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Smallpox vaccines: targets of protective immunity

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Summary

The eradication of smallpox, one of the great triumphs of medicine, was accomplished through the prophylactic administration of live vaccinia virus, a comparatively benign relative of variola virus, the causative agent of smallpox. Nevertheless, recent fears that variola virus may be used as a biological weapon together with the present susceptibility of unimmunized populations have spurred the development of new generation vaccines that are safer than the original and can be produced by modern methods. Predicting the efficacy of such vaccines in the absence of human smallpox, however, depends on understanding the correlates of protection. This review outlines the biology of poxviruses with particular relevance to vaccine development, describes protein targets of humoral and cellular immunity, compares animal models of orthopoxvirus disease with human smallpox, and considers the status of second and third generation smallpox vaccines.

The smallpox vaccine and the eradication of smallpox

From one perspective, the origins of vaccinology and immunology can be traced to the early efforts employed to prevent smallpox (1). Smallpox was a devastating disease: in a naive population, the death rate was estimated to be 50% or greater in the very young and those over 40 years of age, with a lower mortality in the years between (2). However, individuals who recovered from smallpox, easily identified by residual facial scars, were resistant to subsequent occurrences of the disease. Presumably, it was such observations in 10th century China and India that led to prophylactic intranasal or cutaneous inoculations of smallpox scab material, which usually caused a mild infection but prevented a more serious one. Despite the risk of developing smallpox and spreading the disease, this procedure known as variolation was beneficially used in many parts of the world until the 18th century.

In 1798, Edward Jenner, a physician in rural England, described a safer and effective alternative to variolation. He was aware of a belief among country folk that acquisition of cowpox protected against smallpox and also noted that the lesions on the hands of milkmaids that contracted cowpox resembled those produced by variolation. Accordingly, Jenner tested whether the deliberate inoculation of cowpox material would prevent the pustules caused by subsequent variolation, which would be a sign of protection against disease. The success of this prescient experiment led Jenner to speculate that ‘the annihilation of the Small Pox, the most dreadful scourge of the human species, must be the final result of this practice’. We now understand that the viruses responsible for cowpox and smallpox are closely related and provide cross immunity. Fortunately, there were no animal reservoirs of variola virus (VARV), the causative agent of smallpox, and vaccine-resistant VARV did not arise. Through international efforts directed by the World Health Organization, Jenner’s prophecy came true in 1977, when the last natural case of smallpox

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was diagnosed in Somalia. The vaccines used in the smallpox eradication campaign consisted of live vaccinia virus (VACV), though Jenner initially used cowpox virus (CPXV). The eradication of smallpox is one of the outstanding achievements of medicine, which saved millions of lives and allowed the discontinuation of routine smallpox vaccination.

Correlates of smallpox protection

The eradication of smallpox occurred prior to modern advances in virology and immunology, precluding a thorough understanding of the basis for protection following vaccination. The vaccines used in the global smallpox eradication campaign consisted of several related strains of live VACV (Dryvax® New York City Board of Health strain in the USA; Lister in the UK; Temple of Heaven in China, and EM-63 in the USSR) usually administered percutaneously by scarification of the skin with a bifurcated needle or with a jet injector (3). Smallpox vaccine recipients with severe T-cell abnormalities developed generalized VACV infection, whereas agammaglobulinemics did not, pointing to the importance of cell-mediated immunity in controlling the primary infection caused by the live vaccine. A successful vaccination or 'take' in a naive, immunocompetent individual results in VACV replication in the skin producing a papule with surrounding erythema in 3 to 5 days, followed a few days later by a vesicle and then a pustule. A scab forms and separates from the skin after 2 to 3 weeks. Low-grade fever, headache, myalgia, fatigue, and regional lymphadenopathy often accompanies vaccination (4) and correlates with increased levels of cytokines (5–7). A modified or accelerated skin reaction is usually indicative of pre-existing immunity. Protection against mortality due to smallpox is nearly complete for 20 to 30 years and gradually wanes thereafter (2).

In naive individuals, a vaccine take correlated with development of neutralizing antibody to VACV (8), whereas in previously vaccinated individuals successful takes correlated inversely with the pre-existing neutralizing antibody levels (9). A prospective study (10) showed that smallpox contacts with a neutralizing antibody titer of 1:32 or greater were protected against disease. The route of administration of the vaccine is important: the percentage of children developing neutralizing antibody after receiving the vaccine percutaneously and subcutaneously was 83 and 23%, respectively, and this difference correlated with immunity to revaccination (11). In another study, percutaneous administration produced a higher neutralizing antibody response than intradermal or intramuscular administration (12). Neutralizing antibody was detected by days 12 to 15 and was maximal by days 25 to 30 (12,13). Recent studies indicate that $CD4^+$ and $CD8^+$ T cells are also induced by smallpox vaccination (14,15). Studies of elderly individuals demonstrated that the antibody levels determined by enzyme-linked immunosorbent assay (ELISA) or neutralization of VACV persist for many decades, as do memory B and $CD4^+$ and $CD8^+$ T cells (13,16–20). Multiple vaccine boosts, however, do not seem to enhance the long-term antibody levels (17,19). Although not carried out in a controlled manner, several studies suggest that administration of hyperimmune globulin (VIG) provided significant protection to smallpox contacts and alleviation of vaccine complications (21,22). In summary, the data show a positive correlation of vaccine-mediated protection against smallpox with a skin take, neutralizing antibody, and VACV-specific T cells.

Recent interest in new smallpox vaccines

Interest in smallpox vaccines was renewed in recent years due to concerns that a still existing unregistered stock of VARV could be used as a bioweapon, particularly since the unvaccinated population is now fully susceptible to infection (23). Stocks of smallpox vaccine dwindled in the years following eradication and their replenishment has been

advocated. However, the classical method of vaccine production in the skin of animals does not meet current safety standards. In addition, although the vaccine was considered safe when contrasted with the possibility of contracting smallpox, significant numbers of serious adverse events were recorded (24). A safer vaccine could benefit millions of people advised not to take the classical one because they or their contacts have immune deficiencies, eczema, or atopic dermatitis, which make them more susceptible to generalized or progressive vaccinia. In certain situations, a safer vaccine might also be used to prevent disease caused by other OPXVs. Monkeypox virus (MPXV) is a continuing zoonosis in parts of Africa and a potential terrorist agent (25–27). A mild form of MPXV infected 69 people in the United States following importation of African rodents, which comprise the natural reservoir (28). Self-limited human infections with several strains of CPXV transmitted by rats and cats occur in Europe (29) and with feral vaccine-derived VACV in India (30) and Brazil (31,32).

