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Smallpox vaccines
New formulations and revised strategies for vaccination

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Smallpox has been eradicated but stockpiles of the causative infectious agent, variola virus, have been maintained over decades. Today, the threat of accidental or intentional poxvirus release is accompanied by the fact that the existing licensed smallpox vaccines cause rare but severe adverse reactions yet are the only products with approved efficacy against smallpox. New safer vaccines and new strategies of immunization are to be developed to fit to a scenario of emergency smallpox vaccination. However, we still lack knowledge about the pathogen and the mechanisms involved in acquiring protective immunity. Here, we review the history of smallpox vaccines and recent achievements in the development of highly efficacious and safer vaccines and vaccine applications. These include (1) assessment of adequate animal models to study pathogenesis and protective immunity, (2) characterization of the immunity elicited by next generation vaccines, and (3) the investigation of the requirements for rapidly protective vaccination.

Introduction

Smallpox is a human disease caused by *Variola virus* (VARV), a virus species within the genus *Orthopoxvirus* of the large poxvirus family. Smallpox has been, throughout the history, the most prevalent pandemic infectious disease with an enormous mortality worldwide. It is estimated that until the 18th century in Europe around one person in ten died of smallpox. In 1980 the World Health Organization (WHO) announced that the naturally occurring disease Smallpox has been eradicated through a worldwide vaccination campaign with *Vaccinia virus* (VACV) that still today is likely the most successful public health measure of medicine.[1] In the following years all known stocks of VARV, about six hundred from all parts of the world, were destroyed or deposited in two centers in the USA and in Russia. Currently, the Centers for Disease Control and Prevention in Atlanta, GA, and the State Research Center of Virology and Biotechnology in Novosibirsk, Russia, are the only WHO collaborating centers that maintain and work with VARV in defined research projects under Biological Safety Level 4. Indeed, our knowledge about the molecular mechanisms of smallpox pathogenesis is very limited and further studies might be needed to develop new effective antivirals and vaccines. However, the value of research with the smallpox virus is debated because there are extreme limitations to study VARV infections in vitro and in vivo and repeatedly, the call is made for finally destroying the stocks.[2] Along with the successful eradication campaign, population wide vaccinations with VACV were gradually discontinued. A consequence of this was that the great part of the world's populations is now again susceptible to infections with orthopoxviruses. This fact together with recent assumptions that VARV or another poxvirus pathogenic for humans might be used as a bioweapon raised the awareness that human poxvirus infections might reemerge which resulted in an increased interest in the disease and it's countermeasures.

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Poxvirus Infections of Humans

Members of the family *Poxviridae* contain large genomes of double-stranded DNA (in the range from 130,000 to >300,000 nucleotides) and infect vertebrate (*Chordopoxvirinae*) and insect (*Entomopoxvirinae*) hosts. Humans can be infected with various poxviruses from the genera *Orthopoxvirus*, *Parapoxvirus*, *Yatapoxvirus* and *Molluscipoxvirus*. The orthopoxvirus VARV, the causative agent of smallpox, and the Molluscum contagiosum virus are the only poxviruses that infect only humans. Human infections with other poxvirus species including the orthopoxviruses Vaccinia virus (VACV), Cowpox virus (CPXV) and Monkeypox virus (MPXV) are zoonoses (reviewed in ref. 3).

Of the human pathogens, the species VARV is the most contagious and virulent. The obligatory infection of human hosts together with the efficient protective immunity acquired by immunization, were the prerequisites for the successful eradication of VARV without the need to eliminate the virus from natural animal reservoirs. Smallpox is a systemic febrile—rash disease with a mortality rate of about 30% following natural infections. Both upper respiratory tract and skin/contact infections of humans with VARV are preceded by a rather long incubation time of about 14 days (7–17 days), followed by sharp transient increase in body temperature and other symptoms including backache, headache, vomiting and prostration. Several days (3–4) after the onset of fever, centrifugal and synchronized rash develops throughout the skin developing from macular to papular rash. The synchronized macular to papular rash serves

