glycoprotein needed for the optimal production of an infectious form of virus critical for virus spread. Thus, while LC16m8 can grow and make infectious particles, it spreads poorly in cell culture. LC16m8 has been shown to generate protective immunity in mice (24), rabbits (24), and nonhuman primates (25). However, there are two important concerns about this vaccine. Since the key attenuating mutation in B5R is a one-base deletion that results in a frame-shift and early truncation of the B5 protein (23), there is evidence that virus can revert to wild-type during growth (26). An additional concern is that the B5 protein is an important protective target of the humoral immune response to live vaccinia virus vaccination (27,28), and this aspect of protection may be lost in an LC16m8 vaccinated individual.

Modified Vaccinia Ankara

In Germany, modified vaccinia Ankara (MVA) was developed as a highly attenuated potential smallpox vaccine (29,30). This vaccine was obtained after 572 serial passages of the parental vaccinia strain on chick embryo fibroblasts. This resulted in about 25 kilobases deleted and a virus that no longer produced infectious progeny virus in almost all mammalian cell lines. That is, the virus could infect, replicate its DNA, and generate abundant amounts of key viral proteins, but could not assemble into infectious virions in most mammalian cells. Because of the inability to generate infectious virions in human cells, this type of virus would likely be safe to give to many people who have conditions that would not allow routine smallpox vaccination. Therefore, this virus has been intensively studied as a next generation smallpox vaccine that may ultimately gain FDA approval in the United States. The virus has been widely studied and shown to generate antibody responses similar to Dryvax (31), as well as protection in mouse (32,33) and nonhuman primate challenge models (34–36). There is evidence that MVA vaccination results in more rapid protection when compared to a fully replication competent vaccine, like Dryvax (37). While the mechanism for this enhanced early protection by MVA is not entirely known, part of the explanation may be that it induces more rapid immunity (37) because it is given at about a 1000 times higher dose than current replication competent vaccinia vaccines. It also appears that MVA can activate innate immune responses because it is missing genes present in replication competent vaccinia virus that encode proteins that may initially dampen the immune response (38,39).

SUBUNIT-BASED VACCINES

Until recently, it was believed that protection conferred by live vaccinia virus vaccination was predominantly due to anti-vaccinia T-cell responses. This was mainly based on the fact that inactivated smallpox vaccines did not protect against smallpox (1). Thus, it was assumed that live vaccinia virus vaccination protected by potent antiviral T-cell responses. However, the inability of experimental inactivated vaccines to protect may have been due to denaturing of key targets (40) as well as the fact that the vaccine preparations did not contain some critical antigens that are present on a minor population of infectious virus (41,42). Furthermore, in recent years, protection via vaccination with live vaccinia virus has been shown to be dependent on vaccinia specific, $CD4^+$ dependent B-cell responses (43–47). Thus, future-generation smallpox vaccines that are capable of inducing protective antibody responses are viable alternatives to the current live-virus vaccines. One way to induce such antibody responses is to provide protein(s) directly to the immune system to which neutralizing and protective antibodies can be generated. Strategies to present these critical proteins include direct injection of soluble proteins with adjuvants, introduction of recombinant DNA that host cells transcribe and translate, and live or attenuated vectors that deliver poxvirus proteins to the host immune system.

Since poxviruses are large DNA viruses that encode over 200 proteins, the identification of suitable proteins that would generate a protective immune response is complex. Most research has focused on different surface membrane proteins of the two infectious forms of virus, the mature virus (MV) and the extracellular virus (EV) (48,49). Furthermore, including targets against both MV and EV appears to provide the best protection from morbidity and mortality (50–54). Including targets against both forms of infectious virus is believed to provide a way to decrease the infecting inoculum (believed to be mainly MV), and then alter the spread and dissemination of the virus within an infected host (thought to be mainly EV) (55–59). Initial insights into appropriate targets against MV and/or EV proteins were based on the production of antibodies that could neutralize virus in vitro or provide passive protection against vaccinia virus challenge in vivo (50,60–71). Relevant protein targets were also identified by examining what proteins were recognized by vaccinia immunoglobulin (VIG) (27,28,72–74), serum from vaccinia virus vaccinated individuals that was used clinically to treat complications from live vaccinia virus vaccination. Many of the protein targets identified by these approaches are targets of potent neutralizing antibodies. The following sections and tables will cover the most widely studied viral targets and the effort that is being made to combine these targets into effective subunit vaccines.

Protein-Based Subunit Vaccines

The first successful attempt at a subunit vaccine to protect against lethal vaccinia virus challenge was by Lai, et al. in 1991 (75). They intraperitoneally injected purified vaccinia virus A27 protein (an MV protein) generated in *Escherichia coli* and found that the antibody response generated was both MV neutralizing in vitro and 100% protective against a lethal intraperitoneal challenge with vaccinia virus. The EV proteins A33 and B5, produced in baculovirus, were first shown to generate protective immune responses by Galmiche et al. (65). They found that injection of A33 or B5 protein provided 100% protection from lethal intranasal challenge with vaccinia virus. While only B5 vaccination elicited in vitro EV neutralizing activity, the antibodies produced against A33 resulted in ''comet inhibition,'' indicating that they altered the way EV spread in cell culture. Antibody to A33 may also provide protection through the activation of complement (76). Table 1 summarizes the individual orthopoxvirus genes that have been examined as a subunit vaccine. Proteins have been expressed in bacteria (44,47,65,75,77–79), baculovirus (52,53,65,80), and even recombinant plants (81).

While work with individual proteins has helped identify appropriate targets to include in a subunit vaccine, the combination of multiple proteins is believed to provide the optimum protection (Table 2). For example, A33, B5, and L1 proteins have been used in combination to generate a mouse antibody response to both the MV and EV infectious forms of vaccinia virus (52,53). These trivalent subunit vaccines provide 100%