroconversion [24,25]. Although this secondary level of evidence suggested that less-pathogenic strains could provide improved safety without compromising efficacy, the incidence of SAEs, particularly PVE, were too rare to prove statistically significant differences between vaccinia strains. Current assessment of PVE would require clinical trials of the vaccine in millions of recipients in order to assess true differences between vaccine strains.

The attenuated vaccines date back to at least 1931, when an investigator at the Rockefeller Institute (NY, USA) described serial passage of the NYCBOH strain in chick embryo cells [26]. Two strains, CVI-78 and CVII, the latter carried for a total of 235 passages over chick embryo explants, were shown to be effective with reduced adverse events by intradermal inoculation in humans [27]. The CVII vaccine was tested in 60,000 Netherland’s Army recruits and had less local and systemic reactogenicity than other replicating vaccines. However, CVII vaccination resulted in lower neutralizing antibody titers compared with a Lister-inoculated comparator group, and led to the conclusion that, without subsequent challenge with lymph-derived vaccine and induction of a skin-pock lesion, CVII would be ineffective [3]. CVI-78 was resurrected in the early 1970s by the National Institute of Allergy and Infectious Diseases, US NIH, in a study that compared four vaccines: calf lymph- and egg-derived NYOBOH, CVI-78 and Lister. The results again demonstrated that, regardless of the route of administration, induction of neutralizing antibody titers for attenuated CVI-78 were less compared with Lister or NYOBOH (75 vs 93–96% of subjects) and, upon standard challenge vaccination, the CVI-78 recipients were more likely to have primary skin responses, an indicator of nonprotective immunity [5,8,28,29]. Several other attenuated strains, including Darian-derived, temperature-sensitive strains (LC16m8) and G-9, an attenuated variant of the highly pathogenic vaccinia stain Temple of Heaven, were tested in human studies. For the most part, there were improved immune responses compared with prior results observed with CVI strains, although detailed data on G-9 have never been published.

Modified vaccinia Ankara

Modified vaccinia Ankara originates from the dervish vaccinia strain Ankara (chorioallantois vaccinia virus Ankara [CVA]) derived from a pox lesion from a horse in Ankara, Turkey, and maintained in the Vaccination Institution Ankara [30–32]. Attenuated by over 570 continuous passages in primary chick embryo fibroblast (CEF) cells, MVA was used to protect live-stock against orthopoxviruses [33,34] and, later, tested as a pre-immunization to primary vaccination in human populations at risk of complications when using first-generation smallpox vaccines. Early indications that MVA could provide a safer immunogenic smallpox vaccine came from preclinical studies performed in irradiated, immuno-compromised rabbits that demonstrated generation of neutralizing antibodies and protection against vaccinia virus challenge [35]. The reduction in CVA virulence through passage on CEF was reported at passage 371 [36] and the strain was renamed MVA after passage 516 in order to distinguish it from other attenuated vaccinia virus strains [33]. Early clinical development of MVA as a pre-smallpox vaccine in Europe used MVA-517 (corresponding to the 517th passage) as a priming vaccine, followed by vaccination with Lister-Elstree. These studies included subjects at risk of adverse reactions from vaccinia. In 1976, MVA derived from the MVA-571 seed stock (corresponding to the 571st passage) was tested in approximately 120,000 individuals without any reports of SAEs. Several high-risk groups were vaccinated, including young children with skin conditions [37–39]. Rigorous follow-up of those vaccinated was not performed in that setting, as is currently the standard for investigational vaccine protocols.
IMVAMUNE is derived from a seed stock that corresponds to the 597th passage in CEF cells and has undergone several more rounds of limiting dilution compared with the historical MVA strain used in Germany. This MVA strain has multiple cloning sites and has been tested in humans as an experimental recombinant HIV and cancer vaccine at doses up to five-times higher than those used with the IMVAMUNE smallpox vaccine [40,41]. Bavarian Nordic has conducted an extensive preclinical development program that has demonstrated the safety, efficacy and bioequivalence of IMVAMUNE to first-generation smallpox vaccines (e.g., Dryvax and Lister-Elstree). The preclinical program has included a unique challenge model of intratracheal monkeypox and two murine orthopoxvirus challenge models. Human testing is currently ongoing, but data available to date, from Phase I and II studies, suggest that the vaccine can elicit both T- and B-cell immune responses, and the implications of how this compares to replicating vaccinia vaccines are discussed later [42,43].