as a first diagnostic marker of smallpox and human monkeypox. In most cases, rash healed after several days leaving notable pox signs on the skin.[4,5] In the common (about 90% of the cases) ordinary smallpox as categorized by the WHO, case mortality positively correlated with the rash extent (10% to 80% fatality rates, mean 30%). Other less common forms of VARV infection included hemorrhagic disease peaking in about one weak (100% fatality rate) and flat smallpox, a more slowly developing disease course with high fatality rates (>90%).[5-7] Neither the exact cause of death in human smallpox is known yet, nor are the mechanisms underlying the development of the various forms of the disease. This is mainly due to the fact that smallpox was eradicated about 30 years ago, before sufficient medical information on the factors affecting disease severity could be collected. However, the involvement of virus induced immune modulation and immune pathogenesis ("cytokine storm") were suggested to contribute to disease severity, relying on data from animal models for poxvirus infections.[8-10]

The zoonotic disease monkeypox is considered as the most important poxvirus infection in humans since the eradication of smallpox. It is caused by MPXV, a natural pathogen of African rodents being discussed as potential agent of bioterrorism. Unlike the host restricted VARV, MPXV exhibits a broad species specificity and can cause fulminant disease in various animal species including dormice, squirrels, prairie dogs, non-human primates and humans.[11-13] Outbreaks of human monkeypox were reported in Africa (1970–1986, 1996–1997) and in the USA (2003).[14,15] Human monkeypox can be misidentified as smallpox due to the disease pattern with rash and clinical manifestations resembling discrete ordinary-type smallpox and the presence of poxvirus markers (virion morphology, antigen composition) that are indistinguishable by many commonly used methods in diagnosis. Clear discrimination between MPXV and other orthopoxviruses is possible by the use of nucleic acid amplification technologies that detect specific differences in the genomic DNA sequence of the viruses.[16-20] The human infection dose of MPXV is not known. Yet, human monkeypox is believed to result from either respiratory, percutaneous or permucosal exposures. Severe cases occur, and the disease in humans can be fatal (for some outbreaks mortalities >10%). Overall, since the 1970s, reported monkeypox cases in humans have increased, as have outbreaks with reported human-to-human transmission.[15]

Cowpox virus (CPXV) is the orthopoxvirus species endemic to Eurasia and represents a variety of viruses that are natural pathogens of rodents, mainly suggested by epidemiological data. CPXV can infect and cause disease in a very broad range of host species including various mouse and vole species, domestic cats, horses, zoo animals (elephant, rhinoceros, okapi, cheetah), and man. In apparent agreement, in comparison to other orthopoxviruses CPXV genomes encode the broadest set of viral genes to regulate virus-host interactions and might enable CPXV to more efficiently evade the immune control and to more easily adapt to different species[21-23] yet, the contribution of each of those regulatory genes to virulence in the different hosts remains elusive. Human CPXV infections result from contact with diseased animals and are mostly confined to local skin or eye infections (often spread by auto-inoculation). Nevertheless, generalized infections with fatal outcome can occur in immunocompromised individuals and render this virus a potentially harmful human pathogen.[24,25]

Vaccinia virus (VACV) is often confused with CPXV but represents a clearly distinct species in the genus *Orthopoxvirus*. This mix-up evolved from the report of Jenner that he used Cowpox for vaccination against smallpox.[26] Yet, in the 20th century it became known that all available smallpox vaccines were based on VACV[27,28] and whether the virus used by Jenner was a member of the Vaccinia or CPXV species remains elusive. Various strains of VACV have been used to vaccinate against smallpox, other strains have been established as more virulent laboratory viruses such as VACV Western Reserve and VACV IHD-J. VACV inoculation of unimmunized individuals (e.g., either by vaccination or through laboratory accidents) usually results in local infections of the skin or the eye. The disease pattern closely resembles the one observed with local human CPXV infections. However, VACV infection of at-risk (e.g., immunocompromised) individuals, to whom vaccination is contraindicated, may lead to severe disease which might even be fatal.[3,29-32]

Smallpox Vaccine

Throughout history, smallpox recurred in devastating epidemics and caused millions of mortalities worldwide. In the 10th century reports from China describe the first attempts to control the disease by immunization. Hereby, scab material from VARV infected patients was applied by intranasal or dermal inoculation to naïve individuals, a process known as "variolation" or "inoculation". The success rate of those practices is uncertain yet development of smallpox as a result of the inoculation was reported.[5]