Since smallpox has been eradicated, the efficacy of new vaccines must be evaluated indirectly by an analysis of human immune responses supplemented with the use of animal models and surrogate poxviruses to demonstrate disease protection. An understanding of the biology of poxviruses and the targets of the protective immune response can facilitate the development of new generation vaccines. For this reason, the present review includes an outline of the biology of OPXVs with particular relevance to vaccine development, discusses targets of humoral and cellular immunity, compares smallpox with animal models of OPXV disease, evaluates correlates of protection, and provides the status of second and third generation smallpox vaccines. Excellent reviews of smallpox vaccines from different perspectives have been published recently (33,34).

Outline of OPXV replication

Poxviruses comprise a large family of enveloped, double-strand DNA viruses that infect vertebrate and invertebrate species (35). The members of this family are unusual, with regard to other DNA viruses, in their cytoplasmic site of replication. This review focuses on orthopoxvirus (OPXV) genus, which includes VARV, VACV, CPXV, MPXV, and ectromelia (mousepox) virus (ECTV) among others. OPXV double-stranded DNA genomes are about 200,000 bp in length and encode about 200 proteins, most of which have 90% or more sequence identity between species. The complete genome sequences of a large number of VARV isolates and other OPXVs are available. While all OPXVs encode homologous proteins for vital functions such as entry, gene expression, genome replication, and virion assembly, there are differences with regard to proteins involved in immune evasion and other host interactions. In this respect, CPXV has the most complete genome, whereas some immune evasion and host interaction genes are missing or disrupted in VARV, VACV, MPXV, and ECTV. The similarity in the structural proteins expressed by different OPXVs accounts for their immunological cross-reactivity and protection. The viral DNA polymerase has high fidelity resulting in a low mutation rate, consistent with the absence of notable antigenic variation.

The basic infectious poxvirus particle is the mature virion (MV), which consists of a nucleoprotein core containing the genome and a complete early transcription system, flanked by lateral bodies, and surrounded by a lipoprotein envelope (36,37). Mass spectrometry studies indicate that there are more than 80 viral protein components of the MV (38–40), of which more than 20 are associated with the surface membrane (35). The MVs assemble in viral factory regions of the cytoplasm and may remain in the cytoplasm as individual particles or become occluded in dense bodies known as A-Type inclusions in the case of ECTV and CPXV until cell lysis. Free and occluded MVs are thought to be important for transmission between hosts. In addition, a subset of MVs becomes surrounded

by modified Golgi or endosomal membranes, transported to the cell periphery on microtubules, and released by exocytosis as enveloped virions (EVs) (41). The EV consists of an MV with an additional lipoprotein membrane containing eight EV-specific viral proteins, although there are other small differences in protein composition (42). Most of the EVs remain adherent to the plasma membrane and mediate cell-to-cell spread at the tips of long actin-containing microvilli (43,44), which are important for virulence in animals (41). Since the fusion proteins necessary for virus entry are located in the MV, disruption of the EV membrane is required for their exposure (45,46). Studies of VACV entry indicate that fusion can occur at the plasma membrane or in endosomes by a fluid uptake or macropinocytosis mechanism (47–50).

Four proteins enable attachment of MVs to the cell. The A27 protein exists as trimers and hexamers on the surface of MVs (51) and binds heparin and cell surface proteoglycans (52,53). H3, another MV surface protein, also binds heparin and cell surface proteoglycans (54). D8 is an MV surface protein that binds chondroitin sulfate (55,56). The fourth attachment protein, A26, is physically associated with A27 (57,58) and binds cell surface laminin (59). Fusion with the cell membrane and entry of the core into the cytoplasm requires at least 12 additional MV transmembrane proteins that form a complex known as the entry fusion complex or EFC (45,60). Such a large number of entry proteins are unprecedented among viruses. The entry proteins are A16 (61), A21 (62), A28 (63), F9 (64), G3 (65), G9 (66), H2 (67), I2 (not yet shown to be a component of EFC) (68), J5 (60), L1 (69), L5 (62), and O3 (70). The individual roles of the EFC proteins remain to be determined.

The transmembrane proteins exposed on the outer surface of the EVs include A33 (71), A34 (72), A36 (73), A56 hemagglutinin (74), and B5 (75,76). Deletion of any one of the above genes, except for the gene encoding A56, results in decreased virus spread. The A36 protein is particularly important for formation of actin tails (77,78). K2 (79) and the complement regulatory protein C3 (80,81) lack transmembrane domains but are associated with A56 (79). A56 and K2 help to prevent superinfection of cells (82,83), and cell surface C3 enhances protection against complement (81). F13, a palmitoylated protein, is associated with the internal surface of the EV membrane (84,85) and is important for EV formation (86).

Viral gene expression is stringently regulated during infection (87). The early transcription system, which is packaged in infectious virus particles, directs the expression of more than half of the genome shortly after entry of the core (88). Although kinetic analysis reveals two early gene clusters (89), the members of both are synthesized without the need for *de novo* protein synthesis and may all be classified as belonging to the immediate early class as defined for other viruses and phage (90,91). The genes of both early kinetic classes encode proteins involved in host interactions including immune defense (92), DNA replication (93), and gene expression (35). Transcription of the remaining genes is dependent on viral protein and DNA synthesis and they are grouped in intermediate and late classes. The latter encode most of the proteins assembled into virus particles, which are important targets for neutralizing antibodies. Early, intermediate and late genes have distinct promoter sequences that are recognized by stage-specific transcription factors and a multisubunit DNA-dependent RNA polymerase (35). The close spacing of genes and the tendency of transcripts to overlap, particularly at late times after infection, have made global transcript mapping difficult. The problem was only partly overcome by use of tiling arrays (89,94), which have been superseded by higher resolution deep RNA sequencing (91). Further refinements to more definitively map the transcriptional start and stop sites are anticipated. As discussed below, the time of synthesis of proteins may affect their presentation to the immune system.