IMVAMUNE properties
IMVAMUNE possesses interesting properties for a new generation of attenuated smallpox vaccines. IMVAMUNE differs from other MVA strains in that it has undergone extensive plaque purification in CEF cells, is propagated in serum-free conditions and, after passage 570, is a genetically stable virus [44–46]. The attenuation characteristics of this MVA separate it from other MVA strains [101]. Most MVA strains used in research are polyclonal and contain virus subtypes that are genetically similar to a replicating vaccinia strain. These subtype viruses, if virus host-range genes (e.g., KIL) are present and thus enable mammalian cell replication, could result in the emergence of phenotypes identical to replicating vaccinia. Culture replicating subtypes can theoretically emerge in vivo as replicating vaccinia virus, particularly when injected into immunocompromised animals [46,47]. However, rescue studies on host-range defects [48] and injection of MVA at 1000-times the lethal dose of vaccinia failed to induce death in severe combined immunodeficiency (SCID) mice [49]. Although it can be speculated that human use of such MVA variants can lead to the isolation of these virulent strains in vivo, to date this has not been observed in preclinical animal testing or human clinical trials involving multiple MVA-based vaccines. IMVAMUNE is replication deficient in mammalian cells and, in comparison to direct ancestral MVA strains, produces an acceptable safety profile in severely immune compromised animals [100].

Comparison of the genomes of IMVAMUNE and the ancestral vaccinia Ankara strain CVA demonstrates a loss of 15% of the genetic information (~31,000 bp of DNA), resulting from six major deletions in the MVA genome. Deletion of the host-range gene KIL and deletions of other virulence and host-range genes, including the gene for type A inclusion bodies, may partially explain the lack of replication in mammalian cell lines of IMVAMUNE [44,46,50]. Interestingly, even though IMVAMUNE exhibits attenuated replication in mammalian cells, efficient transcription of the viral proteins occurs within host cells and the functional result of the deletions appears to block virus assembly and egress [45,46,51,52]. This latter finding may have effects on comparative immune responses to first- or second-generation vaccines in which vaccinia virus assembly and egress occurs. However, notwithstanding the significant effect of genome deletion on attenuation and reduced virulence, preclinical studies using IMVAMUNE have demonstrated the ability to induce humoral and cellular immune responses to both vaccinia genes as well as cloned target proteins. Human cells infected with replicating vaccinia respond by induction of type I interferons (IFNs) and the expression of a soluble IL-1 receptor [47], a potential virulence factor for certain poxviruses [53,54]. Viral induction of type I IFNs and the expression of the receptor may lead to interference with human immune responses through molecular mimicry of cytokines and immune receptors. MVA does not express soluble receptors for IFN-γ, IFN-α/β, TNF or CC chemokines [50], which could influence comparative effects on innate immune responses [55] or the induction of stable long-term memory responses [47,56].

Manufacturing, potency & administration of vaccine
The formulation of IMVAMUNE used in clinical trials is a purified live vaccine produced under serum-free conditions in CEF cells, supplied as a sterile liquid-frozen preparation and does not contain adjuvants or preservatives. In preparing the vaccine, virus cell suspensions are homogenized by a process that includes a freeze and thaw, Benzonase® and ultrasound treatment. The bulk vaccine is adjusted to a titer of 2 × 10⁹ tissue culture infectious dose (TCID₅₀)/ml, filled into 2-ml injection vials and stored below -15°C as a frozen liquid product. Prior to administering the vaccine, the vial is thawed at room temperature and gently swirled for at least 30 s. The injection volume is 0.5 ml (1.0 × 10⁹ TCID₅₀) per dose and is administered as a subcutaneous injection. IMVAMUNE can also be effectively administered via intramuscular injection.

Stability tests are continuing but current preliminary data support stability for at least 2 years at -15°C. In reference to historical smallpox vaccine guidelines, the WHO’s efforts to develop freeze-dried vaccines (Dryvax, Lister and EM-63) were partially in response to the instability of vaccinia strains in liquid form during extended exposure to climate level temperatures. Long-term stability of IMVAMUNE is under evaluation. In addition, the vaccine, once thawed, must be administered immediately within 12 h; this further distinguishes it from Dryvax and ACAM2000, since both have extended post-thawing half-lives of 1–2 weeks. IMVAMUNE is not stable to refreeze once thawed and would require cold-chain logistics during an epidemic.

Summaries of nonclinical studies
The studies conducted for evaluating the safety prior to initiation of human trials included repeat administrations (subcutaneous and intramuscular) in animal models that demonstrated reversible nondose-limiting injection-site reactions and lymphoid changes. Injection-site expression levels of vaccinia by RT-PCR revealed only one weakly positive reaction at injection sites beyond day 3 (at day 7), suggesting no persistence of virus or genetic incorporation. Teratology studies in both rats and rabbits did not demonstrate
teratogenic or intraterine toxicity, and peri- and postnatal studies did not reveal any toxicity to embryos or developing offspring at doses up to $1 \times 10^8$ TCID<sub>50</sub>.

