antigenically authentic protein to what would be made during a natural infection; (*ii*) By producing the protein within cells, it may generate a stronger T-cell response through normal major histocompatibility complex (MHC) class I presentation; (*iii*) Multiple gene targets can be easily included in a vaccine; and (*iv*) Lyophilized DNA can be stored at room temperature for long periods of time without degradation. These advantages have led a number of laboratories to pursue subunit DNA smallpox vaccines.

Galmiche et al. were the first to demonstrate that protective responses could be generated by A33R or B5R DNA vaccines (65). Similar to what they found with protein vaccination, intramuscularly injecting DNA encoding either the A33 or B5 protein (but without the need for additional adjuvants) resulted in 100% survival of vaccinia virus challenged mice. This work led Hooper et al. to create a bivalent DNA vaccine encoding both the MV and EV proteins L1 and A33. Using a ''gene gun,'' a device created to inject DNA coated on gold beads, they were able to show 100% survival and only mild disease symptoms after challenge by the intraperitoneal route with a lethal dosage of vaccinia virus (50). Hooper et al. later expanded upon this work by including the A27L and B5R genes to make a tetravalent vaccine (51). While they found that a bivalent A27L and B5R DNA vaccine did not give complete protection, the tetravalent vaccine (A27L, A33R, B5R, and L1R) gave complete protection from a lethal intraperitoneal vaccinia virus challenge with only mild disease symptoms (51). Pulford et al. used DNA vaccines against single MV and EV vaccinia virus protein targets to determine if they could provide protection from an intranasal challenge with vaccinia virus (88). In addition to showing that the B5R DNA vaccine offered 100% protection from challenge, they also demonstrated that smallpox subunit DNA vaccines could induce an IFNγ response and a memory response mediated by a $CD4^+$ T-cell population (88). To determine if additional antigen targets would be beneficial in a polyvalent vaccine, Sakhatskyy et al. (89) added a fifth gene, D8L, to the tetravalent DNA vaccine formulation used by Hooper, et al. (51). They found that adding the D8L gene to the other four vaccinia virus genes offered better protection in an intranasal model of challenge than without it, though protection was not 100% (89). Additionally, Sakhatskyy et al. determined that using the VARV homologs of A27L, B5R, and D8L partially protected mice from a lethal intranasal vaccinia virus challenge (79). As discussed previously, the additional use of VARV sequence to construct a vaccine may be important to ensure immune reactivity in the face of a smallpox challenge.

The lack of complete protection from morbidity seen by some investigators with the polyvalent DNA vaccines (Table 2) could be due to the method of DNA delivery and the type of immune response that was generated. To determine if this was the case, Hooper et al. used a novel method of skin electroporation to deliver their DNA vaccine (90). They found that this method of delivery improved the efficacy of their tetravalent DNA vaccine (A27L, A33R, B5R, and L1R), and provided complete protection from challenge in an intranasal model of infection. They found that skin electroporation mimicked to a greater extent the type of antibodies produced during Dryvax vaccination, by inducing more mouse IgG2a antibodies (Th1 response), than the gene gun method of DNA delivery (90). This finding makes the skin electroporation method a more attractive method of DNA delivery than the gene gun method. The mode of vaccination was further highlighted by work in nonhuman primates. Hooper et al. found that they could generate complete protection from an intravenous monkeypox virus challenge using their tetravalent (A27L, A33R, B5R, and L1R) formulation delivered by a gene gun (91). However, Heraud et al. found that when the monkeypox homologs of A27L, A33R, B5R, and L1R were injected as naked DNA, there was no protection from monkeypox challenge (84). Going forward, smallpox DNA vaccines will need to be administered in a way best able to generate a Th1-type immune response that includes both neutralizing antibodies and strong T-cell responses.

Vector-Based Subunit Vaccines

Vectored vaccines utilize a nonpathogenic virus or bacteria to deliver a desired antigen. Because protein and DNA vaccinations have been shown to require multiple vaccinations to achieve protective immunity, vectored vaccines have been pursued as a way to generate a smallpox vaccine that can offer protection in a single vaccination. In a smallpox outbreak setting, it would be important to induce protective immunity as rapidly as possible to avoid spread of the virus. The first laboratory to explore vector subunit smallpox vaccines utilized replicon particles of Venezuelan equine encephalitis virus (92). By expressing A27, A33, and B5, they generated a strong mouse IgG2a antibody response (Th1-type response), and protected mice from a sublethal dose of cowpox virus. Kaufman et al. utilized replication incompetent recombinant Adenovirus serotype 35 (rAd35) vectors expressing A27, A33, B5, and L1 antigens (54). By delivering a single immunization with all four rAd35 vectors, they were able to achieve complete protection in mice from a lethal intranasal vaccinia virus challenge. The rAd35 vaccine generated strong MV neutralizing antibodies that were balanced between mouse IgG2a and IgG1 antibodies (Th1 and Th2 response) (54). Vectored vaccines so far appear to be a promising delivery method for subunit smallpox vaccines, but much work is still needed to determine the immunogenicity and safety profile in nonhuman primates and humans.

CONCLUSIONS

While stockpiling of a live vaccinia virus vaccine grown in cell culture has been successful, significant concerns about the minor and major complications from this vaccine remain, especially in populations that have contraindications for vaccination. More attenuated live vaccinia virus vaccines, which will be much safer to give to a diverse population, will likely be the next new generation smallpox vaccine that gains regulatory approval. However, growing and maintaining a stock of a live virus vaccine, as well as the potential for adverse events, are limitations that fuel the continued pursuit of future generation smallpox vaccines. Subunit vaccines are showing great success. Many possibilities for protective vaccines exist, and future efforts to directly compare different vaccination strategies will be needed. For example, Barefoot et al. chose a single immunogen, B5, and compared multiple vaccination strategies for generating immune responses and examined the level of protection from challenge (93). They found that a heterologous prime-boost combination of recombinant vesicular stomatitis virus (rVSV) expressing B5 and recombinant Venezuelan equine encephalitis virus replicons (VRP) expressing B5 as the most synergistic regimen. A possible scenario is that the best protection from challenge may incorporate a combination