Not all cells support OPXV replication. Importantly for this review, replication of VACV is abortive in mouse and human primary macrophages and dendritic cells but permissive in B cells (95–100). Although VACV is able to enter these non-permissive cells and express early genes, neither viral DNA replication nor late genes are expressed. Unaccountably, intermediate genes are expressed in human macrophages despite the apparent absence of viral DNA replication. Based on flow cytometry studies of human peripheral blood lymphocytes infected with recombinant VACV expressing green fluorescent proteins regulated by an early/late promoter, the levels of expression were monocytes ($CD14^+$) > B lymphocytes ($CD\ 19^+$) = NK ($CD\ 56^+$) > T lymphocytes ($CD3^+$) (101). The block in resting T cells occurs at the virus-binding step, which is overcome upon T-cell activation *in vitro* (102).

Targets of neutralizing and binding antibodies

Some virion surface proteins provide accessible targets for antibodies. As indicated above, the situation is complicated for OPXV because of the existence of two main infectious forms. Antigenic differences between the MV and EV forms of VACV were recognized over 40 years ago and correlated with greater protection by live virus compared to inactivated virus (103–105). Both live and inactivated vaccines generally consist of MVs isolated by disruption of infected cells and further purified. However, whereas live MVs replicate in the animal host and induce antibody to both forms of virus, inactivated MVs only induce antibody to themselves. Depending on the process, it is also possible that inactivation destroys some immunogens of the MV and that the live virus induces protective antibodies to non-virion components as well as to EVs and also stimulates cellular immunity. No successful inactivated OPXV vaccines have been made.

The traditional way of measuring antibodies that neutralize MVs has been by plaque reduction. MVs are incubated with antibodies and then allowed to form plaques in a few days on a monolayer of susceptible cells (106). Faster, higher throughput assays have been devised using recombinant MVs that express β-galactosidase (107) or green fluorescent protein (108,109). EV neutralization can also be measured by a plaque reduction assay, although EVs are more difficult to neutralize than MVs (110,111). One technical problem is that EVs are fragile and the outer membrane is easily disrupted exposing the MV membrane. To alleviate this problem, a monoclonal antibody (mAb) to an MV protein is frequently used to neutralize partially disrupted EVs before adding EV-specific antibody. Alternatively, the ‘comet reduction assay’ is used to measure antibodies that prevent the spread of EVs in cell monolayers. The so-called comets are satellite plaques that form by EVs released from infected cells. Antibodies to EV proteins may reduce the size and number of the comets; however, even high titer antibodies from infected animals cannot prevent cell-to-cell spread as seen by expansion of the primary plaque (112).

The identification of antibodies to specific MV and EV proteins followed progress in dissecting the structure of VACV. Table 1 lists proteins associated with the MV and EV-specific membranes that have been shown to be targets of neutralizing or comet-reducing antibodies. Polyclonal antibodies to the MV membrane-associated proteins A17 (113), A27 (114), A28 (115), D8 (56), H3 (54), and L1 (116) can neutralize VACV. As indicated above, A27, D8, and H3 are involved in attachment of MVs, and A28 and L1 are required for fusion of the viral and cell membranes. Remarkably, H2, another protein required for fusion, interacts directly with A28 (117) and greatly increases the amount of neutralizing antibody that reacts with A28 (118) (Fig. 1). However, immunizations of mice with the genes encoding the other VACV entry proteins did not result in the generation of neutralizing antibody (118).

Neutralizing mouse mAbs that target the A27 (119) and L1 (116,120,121) MV proteins have been described. Several neutralizing mAbs target the N-terminal region of A27 (122). Neutralizing mAbs bind to a native conformation-specific form of L1 with intact intramolecular disulfide bonds (116). Structural studies show that the Fab fragment binds to a discontinuous epitope in L1 consisting of two loops that are held together by a disulfide bond (123). L1 mAb can neutralize VACV after cell attachment, but the mechanism of neutralization is not understood.

Antibodies to two EV-specific proteins (Table 1), A33 and B5, inhibit comet formation and B5 antibody also neutralizes EV infectivity (124). Rodent and human mAbs to the A33 (71,125,126) and B5 (127–129) proteins are capable of neutralizing EV and/or reducing comet formation. The epitopes of these mAbs have not been precisely mapped. However, despite the similar sequences of A33 homologs in OPXV, mAbs can differentiate between the homologs in VACV, ECTV, and MPXV (71,130). Although the proteins of VACV and VARV have a high degree of amino acid identity, there are differences in some that may be relevant for vaccine purposes. Thus, VACV B5 and the VARV homolog B6 have 23 amino acid differences. Similarly, although polyclonal antibodies to B5 cross-reacted with the homologous VARV protein, 10 of 16 mAbs did not (131).

Efforts have been made to more completely characterize the binding antibodies in human or animal sera following immunization with live VACV. In a proteome-wide approach, proteins corresponding to 185 VACV open reading frames were expressed in an *Escherichia coli* cell-free system and printed on a microarray. The microarray was then incubated with sera from humans, rabbits, or mice that were infected with VACV (132,133). Antibodies to about 25 proteins, many of which are unlikely to be involved in virus neutralization, were detected with considerable heterogeneity of individual responses. After the first vaccination, antibodies to six membrane proteins, A13, A17, A33, B5, D8, and H3, were found in 50% or more of the 13 individual human sera tested. After the second vaccination, A27 and L1 were detected. An attempt was made to correlate the neutralizing activity in individual sera with the amount of antibody to 16 membrane-associated MV proteins. A positive correlation was found for H3, A27, D8, A14, D13, and L1 (134). However, depletion of either H3 or L1 antibody or both from sera did not reduce the neutralizing activity. This was true even when H3 was depleted from serum that had detectable reactivity to only H3 and D8. Earlier studies had shown that depletion of either L1 (121) or A27 (135) from pooled human sera also did not significantly reduce the neutralizing titer. In a separate study, modest reductions in MV neutralizing activity was obtained by adding A27 and H3 proteins to human immune sera but not by adding L1 protein (136). These results suggest that there are multiple neutralizing targets on the MV, likely including some that have not yet been identified, or that the targets are complex and comprised of multisubunit structures such as A28-H2 (118) or higher order assemblies.