Three preclinical evaluations provide the primary support for safety and efficacy. To assess the attenuation profile of different strains of MVA, their growth was compared on CEF cells and across a number of mammalian cell lines. The strains included IMVAMUNE (MVA-597), MVA-572 (a 1970s smallpox vaccine used in Germany [57]), MVA-Vero (a MVA-575 strain adapted to Vero cell line [58]) and MVA-I721 (a MVA strain deposited at the Institute Pasteur [accession number I-721]). All four strains replicated in primary CEF cells but demonstrated significant differences in the ability to replicate in mammalian cells, with IMVAMUNE showing the near absence of replication across monkey (CV-1), rabbit (RK-13), murine (AG-101) and human (143-B, HeLa) mammalian cell lines [101]. These results suggest that MVA variants may exist in the MVA-572 and MVA-I721 parental lots. This conclusion is supported by studies inoculating immune-deficient mice (AGR129 strain), which led to in vivo replication and the emergence of altered genetic variants from these MVA strains but not from IMVAMUNE [59]. These results challenge the perception that all MVA strains are similar and suggest that lack of human cell replication, as observed with IMVAMUNE, represents a more stable clonal virus that could be uniquely suited to a human attenuated smallpox vaccine.

The second line of evidence for clinical attenuation was demonstrated using an immune-compromised mouse model inoculated with IMVAMUNE. AGR129 mice have gene deletions that inactivate the function of the IFN system (IFN receptor types I ($\alpha/\beta$) and type II [IFN-γ]) and inactivate the production of IFN-γ on a Rag<sup>−/−</sup> gene-deletion background), which prevents the formation of mature B and T cells. To evaluate IMVAMUNE, seven strains of vaccinia differing in virulence (IMVAMUNE, MVA-572, MVA-Vero, MVA-I721, smallpox vaccines Dryvax and Lister-Elstree, and a highly lethal vaccinia strain Western Reserve [WR]) were administered intraperitoneally at $1 \times 10^7$ TCID<sub>50</sub>. AGR129 mice that were inoculated with IMVAMUNE survived for more than 120 days with no replicating virus isolated from the ovaries (a marker of virus dissemination) at any time point. Animals inoculated with other MVA strains died or demonstrated virus-mediated disease at an average time to disease of 20 days (MVA-I721), 80 days (MVA-Vero) and 81 days (MVA-572) [101]. Recent reports of MVA providing protection against otherwise lethal poxvirus infection in Toll-like receptor-9-deficient mice suggests that MVA recognition and induction of immunity can occur through both Toll-like receptor-9-dependent and -independent pathways [60, 61]. Replicating vaccinia strains Dryvax, Lister-Elstree and WR killed all mice within 8 days of inoculation. The combined immunodeficiency mouse model provides evidence that MVA strains differ in their preclinical safety profiles and there is lack of mammalian cell replication with IMVAMUNE.

Preclinical efficacy was tested using two unique challenge models, employing both the Ectromelia virus (ECTV) and monkeypox virus (MPXV) [43]. Earlier animal studies demonstrated that IMVAMUNE induces an equivalent humoral and T-cell immune responses in mice compared with first-generation smallpox vaccines (Dryvax and Elstree) [42, 62, 63]. In the ECTV challenge model, a single dose of IMVAMUNE protected mice from intranasal challenge with a lethal dose of VV-WR or ECTV that was given 6 weeks after vaccination. Mice challenged 3 days after vaccination with WR virus were also protected, consistent with previous studies using first-generation vaccines and variola or vaccinia challenge [64]. In the nonhuman primate model of intratracheal delivery of MPXV, challenged animals vaccinated with IMVAMUNE were completely protected, with the exception that one animal developed skinpox lesions. Viral loads were even more diminished if IMVAMUNE was followed by conventional inoculation with the Elstree vaccine prior to the challenge. The overall clinical response to vaccination with IMVAMUNE was equivalent to first-generation smallpox vaccines [43]. These challenge studies provide important efficacy data in that the MPXV model mimics natural variola infection, producing a smallpox-like disease (i.e., fever, skin lesions and fibrinonecrotic broncho pneumonia); although 100% of placebo-treated animals die [43], which is in contrast with the lower mortality in human smallpox [65].

**Clinical experience with IMVAMUNE**

IMVAMUNE, as a third-generation smallpox vaccine for special populations at risk of SAEs from replicating vaccinia, must demonstrate a safety profile that succeeds at eliminating common complications associated with replicating vaccinia-based vaccines. For instance, accidental spread of virus to close contacts is not probable with IMVAMUNE due to the lack of replication in human cells and intramuscular inoculation eliminating the development of skinpox lesion. The occurrence of eczema vaccinatum and progressive vaccinia also seem unlikely but these assessments will require continued monitoring in clinical trials and beyond. Assessment of the occurrence of PVE will also require long-term monitoring. The rarity of both PVE and myopericarditis requires large numbers of volunteers to receive IMVAMUNE in order to achieve statistically superior safety assessment over first- or second-generation vaccines. Overall, any comparison trial would require a treatment arm receiving a replicating vaccine. Use of a replicating vaccinia vaccine as part of a trial design to compare adverse events is not acceptable for these special populations.