The outstanding discovery by Edward Jenner published at 1796 was suggesting a possible linkage between the presence of skin and mucosal lesions on cows and on the hands of their caretakers, and the low percentage of smallpox between those caretakers. By skin exposure of young children to liquid recovered from those lesions, he eventually showed that the children were protected from a subsequent challenge with liquid from smallpox lesions. This finding of cross protective immunization among orthopoxviruses led to the invention of the 1st Vaccine—the smallpox vaccine.[26] Sometime between 1796 and the 20th century VACV became the vaccine strain and was used in the massive worldwide vaccination campaign, coordinated by the WHO. The campaign was the 1st and the so far only vaccination campaign which allowed for the eradication of a pandemic disease—smallpox.[5]

The Different Generations of Vaccines

Numerous VACV strains with different biological properties served as first generation vaccines for immunization against smallpox. These viruses were fully replication competent and more or less virulent in man.[5,33-35] Vaccines based on VACV strains Lister/Elstree, New York City Board of Health (NYCBH), EM-63 and Tian-Tan were preferentially used during the smallpox

eradication campaign because of a better safety record than other vaccines based on VACV strains Copenhagen or Bern.[5] These first generation vaccines were produced by several countries on various tissues: e.g., Lister based vaccines were produced on chick chorioallantoic membranes while NYCBH was propagated on calf or water buffalo skin and stored either as wet frozen vaccine or later on as dried stock (Dryvax). Vaccine production was gradually discontinued as the disease was eradicated.[5,33,36,37] The understanding that intentional use of VARV might be possible raised the need for vaccination of first responders and laboratory personnel, as well as to renew vaccine stockpiling.

The historical methods to generate smallpox vaccines cannot accommodate modern guidelines for production of vaccines for human use, leading to development of 2nd generation vaccines (**Table 1**). Those vaccines utilize the same historical vaccine strains Lister and NYCBH with defined manufacturing processes (e.g., Elstree-BN produced from the Lister/Elstree strain by Bavarian-Nordic, Germany and ACAM2000™—produced from the NYCBH by Acambis). Adapting those manufacturing guidelines and alterations are intended to improve several parameters including homogeneity, consistency between lots, and to minimize the theoretical risk of contaminations with adventitious agents. The major advantages of these 2nd generation vaccines is the fact that they are based on the same (Lister) or very similar (NYCBH vs. ACAM2000™) virus strain that was used during the eradication program and therefore they have a proven record for efficacy against human smallpox. This feature clearly contributed to the conditional approval of the use of the 2nd generation vaccine ACAM2000 as substitute for the expired historical vaccine Dryvax.[38] However, 2nd generation vaccines also share the safety profile with the 1st generation vaccines. Due to the risk of severe adverse events a significant part of today's population has contraindications that prevent the application of first and second generation VACV vaccines.

Therefore, in parallel to the production of 2nd generation vaccines, the development of safer 3rd and 4th generation vaccines has been prioritized. An obstacle in the evaluation and licensing of those new vaccines is that smallpox disease in humans no longer exists and for licensing of new drugs against smallpox new regulatory pathways for efficacy evaluation in animal models in combination with clinical testing in humans (safety and potency) needs to be implemented (see animal models). Third generation vaccines are based on live but attenuated VACV with established safety and immunogenicity records from clinical testing in humans (e.g., strains MVA, LC16m8, NYVAC and dVV-L) and 4th generation vaccines are represented by non-infectious subunit vaccines (DNA, protein) (**Table 1**).

