protection from a lethal intranasal challenge with vaccinia virus in BALB/c mice with only mild disease (as measured by weight loss). This protection was achieved with as little as two doses given only two weeks apart and challenge of the mice three weeks after the boost vaccination (53). Addition of the A27 protein to the other three proteins to form a tetravalent vaccine provided little additional benefit in mice (53). This is a remarkable achievement considering that an immune response to only three proteins can provide protection from a virus that encodes 200 proteins. In addition to these four proteins, other MV targets of neutralizing antibodies such as D8 and H3 have been explored, though they have not been shown to greatly enhance the ability of the trivalent protein vaccine (A33, B5, L1) to protect against disease symptoms (77,78).

The focus of protein subunit vaccination has mainly been on the envelope proteins of poxviruses that would target the infectious forms of the virus. However, poxviruses also encode a large assortment of nonstructural proteins that encode immune response modifiers (IRMs) (82). These proteins allow poxviruses to dampen or alter the immune response of the host to more efficiently spread throughout the host and ultimately infect the next host. Xu et al. (83) identified that the interferon (IFN) α/β binding protein encoded by the orthopoxvirus ectromelia virus (ECTV) EVM166 gene was critical for the efficient replication and spread of ECTV within its natural host, the mouse. With this in mind, they vaccinated mice with purified EVM166 protein to induce an antibody response that could neutralize the protein's biological activity. They found that vaccinated mice challenged with a lethal dose of ECTV (by a footpad infection) were protected against death with only mild-to-moderate disease symptoms (83). This was the first demonstration that a nonstructural protein could be used in a subunit vaccine to interfere with the ability of a virus to modulate the host immune response. This approach may be useful in future subunit smallpox vaccines, although it would be critical to determine which IRMs are most important for the replication and spread of smallpox.

While subunit vaccines have shown protection from vaccinia virus challenge, it is also important to show the ability of a vaccine to protect against a viral challenge in its natural host. Thus, the ECTV (mousepox) challenge of mice has been a useful model since ECTV is a natural pathogen of the laboratory mouse *(Mus musculus)*. Fang, et al. found that immunization with two doses of a single EV protein, A33, could partially protect BALB/c mice from death with a lethal dose of ECTV by footpad (47). By combining EV and MV targets, protein vaccinations with A33, B5, and L1 were able to fully protect against an intranasal ECTV challenge with only mild disease symptoms observed (53).

A monkeypox model of poxvirus infection has also been studied using protein vaccination. This model is important because monkeypox represents a known human pathogen, and it is believed that if monkeys can be protected from monkeypox, it is likely that a similar immune response in humans could provide similar protection. Because of the expense of nonhuman primate studies and the need to have a model with a reproducible outcome of death in unvaccinated controls, the monkeypox model in nonhuman primates has focused on a high-dose intravenous challenge (34). There are obvious disadvantages of this model. One disadvantage is that the high-dose intravenous challenge bypasses the natural acquisition and spread of the virus in the host and is thought to reproduce mainly the stage of secondary viremia. Thus, this type of challenge sets a very high hurdle for a vaccine to show protection, since natural acquisition of infection is likely caused by a much lower dose, which may be more easily controlled by vaccination. It is of equal concern that an intravenous challenge may accentuate the protection of vaccines that rely mainly on antibody responses that neutralize the incoming virus. Nevertheless, protein vaccination has been shown to protect monkeys from challenge. Heraud, et al., injected the monkeypox orthologs of A27, A33, B5, and L1 into rhesus macaques and found that these monkeys were completely protected from death with a lethal intravenous challenge with monkeypox, though they exhibited varying degrees of morbidity (84). Similarly, a small pilot study with the vaccinia virus A33, B5, and L1 proteins showed protection from severe disease after monkeypox challenge (80). Future studies using the monkeypox model will need to examine vaccine protection using more natural modes of challenge and will have to determine if adjustments in the vaccine formulation could enhance protection.

Protein vaccination, in general, requires proper formulation to induce an effective immune response to the injected antigens. Varying the source of protein, amounts of protein, site of injection, and adjuvant can all play a role in the ability of the protein vaccination to elicit a potent and effective immune response. Live vaccinia virus vaccination with a fully protective vaccine such as Dryvax resulted in Th1-type cellular and humoral responses (52,85). For protein vaccination, appropriate adjuvants that skew the immune response toward a Th1-type response were shown to produce the best protection from both morbidity and death (53,80,81,84).

While live vaccinia virus vaccination provides cross-protection against various orthopoxvirus infections, there is concern that a subunit smallpox vaccine based on vaccinia virus proteins might miss important epitopes present in the VARV ortholog proteins. Compared to live virus vaccination, the small differences of just a few amino acids between the vaccinia virus and VARV proteins may be amplified in a subunit vaccine that relies on just a few proteins to confer protection. For example, anti-B5 monoclonal antibodies have revealed that there are protective epitopes on the vaccinia B5 protein that are not present on the variola B5 ortholog (86). Similar findings have been reported with differences between the vaccinia virus A33 protein and the monkeypox A33 ortholog (71). Thus, another strategy that is being pursued by many groups is to use the VARV protein orthologs. For example, vaccination with smallpox orthologs of the vaccinia virus A27, B5, and D8 proteins provided complete protection from vaccinia virus challenge (79). Importantly, in this study it was found that the antibodies induced were at least as efficient at binding VARV protein as their vaccinia virus counterparts. Further studies will be needed to determine if VARV proteins can provide greater protection against smallpox virus than vaccinia virus proteins can confer.

DNA-Based Subunit Vaccines

DNA vaccination involves the introduction of recombinant DNA plasmids that encode relevant protein antigens (87). The DNA plasmid is introduced into mammalian cells at the injection site, where the protein is then expressed. This is thought to have a number of advantages over simply vaccinating with a purified protein. (*i*) Using the normal host cell machinery to produce the protein, rather than using bacterial or baculovirus produced proteins, may create a more