Historical data using the MVA vaccine in over 120,000 individuals during the 1970s provide encouraging evidence that MVA as a smallpox vaccine can be safely administered to humans. In 1976, MVA was authorized in Germany as a preimmunization vaccine in combination with Lister vaccine. This two-step inoculation program was thought to diminish potential adverse events, particularly PVE. More than 120,000 subjects received intradermal and subcutaneous injections with a low dose of MVA (1 × 10<sup>6</sup> TCID<sub>50</sub>) before the mass-vaccination program was discontinued [37]. Prior clinical trials described the safety and tolerability of the vaccine and served as the basis for licensure in Germany [66–68]. These studies included data on 7098 subjects (5691 aged <3 years and 1407 aged >3 years). Reactions at the injection site revealed no blistering, pustules or ulcerations, and no cases of PVE or other SAEs were observed. Reactogenicity was limited to approximately
4% with mild fever (>38°C; 2.28%) and approximately 4% with nonspecific systemic symptoms. However, detailed immunogenicity was never sufficiently tested. Administering MVA prior to first-generation smallpox vaccine reduced the frequency of adverse events from the replication-competent vaccinia virus. By contrast, with IMVAMUNE, single-dose vaccination with this earlier CEF-derived MVA elicited only a weak hemagglutinin inhibition and virus-neutralizing antibody titers. MVA prevaccination resulted in a stronger booster effect after vaccination with a replicating vaccinia strain, indicating that priming with MVA induced specific humoral and cellular immune responses.

**Human safety of IMVAMUNE**

To date, more than 2000 subjects have been vaccinated with either IMVAMUNE or recombinant MVA-BN®-based vaccines, including at-risk populations with AD or HIV infection. Tables 1 & 2 provide an overview of ongoing and completed IMVAMUNE clinical studies. The use of MVA-BN-based vectored vaccines in clinical trials is not reviewed here, since these vaccines represent altered viruses and, therefore, comparison and interpretation of the safety and tolerability data can be complex and problematic. In summary, however, administration of MVA-BN-vectored vaccines in HIV-infected subjects has not been associated with dissemination or any severe systemic or neurological events attributable to vaccinia.

**Adverse vaccine reactions**

Clinical trials of IMVAMUNE in subjects over 18 years of age have included HIV-infected subjects and individuals with a history of AD, or active AD. The frequency of reactions occurring in the 1025 subjects enrolled in the completed IMVAMUNE clinical trials is shown in Table 3. Summaries to date suggest that the majority of the events observed in these trials have been classified as being mild to moderate and resolving without sequelae. Reported local and systemic reactions are consistent with prior observations with MVA [69].

Of particular interest are adverse events occurring in special populations with AD (POX-MVA-007 and -008) and HIV-1 infection (POX-MVA-010 and -011), which are two populations contraindicated for first- and second-generation smallpox vaccines. POX-MVA-007 was a Phase I clinical trial that used two injections of IMVAMUNE in adults aged 18–40 years. A total of 60 subjects were enrolled: 15 patients with mildly active AD and 16 patients with a history of AD were compared with healthy subjects (n = 15) and subjects with allergic rhinitis (n = 14). No SAEs or premature study withdrawal events have been reported. Mild-to-moderate transient pain and redness at the inoculation site occurred in nearly all subjects and no differences in the incidence of grade 3 and above adverse reactions during the immediate postvaccination period were observed between AD and healthy controls. A larger open-label comparative (AD vs no AD) trial, POX-MVA-008, is currently ongoing and will recruit up to 530 individuals aged between 18 and 40 years in the USA and Mexico. In trials POX-MVA-010 and -011, the objectives were to compare safety and immunogenicity data in HIV-infected subjects with uninfected subjects. Study POX-MVA-010 included 91 subjects with HIV (30 naive and 61 experienced to vaccinia). To date, there have been no reports of SAEs in the published Phase I studies [70,71]. The company will soon publish complete safety data from the completed larger cohort studies (Chaplin P, Pers. Comm.), which limits their summary in this report. A recombinant BN-MVA containing the HIV nef protein construct has been administered to 14 HIV-infected patients (CD4 > 400/µl), with only mild systemic reactions noted [40].