In contrast to the results with MV neutralization, depletion of B5 from sera greatly reduces *in vitro* neutralization of EVs indicating that this protein is the major target (136,137). Nevertheless, B5 antibody-depleted serum can reduce comet formation indicating that binding antibodies to additional EV proteins are important (137). Moreover, as discussed below, immunization with the A33 EV protein is highly protective in animal models.

Both classical and alternative complement pathways have been reported to enhance the neutralization of MVs *in vitro* (138,139). Complement can enhance the neutralization of EVs by two different methods. Complement in conjunction with antibody to the A33 protein was shown to disrupt the EV membrane and allow access of MV neutralizing antibody (140). In addition, complement was shown to enhance B5 antibody neutralization of EV by opsinization and by lysing VACV-infected cells (141). Depletion of complement in the

severe combined immunodeficiency (SCID) mouse reduced the ability of B5 mAb to protect against a VACV infection; in addition, protection was shown to be isotype dependent (129). The importance of complement in defense against OPXVs is underscored by their encoding a secreted complement regulatory protein that can prevent antibody-dependent complement enhanced neutralization of MV infectivity and is required for virulence (139,142,143). The presence of cellular complement regulatory proteins associated with the EV may similarly provide protection against complement (144).

$CD4^+$ and $CD8^+$ T-cell targets

Consistent with the large number of OPXV proteins, hundreds of VACV $CD8^+$ T-cell epitopes have been identified in humans (145,146), monkeys (147), and mice (148–151), as recently reviewed (152). Furthermore, based on sequence comparisons, a high percentage of the VACV epitopes are predicted to be present in VARV (153). Studies with recombinant VACV demonstrated the importance of early expression for induction of $CD8^+$ T cells (97,154). Relatively more targets are found in VACV proteins expressed early compared to late in infection (145,149,151) and immunoprevalence correlates with mRNA abundance at 4 h after infection (89,153). The early gene bias may be correlated with the abortive infection of dendritic cells by VACV (97,99,100,155), which prevents late gene expression. In addition, cross-competition of $CD8^+$ T cells shapes the immunodominance hierarchy favoring early expression during a boost vaccination (156).

A comprehensive literature compilation and review (157) indicates that 133 VACV proteins are recognized by $CD4^+$ T cells, though the epitopes have not been precisely mapped in many cases (158–164). In contrast to $CD8^+$ T-cell antigens, $CD4^+$ T-cell antigens are directed mostly to late virion proteins, as are antibodies (165).

Smallpox and animal models of orthopoxvirus infection

For an ideal animal model, the disease would closely mimic human smallpox. The transmission of VARV in humans occurred mainly by droplets or aerosol from close contacts. A one to two week incubation period preceded the abrupt onset of fever, headache, and back pain, followed a few days later by eruptive lesions on the tongue and mucous membranes of the mouth and oropharynx and then by maculopapular lesions that spread from the face and extremities and progressed into pustules covering the entire body (166). The delayed time course is thought to represent initial local replication near the site of infection and in local lymph nodes followed by systemic spread with tropism for the skin.

Since the eradication of smallpox, the World Health Organization has tightly regulated experimentation with VARV. Such studies are permitted at only two sites: one in the United States and the other in Russia. VARV is naturally host-restricted to humans and very high doses are needed to cause pathology in cynomolgus monkeys (167). Acute pathology and rapid death occurred when 10^9 plaque forming units (PFU) of VARV were administered intravenously. Virus was detected in lymphoid tissue, skin, oral mucosa, gastrointestinal tract, reproductive system, and liver, correlating with organ dysfunction and multisystem failure. At best, this model mimics the late systemic spread phase of smallpox. Aerosol doses of $>10^8$ PFU caused only mild clinical signs (168). Safety recommendations for other OPXV are described in a publication by the Centers for Disease Control and Prevention and the National Institutes of Health (169).

Human monkeypox is a zoonosis that resembles smallpox clinically but has lower rates of fatality and human-human transmission and differs from it epidemiologically (170,171). In addition, there are differences in the immunomodulatory and host range genes of VARV and MPXV (172). Despite its name, MPXV is widely distributed in a variety of African rodents,

particularly squirrels, which are probably the maintenance reservoir (173,174). Two strains of MPXV are now recognized: the more virulent strain with an estimated mortality rate of 10% originates from the Congo Basin in Africa, and the milder strain, which was imported transiently into the United States in 2003,originates from West Africa (26). Congo Basin strains of MPXV are lethal to cynomolgus monkeys when inoculated by aerosol, intratracheally, subcutaneously, or intravenously with relatively high doses, ranging from 10^5 to 10^7 PFU (175–178). After respiratory inoculation, death in 9 to 17 days is attributed to bronchopneumonia (179). MPXV can experimentally infect and cause disease in a variety of wild rodents including ground squirrels, prairie dogs, and African dormice at relatively low doses ($<10^1 – 10^4$ PFU) (180–186), but these animals are difficult to breed and there is a lack of immunological reagents. Although classical inbred mice are resistant to MPXV (187–189), an extensive search revealed three ‘wild-derived’ inbred strains that are highly susceptible to infection (190). For the CAST/eij mouse strain, the 50% lethal dose of MPXV is 670 PFU by the intranasal route and a log less by the intraperitoneal route, whereas even 10^7 PFU caused no deaths in BALB/c mice (190). The CAST mouse/MPXV model may have advantages for studying correlates of immunity and vaccine efficacy.

