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ABSTRACT

The baculovirus–insect cell expression system is an approved system for the production of viral antigens with vaccine potential for humans and animals and has been used for production of subunit vaccines against parasitic diseases as well. Many candidate subunit vaccines have been expressed in this system and immunization commonly led to protective immunity against pathogen challenge. The first vaccines produced in insect cells for animal use are now on the market. This chapter deals with the tailoring of the baculovirus–insect cell expression system for vaccine production in terms of expression levels, integrity and immunogenicity of recombinant proteins, and baculovirus genome stability. Various expression strategies are discussed including chimeric, virus-like particles, baculovirus display of foreign antigens on budded virions or
in occlusion bodies, and specialized baculovirus vectors with mammalian promoters that express the antigen in the immunized individual. A historical overview shows the wide variety of viral (glyco)proteins that have successfully been expressed in this system for vaccine purposes. The potential of this expression system for antiparasite vaccines is illustrated. The combination of subunit vaccines and marker tests, both based on antigens expressed in insect cells, provides a powerful tool to combat disease and to monitor infectious agents.

I. INTRODUCTION TO RECOMBINANT SUBUNIT VACCINES

Historically, vaccines have been one of the most cost-effective and easily administered means of controlling infectious diseases in humans and animals. Vaccine development has its roots in the work of Edward Jenner (1749–1823) who discovered that man could be protected from smallpox by inoculation with cowpox (Fenner, 2000) and the work of Louis Pasteur (1822–1895) who developed the first rabies vaccine (Fu, 1997). These pioneering efforts led to vaccines against diseases that had once claimed millions of lives worldwide (Andre, 2003). Childhood vaccination programs are now common practice and elaborate vaccination programs have been set up by the World Health Organization (WHO), leading to the official eradication of smallpox in 1979 (Fenner, 2000). Today large parts of the world are also declared poliomyelitis free, and measles is the next target for eradication. Vaccines have controlled major bacterial and viral diseases in humans, and effective vaccines are available against many more (Andre, 2003; Hansson et al., 2000b). Vaccination also protects our livestock and pet animals (Pastoret et al., 1997). For some diseases, however, such as malaria and acquired immunodeficiency syndrome (AIDS), vaccines are desperately sought.

Most human and animal vaccines are based on killed or live-attenuated pathogens. Killed vaccines require the production of large amounts of often highly virulent pathogens and these types of vaccines are therefore risky to produce. Another risk lies in the potential for incomplete inactivation of the pathogens. Inactivation on the other hand affects the immunogenic properties of the pathogen, and hence the efficacy as a vaccine, and it is often difficult to find the balance between efficient inactivation and conservation of immunogenicity. Live-attenuated vaccines consist of pathogens that are reduced in virulence or have been attenuated either by growing them in alternative hosts or under unfavorable growing conditions, or by recombinant DNA technology. These live-attenuated
vaccines can potentially replicate in their host, but are typically attenuated in their pathogenicity to avoid the development of severe disease. Live vaccines elicit humoral and cellular immunity, and may provide lifelong protection with a single or a few doses (Dertzbaugh, 1998; Hansson et al., 2000b; Schijns, 2003). Such long-term protection is advantageous in developing countries where individuals are often only immunized once. A drawback of live-attenuated vaccines is that they can cause side effects, which may be dangerous when used for prophylaxis in immunocompromised persons such as the elderly or individuals with genetic or acquired diseases of the immune system (e.g., AIDS or severe combined immunodeficiency; SCID). Live-attenuated vaccines may also convert to virulent strains and spread to nonimmunized persons as observed during recent poliomyelitis outbreaks (Kew et al., 2004). Adverse effects with both killed and live-attenuated vaccines can also be due to allergic reactions to components of the vaccine such as residual egg proteins in the case of influenza vaccines (Kelso and Yunginger, 2003) or gelatin in the measles-mumps-rubella (MMR) vaccine (Patja et al., 2001).