**Special interest adverse events (cardiac events)**

Due to recent clinical trials with ACAM2000 and Dryvax and First Responder Vaccination Programs employing Dryvax, there is additional concern regarding the association of vaccinia inoculation and the development of myopericarditis after vaccination [17,72–74]. Myocarditis and pericarditis as sequelae of viral infection has, for some viruses, been shown to be autoimmune related and generally occurs in later teen years or young adulthood [22,75,76]. Whether the incidence of vaccinia-related

| Table 1. Summary of completed human trials of IMVAMUNE® attenuated smallpox vaccine. |
|-----------------------------------|-----------------|-----------|----------|--------|--------|
| Study                            | Population      | Dose (route) | Dose of IMVAMUNE | n | GMT* (% positive) | nAb‡ (% positive) |
|-----------------------------------|-----------------|-----------|------------------|---|------------------|------------------|
| POX-MVA-001§ Vaccinia naive       | 10⁶ (sc.)       | 2         | 18               | 39 | 33               |
|                                     | 10⁷ (sc.)       | 2         | 16               | 81 | 50               |
|                                     | 10⁸ (sc.)       | 2         | 16               | 100| 80               |
|                                     | 10⁸ (im.)       | 2         | 18               | 100| 87               |
| Nonvaccinia naive                  | 10⁴ (sc.)       | 1         | 18               | 100| 89               |
| POX-MVA-002§ Vaccinia naive       | 2 × 10⁷ (sc.)   | 2 + Dryvax | 15               | 100| 100              |
|                                     | 5 × 10⁷ (sc.)   | 2 + Dryvax | 15               | 100| 93               |
|                                     | 1 × 10⁸ (sc.)   | 2 + Dryvax | 15               | 100| 87               |
|                                     | Placebo × 2     | 2 + Dryvax | 15               | 84 | 85               |
|                                     | 1 × 10⁸ (sc.)   | 2 + placebo| 15               | 100| 92               |
|                                     | 1 × 10⁸ (im.)   | 2 + Dryvax | 15               | 100| 100              |

*Measuring total anti-MVA IgG by ELISA, percentage responding 14 days after last dose of IMVAMUNE or 14 days after Dryvax alone.

‡Conversion percentages are based on plaque-reduction neutralization titer GMT titer approximately 30 days after last dose.

§See clinical trial [71,72].

GMT: Geometric mean titer; im.: Intramuscular; MVA: Modified vaccinia Ankara; NA: Data not available in peer-review published or reported form; nAb: Neutralizing antibody; sc.: Subcutaneous.
myopericarditis is indeed more frequent than recognized in the past as a consequence of changing vaccination patterns using older subjects is subject to debate. Recently reported incidence rates for developing myopericarditis following vaccination with the first-generation smallpox vaccine Dryvax (10.38 events per 1000 vaccinations) have been a surprise and are alarming [7,78]. Historical data related to myopericarditis must also be interpreted carefully as those studies were prior to the clinical use of echocardiograms and cardiac-specific enzymes. Adverse events from published clinical trials using IMVAMUNE and MVA-BN-based recombinant vaccines have not reported a case of myopericarditis. Further evidence supporting the cardiac safety of IMVAMUNE was obtained from a placebo-controlled Phase II study in healthy individuals (POX-MVA-005), which intensively monitored the risk of myopericarditis developing after vaccination with IMVAMUNE. There have been no cases of myopericarditis reported for this study (Chaplin P. Pers. Comm.).

Immune response to IMVAMUNE in healthy individuals

In order to delineate what is known to date regarding IMVAMUNE immune responses, current and completed clinical trials can be broken down into vaccinia-naive or -experienced subjects with or without either AD or HIV.

| Study         | Population                        | Dose (route) | Doses | n   |
|---------------|-----------------------------------|--------------|-------|-----|
| POX-MVA-004   | Vaccinia-naive                    | 2 × 10^7 (sc.) | 2     | 55  |
|               |                                   | 5 × 10^7 (sc.) | 2     | 55  |
|               |                                   | 10^7 (sc.)    | 2     | 55  |
| POX-MVA-005   | Vaccinia-naive                    | 10^6 (sc.)   | 2     | 183 |
|               |                                   | 10^6 (sc.)   | 1 + placebo | 181 |
|               |                                   | 10^6 (sc.)   | Placebo | 181 |
| Nonvaccinia-naive |                              | 10^6 (sc.)   | 1     | 200 |
| POX-MVA-007   | Vaccinia-naive                    | 10^6 (sc.)   | 2     | 15  |
|               | History of AD                     | 10^6 (sc.)   | 2     | 16  |
|               | Mild active AD                    | 10^6 (sc.)   | 2     | 15  |
|               | Mild allergic rhinitis            | 10^6 (sc.)   | 2     | 14  |
| POX-MVA-008   | Vaccinia-naive                    | 10^6 (sc.)   | 2     | 230 |
|               | AD, vaccinia-naive                | 10^6 (sc.)   | 2     | 300 |
| POX-MVA-010   | HIV-infected naive                | 10^6 (sc.)   | 2     | 30  |
|               | HIV-infected non-naive            | 10^6 (sc.)   | 1     | 61  |
|               | Naive                             | 10^6 (sc.)   | 2     | 30  |
|               | Non-naive                         | 10^6 (sc.)   | 1     | 30  |
| POX-MVA-011   | Naive                             | 10^6 (sc.)   | 2     | 90  |
|               | HIV+ CD4 200–750                  | 10^6 (sc.)   | 2     | 360 |

AD: Atopic dermatitis; MVA: Modified vaccinia Ankara; sc.: Subcutaneous.