ECTV is a sporadic infection of laboratory mice. The classic inbred strains vary greatly in their susceptibility with lethality occurring with doses of 1 PFU or less in the most sensitive strains (191,192). ECTV has been extensively used to study viral pathogenesis, cell-mediated immunity, and genetic resistance to infection. Following low dose inoculation of ECTV into the footpad, the disease course is delayed involving successive local and systemic spread mimicking smallpox though with more rapid severe disease onset and accentuated hepatic involvement (193). Aerosol and intranasal ECTV infection models for ECTV have also been described (171,194,195). In an intranasal mouse (BALB/c and C57BL/6) model, there is a 7-day disease lag period and a 10-day mean time of death (195). Early virus proliferation in the lungs is followed by spleen and liver infection reaching peak values at 8 days.

The natural or original host of VACV is unknown, though vaccine-derived virus is now feral in Brazil and India and occasionally infects humans (196). VACV has a wide host range in the laboratory, and mice that are genetically resistant to ECTV are still susceptible to VACV. For vaccine studies, mice are usually challenged with a pathogenic strain of VACV such as Western Reserve (WR) by the intranasal route, but relatively high doses ($10^4 – 10^6$ PFU) are required, leading to pneumonia and morbidity and death within 7 to 10 days (197–199). In young rabbits, both rabbitpox (RPXV), a variant of VACV, and VACV WR cause a rapidly lethal systemic disease with skin lesions and aerosol transmission when administered by a variety of routes (200).

CPXV is endemic in wild rodents in Europe and central Asia and can be transmitted to cats, pet rats, and other animals to humans (196). In immunocompetent humans with no history of skin disorders, CPXV causes a localized lesion. No human-to-human transmission has been reported, and laboratory workers can be protected by a smallpox (VACV) vaccination. Genetic analysis indicates at least two major CPXV groups and several subgroups of CPXV (29). CPXV has many more immune modulators than other OPXV including VARV. In mice, the 50% lethal intranasal dose of the Brighton strain is similar to that of VACV WR (201). A strain of CPXV isolated from an outbreak in a monkey colony was shown to be highly lethal at relatively low dose in marmosets and may be useful as an OPXV non-human primate model (202,203).

Protective immunity in experimental OPXV infections

Live vaccines

As replication-competent and highly attenuated live vaccines elicit diverse immune responses, multiple factors may contribute to protective immunity. Moreover, the dominant mode of protection may depend on the virus/animal model, type and route of vaccination and challenge, and the interval after vaccination. Several studies, particularly with ECTV, demonstrate that cytokines, macrophages, NK cells, $CD8^+$ T cells, and antibodies are each important for protection and clearing of a primary footpad infection in mice (204–208). In an immunocompetent mouse, either $CD8^+$ T cells or antibody can provide protection (209). However, the importance of interferon, $CD4^+$, and $CD8^+$ T cells for protection against secondary ECTV infections, which is more pertinent in a vaccine context, has been questioned based on the kinetics of the recall humoral and cellular immune responses and studies with immune deficient mice (210–212). Immunized mice genetically deficient in $CD8^+$ T cells or effector function were resistant to secondary infection with ECTV, whereas mice lacking B cells, major histocompatibility complex (MHC) class II, and CD40, which are important for antibody production, were unprotected. Depletion of $CD4^+$ T cells prior to challenge with ECTV, however, did not interfere with protection, whereas depletion of B cells rendered the mice susceptible. Thus, in this model, antibody is necessary and sufficient for protection. The relatively slow kinetics of disease onset after intranasal infection of mice with ECTV has allowed the testing of post exposure vaccination. Significant protection was obtained by intramuscular vaccination with either Lister or MVA vaccine at 2 to 3 days after intranasal challenge with ECTV (195). High dose intravenous administration of MVA is particularly effective for 3-day post-exposure immunization and induces strong innate and adaptive immune responses (213). Studies with genetically deficient mice indicated that NK cells, $CD4^+$ T cells, $CD8^+$ T cells, and antibodies were all important under the latter conditions.

For mice vaccinated with replication-deficient or replication competent VACV and challenged intranasally with pathogenic VACV, antibody and $CD8^+$ T cells contribute to protection, though to different degrees depending on the experimental protocol (214–216). B-cell-deficient mice unable to generate antibodies as well as β2-microglobulin-deficient mice unable to express MHC class I molecules for a $CD8^+$ T-cell response were protectively vaccinated. However, mice with decreased $CD4^+$ T cells or MHC class II expression and double-knockout mice deficient in MHC class I- and II-restricted activities were poorly protected or unprotected. Similarly, skin-scratch vaccinated B-cell-deficient μMT and T-cell-depleted mice were protected against a respiratory challenge with VACV (217). Thus, $CD8^+$ T cells and antibody provide overlapping modes of vaccine protection in these mouse models. In the VACV WR challenge model, post exposure vaccination is not effective, probably because of the very rapid kinetics of disease and death (218).

Monkeys depleted of B cells to prevent an antibody response following live vaccination were susceptible to intravenous MPXV challenge, whereas vaccinated animals depleted of $CD4^+$ and $CD8^+$ T cells prior to challenge were resistant, indicating the dominant role of antibody under these conditions (219).

Protein immunizations

Protection provided by immunization with proteins is usually attributed to antibody. Six MV and two EV proteins are known to be targets of neutralizing or comet-reducing antibodies (Table 1) and immunizations with four of these purified proteins have been shown to protect mice against lethal challenge with VACV. Two MV proteins, A27 and H3, have roles in virus attachment, whereas L1 is involved in membrane fusion and entry as described in a

preceding section. Recombinant A27 (220) and H3 (221), made in *Escherichia coli*, protected mice against VACV challenge by intraperitoneal and intranasal routes, respectively. In another study, however, mice immunized with a soluble recombinant A27 made in insect cells were not protected against a subsequent intranasal VACV challenge, despite the production of neutralizing antibody (222). The third MV membrane protein tested, L1, was also made in insect cells and provided protection against a lethal intranasal VACV challenge (223). The remaining two proteins tested, A33 and B5, are components of the EV membrane. B5 and A33 made either in *E. coli* or as secreted forms in insect cells were protective in VACV intranasal and ECTV footpad infection mouse models (124,223,224).