Modified Vaccinia virus Ankara (MVA) is a highly attenuated strain of VACV that was originally developed by >500 passages in chicken embryo fibroblasts for use as safer vaccine during the last decades of the smallpox eradication campaign.[39] MVA was tested by the Bavarian State Vaccine Institute in Munich as a basis for a new procedure of primary smallpox vaccination.[40] From 1968 to 1988, MVA immunizations were administered to more than 100,000 individuals in Germany without significant adverse events. Molecular characterization of the MVA genome revealed that during the attenuation process MVA has suffered several large deletions in the terminal parts of its genome and collected many point mutations in comparison to conventional VACV strains.[41,42] Most of the genomic alterations in MVA affect regions of the VACV genome that contain non-essential genes for counteracting antiviral innate host responses and for safeguarding broad host cell tropism of VACV. In consequence, MVA shows a characteristic growth deficiency phenotype upon in vitro infection with unimpaired viral protein synthesis in all human cells being tested.[41,43-45] Moreover, the in vivo inoculation of MVA goes along with a strong stimulation of innate host responses resulting in the synthesis of type I interferons, CC chemokines, and in the attraction of host leukocytes to the site of injection.[46,47] Since other studied VACV strains seem not to have similar immunostimulatory capacities this feature is unique to MVA and might contribute to the potency of MVA based vaccines.[47,48] With regard to preclinical safety evaluation the avirulence MVA was reconfirmed in various mouse and non-human primate models being designed for pathogenicity testing.[41,49-51] In animal models, MVA has been extensively studied as candidate vector vaccine against various infectious diseases including influenza, AIDS, measles, hepatitis C, SARS, tuberculosis, malaria or leishmaniasis, and multiple recombinant MVA vaccines have proceeded to clinical phase testing (reviewed in refs. 52–55). MVA as orthopox vaccine has been shown to induce solid protective immunity against lethal challenges with VACV, CPXV or ECTV in mice[56-58] and against monkeypox in cynomolgous macaques.[59,60] For development of a recent MVA smallpox vaccine (IMVAMUNE®) a large-scale production process in chicken embryo fibroblasts has been established and extensive clinical testing is ongoing. The data from testing the vaccine in close to 2,000 individuals including patients with contraindications for immunization with conventional VACV confirms the excellent safety profile of MVA.[61-63] Importantly, thorough evaluation of the immunogenicity of MVA vaccine in humans will be crucial to allow for a licensing process together with efficacy data obtained in animal models.[64]

LC16m8 is a Japanese vaccine strain originating from an attenuated plaque isolate of the VACV strain Lister.[65] Attenuation was specifically linked to inactivation of the B5R open reading frame resulting in inefficient production of extracellular enveloped virions which represent the form of infectious VACV being considered most important for in vivo dissemination.[66] Unlike MVA, LC16m8 can productively replicate in a broad range of host cells and the genome of the virus does not contain other major alterations in comparison to non-attenuated VACV strains.[67] These features might increase the risk of adverse reactions during mass vaccinations. On the other hand, the growth capacity of the virus should increase the antigenic mass being produced in vivo and presented to the immune system. In the late 70s the clinical testing of a LC16m8 in more than 100,000 children in Japan indicated few mild adverse reactions[68] which raise the question of whether LC16m8 is safe enough for at risk individuals. More recent clinical data suggest that LC16m8 has a better safety profile compared to the parental Lister strain.[69] In up-to-date preclinical studies LC16m8 vaccine was shown to induce levels of immunity very comparable to those elicited with Dryvax vaccine

Table 1. Current and future smallpox vaccines

Type	Product	Parental strain	Description	Produced on	Advantage	Disadvantage
1st generation	Dryvax (Wyeth), Sanofi Pasteur (SPSV)	NYCBH (New York City Board of Health)	Historical vaccines	Lymph, skin lesions	Historical experience in smallpox eradication	Rare but severe Adverse reactions
	Lister, Elstree/ RIVM	Lister/Elstree		Egg Chorioalantoic membranes		
2nd generation	ACAM 2000	NYCBH (New York City Board of Health)	Historical, Plaque purified	Cultured cell-lines (e.g., Vero)	Historical experience Improved manufacturing process replication competent—easier to produce	Rare but severe Adverse reactions
	Elstree BN etc.,	Lister/Elstree	Historical, improved manufacturing process	Primary chicken embryo fibroblasts		
3rd generation	MVA		Non-replicating	Primary chicken embryo fibroblasts	Excellent clinical Safety & Immunogenicity, less immune modulatory genes	Unproven efficacy, non replicating—more costly to produce
	LC16m8	Lister	Deletion in B5R, minimal EV release	Cultured cell-lines	Improved Safety over 1st and 2nd generations	Unproven efficacy
	NYVAC	Copenhagen	Deleted immune modulatory genes		Safe (theoretical)	
	dVV-L	NYCBH	Deleted Uracil DNA glycosylase		Safe (theoretical), can be produced in a complementing cell-line	Unproven efficacy nonreplicating
4th generation	Subunit	Not relevant	Up to 4 antigens used as DNA/protein or replicons	Cultured cell-lines or recombinant expression vector	Safe (theoretical)	Unproven efficacy, few antigens—may weaken its potency.

and to efficiently protect animals against lethal poxvirus challenge.[70] Therefore, this vaccine continues to be evaluated as a promising 3rd generation vaccine.