POX-MVA-001, -002, -004 & -005

These four trials represent the initial IMVAMUNE trials in adults. Tables 1 & 2 depict the design of each of these trials in terms of groups, dose tested, prior vaccinia status and numbers tested. POX-MVA-002 was independently conducted by the National Institute of Allergy and Infectious Diseases; Bavarian Nordic sponsored the others. Tables 1 & 2 presents an incomplete summary of the humoral and cellular responses reported to date. We are only able to report complete data for two trials: POX-MVA-001 and -002. Historically, a plaque-reduction neutralization titer (PRNT) greater than 1:40 is considered a positive humoral response. In Table 4, comparative IFN-γ-ELISPOT (>15 spots/million peripheral blood mononuclear cells [PBMCs] considered positive) using a standardized assay across all vaccines depicted offers an assessment of the T-cell responses observed with IMVAMUNE compared with that observed with first-generation Dryvax, experimental cell-cultured smallpox vaccine and ACAM2000 [78–82]. This assay was employed across clinical trials of smallpox vaccines, utilizing the same reagents, assay controls and methods, thus enabling comparison of IFN-γ T-cell responses across vaccine candidates [70,79–82].

In the POX-MVA-001 trial, 86 male subjects aged between 18 and 55 years were vaccinated and stratified based on the presence or absence of a prior history of smallpox vaccination. The first four dosing cohorts were naïve to smallpox vaccination and received the vaccine on days 0 and 28. A fifth cohort of subjects, who had been previously vaccinated against smallpox, received the vaccine on days 0 and 18. Seroconversion rates for developing myopericarditis following vaccination with IMVAMUNE. There have been no cases of myopericarditis reported for this study (Chaplin P. Pers. Comm.).
observed with Dryvax and ACAM2000 or in the POX-MVA-002 trial, which directly compared IMVAMUNE with Dryvax. These early results, from POX-MVA-001 and POX-MVA-002, raise the interesting question as to whether some degree of replication may be an important determinant for neutralizing antibodies [70,80–84]. This premise is supported by the work of others demonstrating that various forms (extracellular) of vaccinia virus are typically generated after infection of human cells. These forms are important in the generation of hemagglutinin and neutralizing antibodies to the virus, which may be critical early in protective immunity [85–87]. Further work is needed to clarify this issue.

A peak CMI response was reached approximately 2 weeks after the second vaccination (day 42) for groups 1–4 in the POX-MVA-001 trial. While a cell-mediated response was detected in five subjects with pre-immunity on day 0 (group 5), those subjects showed a booster response on day 28 following a single vaccination with IMVAMUNE, with a mean T-cell count of 109 cells per million PBMCs. This would indicate that IMVAMUNE was able to stimulate the memory T-cell response induced by a previous replicating smallpox vaccination. In vaccinia-naive subjects, dose responses were observed, with better responses being recorded using the higher doses of IMVAMUNE. At the highest dose (groups 3 and 4), the cell-mediated responses measured on day 42 were in the same range (102 and 152) as subjects with pre-existing immunity (group 5) on day 28. A correlation analysis showed that the T-cell response measured in the ELISPOT was highly correlated to the results of the PRNT and ELISA antibody tests (data not shown) [70,71]. These Phase I results confirm dose-finding data from the study POX-MVA-004 (see later), which demonstrated that a dose of 1 × 108 TCID50 IMVAMUNE was well tolerated and the most immunogenic dose evaluated in healthy subjects [70].

Two larger multicenter Phase II clinical trials in subjects with AD (POX-MVA-008) or HIV-1 infection (POX-MVA-011) are underway to evaluate the immunogenicity and safety of two doses of 1 × 108 TCID50 of IMVAMUNE subcutaneously, given 1 month apart. The primary study objective of the POX-MVA-008 trial is to assess the humoral immune response (measured by ELISA) induced by IMVAMUNE in subjects with AD and, in the POX-MVA-011 trial, safety of the vaccine in HIV-infected subjects. Data on the POX-MVA-011 trial is expected later this year.