Immunizations with single proteins can prevent death, but weight loss and other signs of disease still occurred under most conditions. Immunization with a combination of proteins, particularly at least one MV and one EV protein provides greater protection (223,225–227), as illustrated in Fig. 2. The trivalent vaccine used in the latter study consisted of secreted forms of one MV protein (L1) and two EV proteins (A33 and B5) made in insect cells and mixed with adjuvant. This combination also provided protection against MPXV in monkeys (225), as did a quadravalent combination also containing the A27 MV protein with alum and CpG (228). Although the above animal model experiments were carried out using VACV proteins, there could be an advantage to using the corresponding VARV proteins if they were to be developed as vaccines (131).

DNA immunizations

Immunizations with DNA encoding OPXV proteins may induce antigen-specific $CD8^+$ T cells in addition to antibodies. Conclusions regarding the synergistic effects of targeting MV and EV proteins were obtained using vaccines comprised of DNA encoding the MV proteins A27, L1, D8 and the EV proteins A33 and B5 in mouse and monkey studies (125,229–231). Partial protection was also obtained with DNA encoding the MV proteins A28 plus H2 (118), but these were not tried in conjunction with DNA encoding EV proteins. Priming with a DNA vaccine and boosting with subunit proteins made in bacteria provided better protection in a MPXV model than either alone (232). Alphavirus replicons have also been used to deliver DNA (233). Additional vaccine candidates were obtained by a functional screening of a synthetic cowpox virus genome library in C57Bl/6 mice (234).

Passive antibody

Immune globulin from humans receiving smallpox vaccine (VIG) can provide protection when given to immune competent mice before or shortly after challenge with VACV or ECTV and prolong survival of SCID mice (235,236). Polyclonal antibodies to the L1 MV and B5R and A33 EV proteins protect against intranasal infection by VACV WR (124,237). Moreover, combinations of antibodies, either polyclonal or monoclonal, to MV and EV proteins provide superior protection than VIG or antibodies to single proteins or multiple EV proteins in immune competent or SCID mice (237) (Fig. 3). Interestingly, antibody to the L1 MV membrane protein or the A33 EV protein each provided protection against lethality but not weight loss when given post challenge, although one might have predicted that the latter would be superior because spread is mediated by EVs (237). Both human-like chimpanzee and fully human mAbs to MV (L1 or H3) and EV (A33 or B5) proteins also provide better protection than VIG in mouse models (126,128,238). In addition, mAbs could have safety advantages over VIG, which is made from pooled human sera. Although not permitted under present World Health Organization guidelines, the replacement of individual VACV or ECTV envelope proteins with VARV homologs might be useful for evaluating protection by mAbs in small animal models and, under appropriate containment conditions, safer than evaluating mAbs in VARV non-human primate models.

Peptides containing CD8+ T-cell epitopes

Several studies have shown that peptides corresponding to $CD8^+$ T-cell epitopes can partially protect mice in a lethal VACV intranasal or ECTV challenge model (148,152,239). In one large study (152), 49 different H-2b restricted epitopes were tested. The epitopes varied greatly in their ability to confer protection, ranging from complete protection with minimal disease to no protection at all. Binding affinity partially predicted protection efficacy, but epitope immunogenicity and recognition of infected cells offered the best correlation.

Status of second and third generation smallpox vaccines

Although the smallpox vaccine was extremely effective, the method of production in the skin of animals does not meet current safety standards. This problem was overcome by upgrading to tissue culture production for second-generation smallpox vaccines (e.g. ACAM2000 and Elstree-BN produced from the New York City Board of Health and Elstree/Lister strains, respectively), which are comparable in immunogenicity to the parent vaccines (240). ACAM2000 is derived from a single plaque, which was chosen as representative after tissue culture and animal testing. However, these second generation vaccines are also likely to retain the risk of serious side effects, particularly for recipients with an immunodeficiency, eczema, and certain other conditions. For these reasons, there are ongoing efforts to develop and license highly attenuated vaccines.

With few exceptions, the strains of VACV used for vaccination during the smallpox era were grown in animal skin, thereby preserving their virulence to some degree. Efforts were made to attenuate VACV by repeated tissue culture or chorioallantoic membrane passage, and several such strains were immunogenic and produced milder skin lesions in humans but were not evaluated for protection against smallpox (3). Two such strains, LC16m8 and MVA, have been reexamined in recent years as third generation smallpox vaccines and as recombinant vaccines for other infectious diseases and cancer.

LC16m8 was derived from a small plaque isolate of VACV Lister (241) and used to immunize more than 100,000 infants from 1973–1975 as well as a large number of adults from 2,002–2,005 in Japan without serious side effects. In the latter study, there was a high percentage (>90%) of vaccine takes and neutralizing antibody determined by plaque-reduction assay (242). The small plaque size and attenuation is due to a truncation of the B5R gene encoding an EV-specific membrane protein (243) (Table 1). Although the B5 protein is an important target of EV-neutralizing antibody, the protection conferred by vaccination with LC16m8 was equivalent to that produced by traditional licensed vaccine strains for mice challenged intranasally with VACV, rabbits challenged intradermally with RPXV, mice challenged with ECTV by aerosol and monkeys challenged intravenously with MPXV (244–246). In mice, LC16m8 elicited antibody and T cell responses that were similar to the previously licensed Dryvax vaccine (244). Apparently, the modest attenuation due to the mutation of the B5 protein does not greatly impair immunogenicity in humans or protection in animal models.