The development of vaccines is not easy for all infectious diseases and the medical and veterinary world is challenged frequently by the emergence of novel diseases such as AIDS, severe acute respiratory syndrome (SARS), and West Nile virus infection. The vaccine industry is under constant pressure for rapidly changing pathogens, for which large amounts of vaccines are needed annually, such as influenza viruses (Palese, 2004), and flexible vaccine production techniques are required. For several infectious diseases vaccines cannot be developed using conventional approaches, for instance due to a lack of appropriate animal production systems or the high-mutation frequency of the pathogen (Human immunodeficiency virus (HIV), malaria). A vaccine against the H5N1 influenza strain that is currently epidemic in Asian poultry could not be produced the classical way, by using embryo-nized chicken eggs without reducing the virulence of the virus by reverse genetics, due to high-mortality rates of the chicken embryos (Horimoto et al., 2006).

Recombinant protein production systems may provide good alternatives for the development of vaccines that are more difficult to produce in vivo for manufacture of so-called subunit vaccines. A pathogen consists of many proteins, frequently with carbohydrate moieties, but these are not all equally important for generation of an adequate immunological response. Subunit vaccines contain the immunodominant components of a pathogen and in the case of viral vaccines these are often (glyco)proteins of the viral coat or envelope such as the hepatitis B surface antigen (Valenzuela et al., 1982) or the classical swine fever virus (CSFV) E2 glycoprotein (Bouma et al., 1999). Viral coat proteins
sometimes form virus-like particles (VLPs) when expressed in heterologous systems (Brown et al., 1991), which are often immunogenic and may induce both humoral and cellular responses. The subunit vaccine against hepatitis B produced in yeast is highly successful. An extreme example of subunit vaccines are peptide-based vaccines which consist of small amino acid chains harboring the part of the antigenic protein that is recognized by antibodies. Typically, subunit vaccines do not contain the genetic material of the pathogen or only a small part thereof. Therefore, these vaccines cannot cause disease and do not introduce pathogens into nonendemic regions. An additional advantage of subunit vaccines is that they can be used in combination with specific marker tests, which make it possible to differentiate infected from vaccinated animals, the so-called DIVA vaccines (Capua et al., 2003; van Oirschot, 1999); an important issue in monitoring virus prevalence and virus-free export of animals and their products.

Immunogenic subunits can be isolated chemically from the pathogen, such as the purified capsular polysaccharides present in the Streptococcus pneumoniae vaccine (Pneumovax23; Merck). This process still requires the production of virulent pathogens, which is not without risk. An alternative is the use of recombinant DNA technology to produce protein subunits in a heterologous system, and a variety of expression systems are available (Clark and Cassidy-Hanley, 2005; Hansson et al., 2000b). The yeast system Saccharomyces cerevisiae for instance is used to produce the hepatitis B subunit vaccine (Valenzuela et al., 1982), which is currently the only licensed recombinant subunit vaccine for human use. The yeast Pichia pastoris is used for production of the antitick vaccine Gavac™ (Canales et al., 1997), which protects cattle against the tick Boophilus microplus, the transmitter of Babesia and Anaplasma parasite species. Insect cells are used to produce vaccines against classical swine fever or hog cholera (Depner et al., 2001; van Aarle, 2003). For the production of recombinant proteins in higher eukaryotics, mammalian, insect, and plant expression systems are available that either use transgenes or viral vectors for protein expression. Plants have been recognized for the production of so-called edible subunit vaccines to be administered by ingestion of vegetable foods (Ma et al., 2005; Streatfield and Howard, 2003).

This chapter concentrates on the use of cultured insect cells or larvae in combination with baculovirus expression vectors for the production of subunit vaccines. The baculovirus expression system is an accepted and well-developed system for the production of viral antigens with vaccine potential (Dertzbaugh, 1998; Hansson et al., 2000a; Vlak and Keus, 1990). This system has also been explored for development of vaccines
against protozoan parasites (Kaba et al., 2005) and for therapeutic vaccines against tumors. A vaccine against prostate-cancer (Provenge) is in phase II/III clinical trials and is based on combining recombinant prosthetic acid phosphatase (characteristic of 95% of prostate cancers) with the patient’s own dendritic cells before immunization (Beinart et al., 2005; Rini, 2002). Trials have also been initiated for a prophylactic vaccine using VLPs produced in insect cells against cervical cancer caused by Human papillomavirus (HPV) 16 (Mao et al., 2006).