Table 3. Comparison of IMVAMUNE®/ACAM2000™/Dryvax® adverse events (possibly related) occurring in at least 5% of trial subjects.

| Adverse event characterization | IMVAMUNE (n = 1025) (n; %)* | ACAM2000 (n = 873) (n; %)† | Dryvax (n = 289) (n; %)‡ |
|------------------------------|-----------------------------|---------------------------|-------------------------|
| **Blood and the lymphatic system disorders** | | | |
| Lymphadenopathy | 13 (1) | 72 (8) | 35 (12) |
| Lymph node pain | 1 (0.1) | 494 (57) | 199 (69) |
| **Nervous system disorders** | | | |
| Headache | 280 (27) | 433 (50) | 150 (52) |
| **Respiratory, thoracic and mediastinal disorders** | | | |
| Dyspnea | 0 (0) | 39 (4) | 16 (6) |
| **Gastrointestinal disorders** | | | |
| Nausea | 105 (10) | 170 (19) | 65 (22) |
| Diarrhea | 8 (1) | 144 (16) | 34 (12) |
| Constipation | 0 (0) | 49 (6) | 9 (3) |
| Vomiting | 1 (0.1) | 42 (5) | 10 (3) |
| **Skin and subcutaneous tissue disorders** | | | |
| Erythema | 1 (0.1) | 190 (22) | 69 (24) |
| Rash | 3 (0.3) | 94 (11) | 30 (10) |
| **Musculoskeletal, connective tissue and bone disorders** | | | |
| Myalgia | 103 (10) | 404 (46) | 147 (51) |
| **General disorders and administration-site conditions** | | | |
| Injection-site erythema | 827 (81) | 649 (74) | 229 (79) |
| Injection-site pain | 887 (87) | 582 (67) | 208 (72) |
| Injection-site pruritus | 211 (21) | 804 (92) | 277 (96) |
| Injection-site swelling | 692 (68) | 422 (48) | 165 (57) |
| Fatigue | 316 (31) | 423 (48) | 161 (56) |
| Malaise | 5 (0.5) | 327 (37) | 122 (42) |
| Rigors | 31 (3) | 185 (21) | 66 (23) |
| Exercise tolerance decreased | 0 (0) | 98 (11) | 35 (12) |
| Feeling hot | 1 (0.1) | 276 (32) | 97 (34) |

*Summary of published and unpublished data from incompleted and reported IMVAMUNE clinical trials.
†Prescribing information for ACAM2000, August 2007.
‡Modified from [91].

**Expert commentary**

Derived from MVA, IMVAMUNE is a highly attenuated strain of vaccinia that is unable to replicate in human cells and, therefore, cannot be transmitted or cause dispersed vaccinia infection. The extensive nonclinical development has shown the vaccine to

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induce protective immunity in two important animal challenge studies: immune-compromised mice and primates challenged with MPXV. The issue of durable immunogenicity, both humoral and cellular, requiring the need for repeat inoculations to boost responses, raises some concerns and challenges to develop a best-use strategy during an outbreak scenario. In addition, patients with AD inoculated with vaccinia represent a unique population with altered skin host-defense mechanisms [88], and how these alterations might affect immunogenicity of IMVAMUNE remains to be determined. Finally, MVA as a vaccine strain does not produce the typical skinpock lesion, which eliminates a useful marker of successful vaccination for field assessment of protection during outbreaks.

Recently, antibody profiling across species (including humans) for MVA, Dryvax and WR strains demonstrated that binding antibodies to select structural vaccinia proteins were similar across species [53]. Although this study has some limitations with respect to the panel of vaccinia proteins selected, the data demonstrate remarkable similarity of binding profiles, suggesting that the 3% genetic variation observed between MVA and Dryvax may not significantly alter the repertoire of humoral immune response. However, the deletion mutations of specific proteins in IMVAMUNE could alter the magnitude of protective levels of humoral or cellular immunity, the ability to neutralize variola major and the durability of response. These issues will require further study focusing on the site-directed nature of the neutralizing antibody response. Of concern remains the lower neutralizing geometric mean titer in MVA-vaccinated individuals and whether this reflects altered response to early vaccinia antigens, which others have suggested may be directed to specific vaccinial proteins: B5R, D8, A27, D13 and A14 [54,55]. Development of third-generation smallpox vaccines that target the key antigens required for neutralization of vaccinia forms intracellular mature virus and extracellular mature virus are new candidates entering human testing and may provide a unique boost combination with IMVAMUNE. Such boosting may ultimately contribute to more durable neutralizing antibody titers and T-cell memory. Of course, all of the previous commentary on immunogenicity and durability really depends on IMVAMUNE protection against variola; only studies or experience with variola will reveal this answer. So far, there is the expectation that IMVAMUNE will be effective.

### Five-year view

To date, more than 1900 subjects have been vaccinated with IMVAMUNE and recombinant MVA-BN-based vaccines; no cases of myopericarditis have been observed. Therefore, these early clinical studies suggest that IMVAMUNE may offer a safer cardiac profile, that is, have a much lower rate of cardiac events compared with Dryvax and ACAM2000. Whether the unique absence of replication in human cells translates to an improved safety profile remains to be determined by expanded clinical testing and usage. In addition, the immunogenicity data generated to date suggest that both humoral and cellular immune responses are lower, particularly following one dose. Future considerations with regards to IMVAMUNE include recent development and testing of smallpox vaccines based on DNA plasmids and recombinant protein vaccines, enabling higher antigenic content and use of adjuvants to boost neutralizing antibodies to targeted vaccinia proteins. The role of IMVAMUNE may emerge as a prevaccination, which could be subsequently boosted with newer vaccine designs. Although the limited data thus far suggest two doses of IMVAMUNE induces sufficient antibody and cellular immune responses in the immediate vaccination period, the decay of the response compared with replicating smallpox vaccines needs further study to support IMVAMUNE use as a vaccine that would provide 3–5 years of protection. This concern is supported by recent data comparing three nonreplicating smallpox vaccines in mice that failed to demonstrate long-term (150 days postvaccination) protection against intranasal challenge with cowpox virus [89].