NYVAC is an attenuated candidate vaccine virus originally derived from the Copenhagen strain of VACV to serve for vector vaccine development. It was generated by deletion of 18 non-essential genes suspected to encode viral virulence or immune evasion factors.[71] The virus poorly replicates in murine and human cells but can efficiently grow in some mammalian and avian cell-lines. In the past years, NYVAC vaccines have also been evaluated as 3rd generation smallpox vaccines. Preclinical evaluation of NYVAC by vaccination of immune-suppressed macaques followed by boost with a replication competent vaccine (Dryvax) demonstrated induction of immune responses and the ability to control the replication of Dryvax in immuno-compromised individuals. However, this prime-boost regime did not confer protection from subsequent MPXV challenge raising concern about the efficacy of the strategy of prime-boost vaccination in immunocompromised humans.[72,73] Moreover, there is recent evidence from immunizations in humans suggesting that NYVAC induces significantly lower levels of humoral immunity than conventional Lister or Dryvax vaccines.[74]

dVV-L is an additional replication defective 3rd generation vaccine candidate. It was generated by genetic modification of the VACV Lister strain through deletion of the Uracil-DNA-glycosylase (UDG) gene—an essential component in poxvirus replication.[75] Productive growth of dVV-L relies on cell-lines capable of complementing the UDG function bearing the advantage of reducing the risk for adventitious agents by using defined and approved cell-lines. dVV-L induces immune response and protective immunity comparable to MVA and a good safety profile in immune-compromised animals. Moreover, solid protection of mice against lethal challenges with CPXV or ECTV could be demonstrated.[58]

In addition to developing live attenuated VACV vaccines, there were efforts made to also derive novel orthopox-specific subunit vaccines. These 4th generation vaccines comprise of few (in most cases 1–4) viral antigens as proteins or genes expressed from DNA or recombinant viruses or replicons. Of several antigens that were investigated, four, namely B5, L1, A33 and A27, were the most used in combination,[76-89] which proved to be effective in several animal models including monkeypox infected non-human primates.[82]

Vaccine Potency, Adverse Events and Control of these Complications

All smallpox vaccines used during the eradication campaign were live VACV. Vaccination success was determined by the appearance of "clinical take"—the typical pustule at the site of vaccination.[5]

Additional parameters including the increase in hemagglutination inhibiting or neutralizing antibodies were not tested in all vaccinees, yet neutralizing antibodies appeared to better correlate with "vaccine take".[5-7] The efficient eradication of smallpox following vaccination allowed correlating vaccination efficiency and protection. Nowadays, utilizing the historical vaccine strains to vaccinate first responders and laboratory personnel, the appearance of the typical "clinical take" is still the hallmark of vaccination efficacy. Yet, additional parameters are being collected to have additional more quantitative parameters indicating the efficiency of vaccination. The measurement of neutralizing antibodies to VACV is still being considered a sensitive and reliable method for efficacy testing and serves as a reference for evaluation of additional immune parameters (e.g., binding antibodies by ELISA).[90-94] These methods are also being used to address specific cases of atypical "clinical take" e.g., no-response in 1st and revaccinated individuals.[93] Apart of humoral response the contribution of the various components of the immune system including T cells (CD4, CD8), dendritic cells, natural killer cells, neutrophils and cytokines to the acquired immunity and protection is studied in vaccinated humans and animal models.[62,95] In most individuals, the classical vaccination with VACV proceeded through the standard stages of the development of a vaccine lesion without major complications. However, VACV can rarely induce severe post-vaccinal complications[33,96,97] including eczema vaccinatum, progressive vaccinia, generalized vaccinia, post vaccinal encephalitis and myocarditis. The incidence of these complications is about 10 times higher in primary vaccination then in revaccination.[35,36]