Each expression system has advantages and drawbacks (Table I) and the system of choice depends very much on the specific requirements for a particular vaccine and is often based, at least partly, on trial and error. Before a definitive choice can be made, the expression levels achieved, the adequacy of posttranslational modifications, the immunological performance, the possibilities for scale-up, the costs, the risk of contamination, the method of administration, and legal aspects must all be taken into account.

| Processing/feature | E. coli | Yeast | Mammalian cells | Insect cells | Plants |
|-------------------|---------|-------|-----------------|--------------|--------|
| Glycosylation     | –       | +     | +++             | +            | +      |
| Phosphorylation   | –       | +     | + +             | +            | +      |
| Acylation         | –       | +     | + +             | +            | +      |
| Amidation         | –       | –     | + +             | +            | –      |
| Proteolysis       | +/-     | +/-   | + +             | +            | +      |
| Folding           | +/-     | +/-   | + +             | +            | +      |
| Secretion         | +/-     | +     | + +             | +            | +/-    |
| Serum free        | Not relevant | Not relevant | + | + | Not relevant |
| Yield (% dry mass)| 1–5     | 1     | <1              | Up to 30     | <5     |
| Scale-up          | +++     | +++   | +               | +            | +++    |
| Downstream        | +       | +     | ++              | ++           | – –    |
| processing        |         |       |                 |              |        |
| Costs             | Low     | Low   | High            | Intermediate | Low    |
| Safety            | + +     | + +   | + +             | ++           | + +    |
| Versatility       | +       | +     | ++              | ++           | +      |

*Adapted from Vlak and Keus, Baculovirus Expression Vector System for Production of Viral Vaccines, Advances in Biotechnological Processes 14, pp. 19–28. Copyright © (1990, John Wiley & Sons, Inc.). Reprinted with permission of John Wiley & Sons, Inc.*
II. THE BACULOVIRUS–INSECT CELL EXPRESSION SYSTEM FOR VACCINE PRODUCTION

A. Characteristics

The baculovirus–insect cell expression system (Smith et al., 1983) has been developed for the production of biologically active (glyco)proteins in a well-established and safe eukaryotic environment (Kost et al., 2005). The family Baculoviridae contains rod-shaped, invertebrate-infecting viruses, which have large double-stranded, covalently closed circular DNA genomes (Table II). The members of this large virus family are taxonomically divided into the genera Nucleopolyhedrovirus (NPV) and Granulovirus (GV), based on occlusion body morphology (Theilmann et al., 2005). NPVs express two genes, polyhedrin and p10, at very high levels in the very late phase of infection. The polyhedrin protein forms the viral occlusion bodies or polyhedra and p10 is present in fibrillar structures, which function in polyhedron morphology and in breakdown of infected cell-nuclei to release the polyhedra (Okano et al., 2006; Van Oers and Vlak, 1997). These two genes are not essential for virus replication in cell culture and, therefore, their promoters are exploited to drive foreign gene expression, which forms the basis for the baculovirus–insect cell expression system. Since baculoviruses are rod-shaped, large amounts of foreign DNA can be accommodated within the virus particle, in contrast to vaccinia and especially adenovirus expression vectors (Table II).