### Table 4. Comparison of cell-mediated immunity induction for smallpox vaccines using a standardized IFN-γ-ELISPOT assay in human trials in naive subjects.

| Vaccine name and study* | n | Vaccine titer | Route | (+) ELISPOT (%) | Mean SFCs per 10⁶ PBMC 30 days post first dose | Mean SFCs per 10⁶ PBMC 30 days post second dose |
|-------------------------|---|---------------|-------|------------------|-----------------------------------------------|-----------------------------------------------|
| Dryvax* † | 90 | 1 × 10⁸ PFU/ml | id. | 98 | 402 | NA |
| IMVAMUNE* | 21 | 1 × 10⁸ TCID₅₀ | im. | 95 | 100 | 286 |
| ACAM2000™ | 30 | 1 × 10⁸ PFU/ml | id. | 99 | 442 | NA |
| ACAM1000 | 30 | 1 × 10⁸ PFU/ml | id. | 100 | 331 | NA |
| CCSV | 40 | 1 × 10⁷ PFU/ml | id. | 99 | 251 | NA |
| Controls§ | 10 | Naive controls | | 0 | 5.8 | 4.1 |

Variation of response was 0–15 SFC for a given well run in triplicate. Value is the mean across five studies in the table, except for 30 days post-second dose, where data are mean for IMVAMUNE study alone.

*Studies cited are IMVAMUNE [71], ACAM1000 [81], CCSV [80] and ACAM2000.

†Dryvax® (n) data: all subjects comparatively injected with Dryvax in the studies of experimental vaccines in the table.

§Nonimmunized controls were used in the assay. One donor was used for every three plates. Summary data is for all studies and represents repeated measures for ten donors.

CCSV: Cell-cultured smallpox vaccine; id.: Intradermal by bifurcated needle; im.: Intramuscular injection; NA: Not assessed (only one dose administered); PBMC: Peripheral blood mononuclear cell; PFU: Plaque-forming unit; SFC: Spot-forming cell; TCID: Tissue culture infectious dose.
Perhaps a schedule that includes periodic IMVAMUNE booster vaccinations will solve this issue. On the other hand, in a recent nonhuman primate study, MVA did prevent infection with vaccination 4 days prior to a monkeypox challenge, while Dryvax required vaccination 6 days prior to challenge to be effective [60,90]. These primate-challenge models emphasize differences between MVA and replicating vaccinia smallpox vaccines that need to be considered in the strategies for human use in the pre- and postexposure settings.

Finally, we believe that the IMVAMUNE program is well guided, providing the necessary information needed to evaluate the vaccine for US FDA approval for special populations in an emergency.

Key issues

- IMVAMUNE®, an attenuated vaccinia strain, has been shown not to replicate in human cell lines, reducing the risk of transmissibility to close contacts—a key new safety advantage.
- The vaccine is a modern cell culture-derived vaccine, developed for use in special populations at risk of complications from replicating vaccinia-based smallpox vaccines.
- In 2007, a report of Phase I studies comparing immunogenicity of IMVAMUNE to Dryvax revealed that at dose of 1 × 10⁷ TCID₅₀ IMVAMUNE could elicit robust humoral and cellular immune responses, albeit lower than Dryvax. The duration of protective antibody and T-cell responses are unclear and requires further evaluation in both low- and high-risk populations.
- Numerous registration trials in special at-risk populations (atopic dermatitis and HIV-1 infected) are ongoing or planned, and key data from these trials are expected in 2009.
- To date, in limited small trials, IMVAMUNE has been well tolerated and no myocardial events have been reported.
- Cold-chain and two-dose requirements with respect to IMVAMUNE may result in logistic problems during outbreak scenarios.
- The important unfilled niche in the supply of smallpox vaccine in the event of a bioterrorism act is to protect populations for which replicating vaccinia-based vaccines are contraindicated. IMVAMUNE based on replication-deficient modified vaccinia Ankara provides an attenuated smallpox vaccine for these special populations. Such a vaccine should strive for single-dose immunogenicity comparable to first-generation vaccines, a reasonable duration of protective immunity, as well as a superior safety profile. As additional safety and immunogenicity data from IMVAMUNE clinical trials become available, the capabilities and role for IMVAMUNE will be resolved.

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