MVA, a host-range restricted strain of VACV derived by >500 serial passages in chick embryo fibroblasts, was administered to >100,00 individuals in Germany without significant adverse reactions (247,248). MVA replicates well in avian cells but very poorly in most mammalian cells with a few exceptions (249–252) and is extremely attenuated in immunocompetent and immunodeficient animals (215,253,254). Despite the host range restriction, early and late viral protein synthesis is unimpaired in non-permissive human tissue culture cells and the observed defect is in virion maturation (255). The complete genome sequence revealed the presence of six large deletions and numerous mutations with

a loss of approximately 30 kbp compared to the parent strain (256). Clonally purified MVA isolates are stable and are not pathogenic for severely immunodeficient SCID and AGR129 mice (215,257). Marker rescue studies indicate that the host range restriction is primarily due to 2–3 mutations near the left end of the MVA genome but the genes disrupted were not precisely identified (258). The failure to mimic the host range defect of MVA by producing the six large deletions in the parent VACV (259) or restoring the host range of MVA by replacing the F11L and K1L genes (260) are consistent with the earlier marker rescue data mentioned above. The mutations in MVA involve cell signaling and immune defense as well as host range functions. In contrast to other strains of VACV, MVA induces NF-κB activation (261–263), type I interferon (264,265) and chemotactic factors that enhance immigration of leukocytes to the site of infection (266), which may contribute to immunogenicity.

Mice inoculated intramuscularly with MVA developed virus-specific $CD8^+$ cells and antibodies to purified virions and membrane components of the intracellular and extracellular forms of vaccinia virus in a dose-dependent manner (215). Moreover, after one or two inoculations of MVA, the T-cell numbers and antibody titers equaled or exceeded those induced by skin scratch inoculation of Dryvax and were protected against an intranasal challenge with VACV (215). Both skin scratch, which corresponds to the conventional method of smallpox vaccination, and intradermal vaccination have been reported to provide better immunogenicity and a dose-sparing effect compared to the intramuscular route (217,267). Other studies also found comparable protection of mice with MVA and the Lister strain of VACV, though there was less of a difference in dose requirements between the two (268). Post-exposure vaccination was also protective in the ECTV mouse model (195,213).

Consistent with these mouse studies, monkeys immunized with one dose of Dryvax, one dose of MVA followed by Dryvax or two doses of MVA had similar antibody responses to MV (L1) and EV (A33 and B5) protective antigens, neutralizing antibody, and $CD8^+$ T-cell responses and were similarly protected against severe MPXV infection as judged by virus load and clinical signs (Fig. 4), though there were more skin lesions in the MVA only immunized animals (176). Additional inoculations of MVA may further increase antibody responses (269). The pre-immunization with MVA reduced the size of a subsequent Dryvax take without reducing the final immune responses (176). After a single injection, MVA produced more rapid protection than Dryvax, which was attributed to the higher dose and corresponding more rapid antibody response with the former (270).

In humans receiving MVA, there was also a dose-dependent effect on antibody production but not for T-cells, and intradermal inoculation provided a dose-sparing effect in one study (271,272). Serum from individuals receiving two doses of MVA had as high or higher neutralizing antibody to variola virus as Dryvax. As in monkeys, prior immunization with MVA reduced human skin lesions produced by Dryvax, suggesting that priming with MVA might reduce complications of a replicating vaccine (273). The extreme attenuation of MVA plus its good immunogenicity support the further evaluation of the vaccine. Disadvantages of an MVA vaccine include the current use of primary chick cells for propagation and high dose requirement when given intramuscularly.

In addition to the third generation smallpox vaccines described above, VACV mutants and recombinants designed to enhance safety or immunogenicity have been described but not yet clinically tested. Some examples are deletion of the A41L gene (274) or E3L gene (275) and insertion of the IL-15 gene (276). Another approach to enhance specific immunity, not permitted by present World Health Organization guidelines, would be to replace certain envelope protein genes of a highly attenuated virus such as MVA with VARV homologs.

Concluding remarks

The correlates of protective immunity to smallpox cannot be known with certainty since the disease was eradicated prior to the development of modern immunology. Live VACV elicits T-cell and antibody responses to a plethora of protein targets in the virus. Consequently, new vaccines must mimic the broad immune responses elicited by live VACV in humans and/or provide potent protection against related OPXVs in relevant animal models. Although $CD8^+$ T cells are important in primary infections, antibodies appear to have the dominant role in secondary infection and following vaccination. Targets of protective antibody have been identified by immunization with purified proteins, DNA encoding specific proteins, and with monospecific or monoclonal antibodies. Combinations that elicit or include antibodies to both MV and EV proteins have proven superior to single proteins in animal models. Nevertheless, there are still gaps in our understanding since attempts using purified proteins to deplete neutralizing antibody from the sera of individuals that have received live VACV have been unsuccessful or only partially successful. Efforts to produce a safer yet efficacious smallpox vaccine have centered on strains of VACV that have been attenuated through tissue culture passage. Strains of VACV that have been genetically modified by recombinant methods might have advantages but it is unclear whether there is sufficient motivation to move them forward to licensure.

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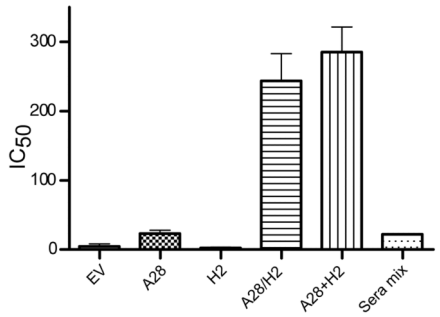

Fig. 1. Synergistic induction of neutralizing antibody following immunization with DNA encoding VACV A28 and H2 proteins

Plasmids expressing codon optimized A28 and H2 were adsorbed to gold beads and injected 4× at intervals into mice using a gene gun. Sera were tested for ability to neutralize VACV MVs and the half maximal inhibitory concentration (IC_{50}) values are indicated. EV, gold bead without empty vector plasmid; A28, gold bead with A28 plasmid; H2, gold bead with H2 plasmid; A28/H2, A28 and H2 plasmids on same gold bead; A28 + H2, mix of A28 and H2 beads injected into same site. Sera mix refers to a mixture of sera from mice immunized with A28-gold beads and mice immunized with H2-gold beads. Adapted from (118).

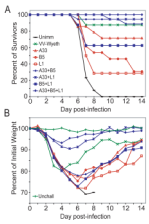

Fig. 2. Protection of mice, immunized with a single recombinant protein or combinations of recombinant proteins, to an intranasal challenge with 2 × 10^7 PFU of VACV strain WR

Mice were immunized 4× with 10 μg of an individual recombinant protein or with mixtures containing 10 μg of each recombinant protein or with VACV Wyeth (derived from Dryvax). The percent of surviving mice (A) and percent of initial weight (B) are averages from two separate experiments with a total of 13 to 19 mice in each group. Abbreviations: Unimm, unimmunized; Unchall, unchallenged. From (223).