The type member of the NPVs is Autographa californica multiple nucleopolyhedrovirus (AcMNPV), a virus with a genome of 133 kilobase pairs (Ayres et al., 1994). This baculovirus is routinely used for foreign gene expression. The baculovirus Bombyx mori NPV is being used for vaccine purposes to a much lesser extent (Choi et al., 2000; Mori et al., 1994). Baculovirus expression vectors replicate in cultured insect cells or larvae and high yields of heterologous protein are generally obtained when the strong viral polyhedrin and p10 promoters are exploited (King and Possee, 1992; O’Reilly et al., 1992). The insect cell lines used in the baculovirus expression system are derived from lepidopteran insects (moths) and are most often Spodoptera frugiperda lines (Sf9 or Sf21) and Trichoplusia ni (High Five™) cells, which can be used in combination with AcMNPV-based vectors. B. mori cells (e.g., Bm5) are used for BmNPV. Insect cell lines vary in their characteristics in terms of growth rate, protein production, secretion efficiency and glycosylation pattern, and interference with viral genome stability (Pijlman et al., 2003b; Vlak et al., 1996). These insect cells are
| Feature                                      | Baculovirus<sup>b</sup> | Adenovirus | Vaccinia |
|----------------------------------------------|--------------------------|-------------|----------|
| Virus morphology                             | Enveloped, rod shaped    | Nonenveloped, icosahedral | Brick shaped |
| Genome structure                             | Circular dsDNA           | Linear dsDNA | Linear dsDNA |
| Genome size                                  | 130 kbp                  | ±35 kbp     | 190 kbp  |
| Expandability                                | Large                    | Low         | Intermediate |
| Particle dimensions                          | 30–60 × 250 –300 nm      | 80–110 nm   | 250 × 250 × 200 nm |
| Replication site                             | Nucleus                  | Nucleus     | Cytoplasm |
| Replication in humans                        | None                     | Replication competent or defective | Yes |
| Progeny virus                                | Budding BVs/lysis ODVs   | Accumulation in the nucleus | Exocytosis/lysis |
| Pathogenicity for mammals including humans   | Nonpathogenic            | Low due to host defense and attenuation | Reduced with modified strains |
| Immunological complications                 | Complement inactivation  | Strong protective responses of the host | – |
| Immunological history                        | –                        | Preexisting immunity due to natural infections | Preexisting immunity due to smallpox vaccination |
| Protein production system in cell lines      | Yes                      | Less frequently | Yes |
| Applications:                                |                          |             |          |
| Antigen display vector                       | Surface display vectors  | No          | No       |
| Carrier DNA vaccine vector                   | Yes                      | Yes         | Yes      |
| Gene therapy                                 | +                        | ++          | –        |
| Vaccine examples                             | Therapeutic prostate cancer vaccine (see text for further information) | Immunomodulators, therapeutic cancer vaccines | Mucosal immunity against tuberculosis and HIV |

<sup>a</sup> Gherardi and Esteban, 2005; Russell, 2000; Young <em>et al</em>, 2006; Universal data base of International Committee on Virus Taxonomy (http://www.ncbi.nlm.nih.gov/ICTVdb/index.htm; January 2006).

<sup>b</sup> AcMNPV, <em>Autographa californica</em> multiple nucleopolyhedrovirus.
relatively easy to maintain and many grow equally well in suspension in large volumes (up to 2000 L reactions) and at high densities as on solid supports, and can be cultivated in serum-free media which facilitates purification of recombinant proteins. Unlike mammalian cells, they do not require CO₂ and can easily withstand temperature fluctuations. An extra advantage is that the chance of contamination with human or mammalian viruses, especially in serum-free cultures, is small compared to mammalian production systems because these vertebrate viruses do not replicate in lepidopteran cells. These cells do not support the growth of mammalian mycoplasmas either. Instead of insect cells, whole insect larvae may be used as live bioreactors for vaccine production. The use of whole insect larvae has the advantage that the simple insect-rearing technology and downstream processing can be exploited. Such in vivo production could be performed by small-scale local industries, especially if the larvae can be fed directly to animals such as for an experimental Newcastle disease vaccine for chickens (Mori et al., 1994). Such vaccines are less well defined however and quality control may therefore be more difficult to achieve.

Expression of proteins in insect cells allows for appropriate folding, posttranslational modification, and oligomerization and therefore, biological activity is normally preserved. Protein glycosylation in insects and mammals is not identical though: the N-glycan–processing pathway in insects results in glycoproteins with paucimannose glycan groups, in contrast to mammalian glycoproteins which contain complex sialylated glycans (see also Harrison, this volume, pp. 159–191). The exact glycan composition varies between different insect cell lines (Kost et al., 2005; Tomiya et al., 2004). In general, glycan groups are not very immunogenic and therefore this does not seem to be a major disadvantage for subunit vaccines. In situations where more authentic glycosylation is required, for instance for preserving functional activity, transformed “humanized” insect cell lines expressing mammalian glycosylation enzymes are available (Jarvis, 2003; Kost et al., 2005; Tomiya et al., 2004). For some insect cell lines it has been reported that fucose groups are added to N-glycans. The impact of this remains to be determined, but since fucans may cause allergic reactions, it may be a point for consideration when choosing an insect cell line for vaccine production (Long et al., 2006; Tomiya et al., 2004).