Eczema vaccinatum (EV) correlates with history of atopic dermatitis (AD) and other severe skin allergies. Recent studies suggest that a Th2 biased immune response in AD render those individuals to inefficiently control viral propagation.[96] EV can occur by dissemination following vaccination or through skin contact of the AD individual with the primary vaccination site of an in-contact vaccinee. EV is characterized by local or generalized papular, vesicular or pustular rash as well as other systemic illness (fever, malaise etc.,). The occurrence of EV varies and severe and fatal cases were reported. There is no approved protocol for treatment but a recent severe case including multi organ failure was treated with a combination of enormous amounts of vaccinia immune globulin (VIG) and two antiviral drugs Cidofovir and ST-246 and eventually recovered after 48 days of hospitalization.[32]

Progressive vaccinia (PV) is a rare but severe complication associated with T cell deficiency. Most PV cases were fatal. PV is characterized by the development of a progressive often necrotic lesion at the vaccination site without signs of healing. Early after vaccination patients can lack symptoms of inflammation known as "clinical take" but later on the disease progresses.[98] There is no approved treatment of PV and the last reported severe case was treated with enormous amount of the combination of VIG and two antiviral drugs Cidofovir and ST-246. He eventually recovered after about two month of hospitalization experiencing multi organ failure and amputation of both legs.[29]

Post-vaccinal encephalitis (PVE) is a rare severe complication with fatality rate of about 1 death per million vaccinees. No predisposing factors are known and the mechanism involved (viral proliferation or immune mediated damage) is unknown, yet improper immune response or poor maturation of the immune system in infants are suggested to be implicated. The symptoms include headache, fever, vomiting, confusion and coma. No treatment is currently approved for PVE. Other major adverse reactions of vaccination include myocarditis and pericarditis.[30,35] During the US smallpox vaccination campaign in 2003, there were 21 cases of sympthomatic myocarditis/pericarditis among 37,900 vaccinees (5.5 per 10,000) following administration of Dryvax vaccine.[99]

Autoinoculation of vaccinia virus from the vaccination sites to other organs of the vaccinee or of in-contact person may lead to vaccinia replication in the eye, face, mouth, lips and genitalia. Of those, infections of the eye may lead to permanent defects including blindness.

Animal Models

Since VARV is highly restricted to humans and the naturally occurring infection has been eliminated with smallpox eradication, the knowledge about the basis of pathogenesis and protective immunity against smallpox was collected without the current understanding of molecular virology and the host immune system. Thus, infections of animals with orthopoxviruses closely related to VARV, mainly using challenge models based on VACV and ectromelia virus (ECTV), greatly contributed to study disease patterns and poxvirus virulence factors.[28,59,72,100-104] In the more recent past, substantial achievements have been made in the characterization of poxvirus animal models for vaccine and drug testing. This was in part a consequence of the announcement of the US Food and Drug Administration (FDA) to allow for the use of efficacy data from animal models data ("animal rule") during drug licensing procedures.[105] This new regulation should serve as a pathway for regulatory review when human efficacy studies are not ethical or feasible, as it is the case for the development of new vaccines and drugs against human smallpox. In addition, the European Medicine Agency has recommended the use of orthopoxviruses other than VACV to assess protective capacity of VACV immunization in animal models giving emphasis to infections with viruses such as ECTV, CPXV and MPXV.[106] To match other features seen with human smallpox efforts were to optimize the models with regard to the requirement for low infectious dose, the development of systemic illness, and the possibility to use respiratory routes of infection (reviewed in ref. 107).

The infection of mice with ECTV is considered an excellent surrogate model for smallpox because a fatal systemic disease is produced by very low infectious doses of virus (reviewed in ref. 108). Importantly, in comparison to the VACV-based mouse challenge model, ECTV infection of BALB/c or C57BL/6 mice is characterized by an extended asymptomatic incubation period.[109,110] This rather long disease free period seems vital to allow for the demonstration of protective post-exposure vaccination with VACV.[109,111] In contrast, respiratory challenge infection with VACV produces a fulminant acute disease which appears to hamper the feasibility of therapeutic immunization.[112]

Also new MPXV models, including those for respiratory challenge infections, have been successfully established using Cynomolgous macaques (*Macaca fascicularis*).[13,59,101,113] Rather high amounts of MPXV (doses of >10^6 plaque-forming units) are usually needed to produce a highly acute severe systemic disease in monkeys. This potential disadvantage of the non-human primate model has been addressed by the study of MPXV infections in small animal species including prairie dogs (Cynomys sp.),[12,114,115] ground squirrels (*Spermophilus tridecemlineatus*),[11] and African dormice (*Graphiurus kelleni*).[116] These rodents were shown to be highly susceptible to MPXV respiratory infection resulting in lethal systemic infections after a protracted incubation period. Thus, these new models appear to mimic human smallpox, and despite still lacking tools for immune analysis, they might prove useful for investigations of antipoxvirals and next generation orthopoxvirus vaccines.