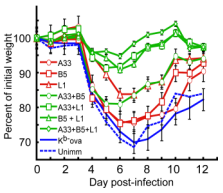

Fig. 3. Protection of mice, passively immunized with a single mAb or combinations of mAbs, to an intranasal challenge with 2×10^7 PFU of VACV strain WR

Groups of four 14-week-old female BALB/c mice were immunized intraperitoneally with 100 μg of A33, B5, or L1 mAb or combinations as indicated. All immunized mice survived except for one mouse that received anti-B5 alone and all control mice that received mouse anti-K^b-ova mAb. Percent of initial mean weights +/− SEM are shown. Modified from (237).

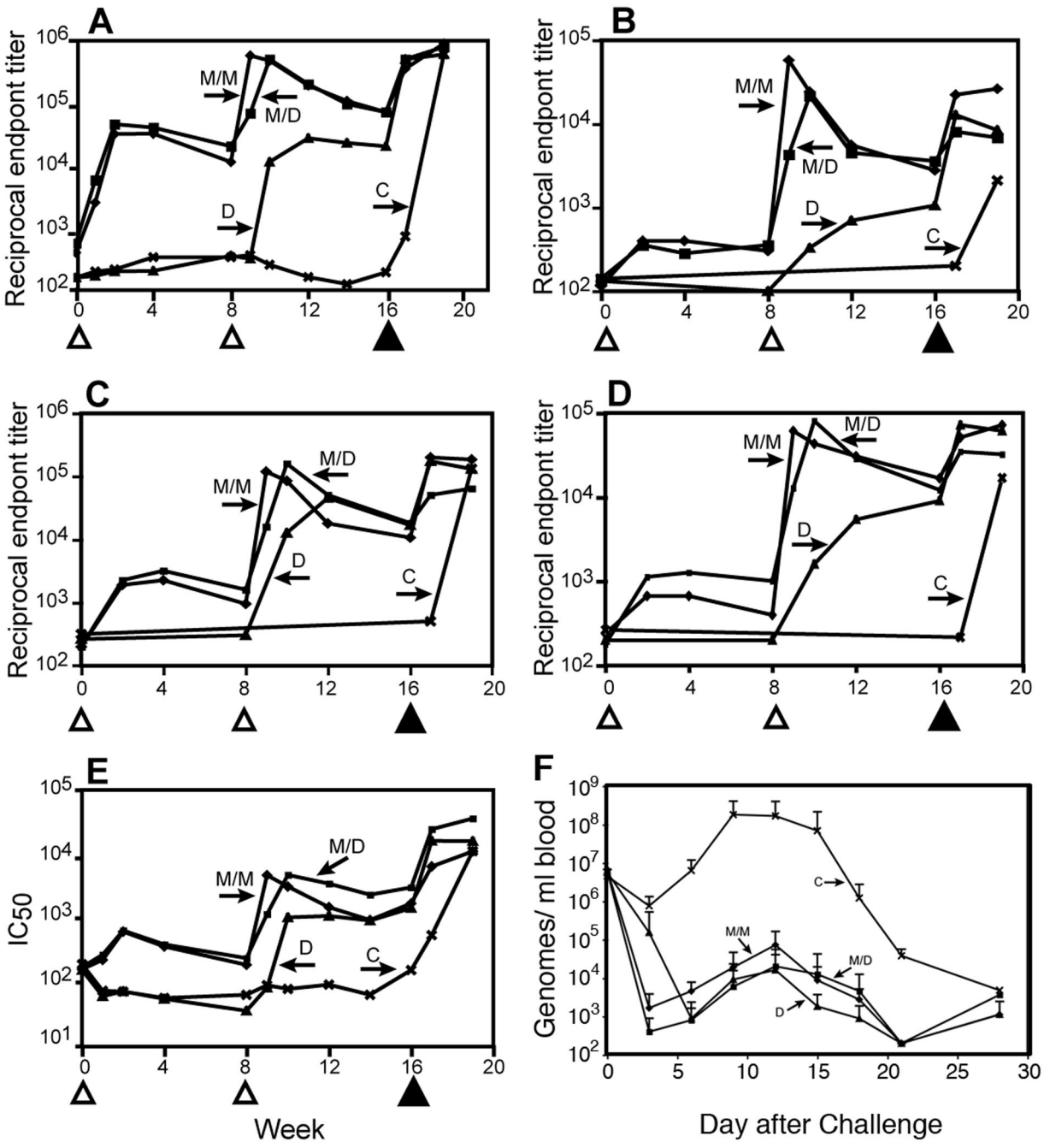

Fig. 4. Comparisons of antibody responses and protection induced by prophylactic immunization with MVA and Dryvax

Monkeys were injected intramuscularly with 10^8 PFU of MVA at 0 time and boosted at 8 weeks with MVA (M/M) or by skin scarification with Dryvax (M/D) or with Dryvax alone at 8 weeks (D) or were unimmunized (C). Antibodies that bound to immobilized purified VACV (Panel A), L1 protein (Panel B), A33 protein (Panel C), B5 protein (Panel D) or neutralized VACV infectivity (Panel E) are shown. At 16 weeks, the monkeys were challenged intravenously with a lethal dose of MPXV and the viral load was determined as the number of MPXV genomes (Panel F). Modified from (176).

Table 1

Targets of neutralizing and protective antibodies

Protein	Location	Role	Neutralize	Comet inhibit	Protection
A17	MV	Assembly, fusion	MV	−	NT[a]
A27	MV	Attachment	MV	−	+
A28	MV	Entry/fusion	MV	−	+
D8	MV	Attachment	MV	−	+
H3	MV	Attachment	MV	−	+
L1	MV	Entry/fusion	MV	−	+
A33	EV	Spread	−	+	+
B5	EV	Spread	EV	+	+

[a]Not tested