Intranasal and aerosolized infections of mice with CPXV, the natural orthopoxvirus being endemic in Eurasia, are also suggested as suitable models for assessment of new orthopoxvirus specific drugs;[117-120] yet the requirement for a relatively high viral dose to induce lethal disease as compared to human smallpox should be considered. Similarly, rabbitpox virus, representing a collection of rabbit adapted strains of VACV, can be applied to induce relatively rapid and fatal disease in New Zealand White rabbits (*Oryctolagus cuniculus*) after intradermal or aerosol challenge with a very low infection dose.[121,122] Yet, dermal lesions which are the hallmarks of smallpox are less obvious in this model, and the disease progresses with a very short incubation period compared to human smallpox.

Overall, animal models for orthopoxvirus infections differ to some extent from human smallpox with regards to the lethal dose, disease profile following infection trough various routes and the interrelations between the host and the viral defense mechanisms.

Future Directions

The potential reemergence of orthopox-specific human diseases calls for the development of adequate countermeasures. Today, there are only few individuals in few countries with complete orthopox-specific immunity due to recent vaccination, and, most of the world population is at risk of developing smallpox if VARV is released. This situation is very different from the situation 30 years ago where global population-wide immunizations were conducted during the final stages of the smallpox eradication program. Thus, the response to a suddenly emerging orthopoxvirus infection must include mass primary vaccinations in an emergency situation. Ideally, such vaccination campaigns should be based on the use of a modern standard, highly immunogenic and safe vaccine, and should allow for rapid containment and eradication of the emerging infection.

However, the future of smallpox vaccines is still unknown. At present, 2nd generation vaccines are being produced to replace historical stocks of smallpox vaccines. However, as exposure to smallpox virus is considered a rather unlikely event, the risk of developing severe adverse reactions following vaccination with currently licensed vaccines does not readily justify pre-event vaccinations at a large scale. For certain individuals with high probability of potential exposure (e.g., military forces, laboratory and medical staff) the risk versus benefit calculation might be different. Importantly, this unsolved debate, and the estimated higher prevalence of immune deficiency in the world population than 30 years ago, drives the development of safer vaccines and the search for improved vaccination regimens to achieve as most rapid protection as possible.

The licensing of safer new 3rd generation vaccines based on MVA and LC16m8 is expected, yet their approval relies on the confidence in their efficacy in comparison to the historical vaccine strains as an efficacy reference. Nowadays, in the absence of circulating smallpox, new parameters for efficacy and new immune correlates of protection must be defined. The assurance for efficacy will rely on the use of data from human clinical trials comparing the immunogenicity of historical vaccines to the one of new candidate vaccines. This information needs to be supplemented with data from efficacy studies in animal models for orthopoxvirus infections. Historically, successful vaccination with the historical strains was qualified by monitoring for appearance of "clinical take"—a parameter successfully used during smallpox eradication. This simple parameter is no longer relevant when utilizing new replication-deficient VACV (like MVA) or subunit vaccines and new immune parameters for successful vaccination must be defined. Acquiring protective immunity by rapid emergency vaccination or by other means of treatment is of particularly high priority. Anecdotal reports from immunizations during the smallpox eradication campaign indicate that post exposure vaccination can protect against severe disease if applied up to 4 days post exposure. Recently, the efficacy of vaccination shortly before or post exposure was demonstrated in animal models.[60,109,111] Future research needs to carefully address the evaluation of immune correlates for rapid protection and to investigate the usefulness of new approaches and schedules for emergency immunization in human clinical trials. These activities should be supplemented by the evaluation of other options for therapeutic intervention. Recently, passive immunization of mice with VIG was shown to provide protection when applied post exposure and the possible interference with co-administrated active vaccine were excluded.[123] New studies with VIG and other investigational antivirals must aim to evaluate the efficacy of those products alone and in addition to emergency vaccination.

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