B. Baculovirus Vectors

Originally, the baculovirus expression system was based on the allelic exchange of the baculovirus polyhedrin gene for a heterologous
gene by recombination in insect cells (Smith et al., 1983). In a similar way, baculovirus vectors have since been developed which exploit the nonessential very late baculovirus p10 promoter (Vlak et al., 1990; Weyer and Possee, 1991). Vectors that leave the polyhedrin gene intact can be used for the production of recombinant proteins in insect larvae (Fig. 1). The in vivo recombination protocol was improved by using linearized viral DNA in the allelic replacement, which resulted in dominant selection and much higher percentages of recombinant viruses (Kitts et al., 1990; Martens et al., 1995). In vectors of this type (BacPAK™ vectors, BaculoGold™, Bac-N-Blue™) the linearized viral DNA carries a lethal deletion (ORF1609) and becomes replication competent only after recombination with a transfer plasmid carrying the foreign gene, thereby restoring the deletion (Kitts and Possee, 1993). Baculovirus vectors based on Gateway technology (BaculoDirect™) are linear baculovirus vectors in which foreign genes are introduced through site-specific in vitro recombination.

At about the same time, another efficient and rapid method for generation of recombinant baculoviruses was developed (Luckow et al., 1993) that employed transposition of a foreign gene expression cassette from a donor plasmid into a bacterial artificial chromosome (BAC) which contains the entire AcMNPV genome (bacmid). In this system (Bac-to-Bac™) recombinant baculovirus genomes are generated in Escherichia coli and then used to transfect insect cells to obtain recombinant baculovirus particles. After generating high-titer virus stocks, insect cells are infected to produce recombinant proteins. With the bacmid-based methodology the time to generate recombinant viruses is reduced considerably. Another advantage is that the recombinant bacmid can be stored in E. coli and recovered when needed. A disadvantage is that the bacterial gene cassette present in bacmid-derived viruses may easily be lost during virus passaging (Pijlman et al., 2003a). In addition to AcMNPV, bacmids have also been constructed for Spodoptera exigua MNPV and Helicoverpa armigera SNPV (Pijlman et al., 2002; Wang et al., 2003). The most recent method combines bacmid technologies with allelic replacement (FlashBac™; Oxford Expression Technologies) and thereby removes the BAC sequences from the viral genome. This latter system is especially suitable for high-throughput screening.

Over the years, novel baculovirus vectors have been developed with special features and for more specific applications: transfer vectors have been modified to express polyhistidine-tagged proteins for easy purification (pFastBac-His™). Transfer vectors with dual, triple, or quadruple promoters usually p10 and polyhedrin, have been developed
FIG 1. Flow chart showing four different methods to make a vaccine based on your favorite gene (YFG) in the baculovirus expression system: (1) protein expression in insect cell bioreactors using the polyhedrin locus for expression, (2) protein expression in insect larvae leaving the polyhedrin gene intact; expression is driven either by a duplicated p10 promoter or by the original p10 promoter, (3) baculovirus surface display methods where YFG is fused to GP64, and (4) DNA vectors with a mammalian promoter (mp) for synthesis of your favorite gene product (YFGP) in the target species. Subsequent steps in the process are: (A) selection and PCR amplification of YFG, (B) cloning into the appropriate baculovirus vector, (C) generation of recombinant BV particles or occlusion bodies, (D) production in insect cells in bioreactors or larvae, (E) purification of recombinant YFGP or collection of BVs loaded with either YFGP or YFG, and (F) delivery of prophylactic or therapeutic vaccines.
for allelic replacement (Belyaev and Roy, 1993; Weyer and Possee, 1991); dual (pFastBacDual\textsuperscript{TM}) and quadruple vectors (Tareilus et al., 2003) have also been developed for bacmid technology. Such multiple vectors can be used to express various proteins simultaneously, and hence are useful for producing multimeric complexes, including viral capsids consisting of more than one viral protein (Belyaev and Roy, 1993). Balancing expression levels is sometimes a problem in these vectors and may require coinfection with a vector expressing only the dominant protein, or a modification of the promoters. One of the promoters in multiple promoter vectors may be used to express a reporter gene, such as green fluorescent protein (GFP), which makes it easy to follow the infection process in cells, perform virus titrations, and track baculovirus infection in the insect (Cha et al., 1997; Kaba et al., 2003). The recently developed vector system (UltraBac) uses the baculovirus late basic protein (P6.9) promoter to express GFP together with the foreign gene to allow earlier monitoring of infection (Philipps et al., 2005).

Baculovirus surface display vectors (Grabherr et al., 2001) expose the antigen on the surface of budded baculovirus particles. This is achieved by fusing the foreign antigen to the baculovirus envelope glycoprotein GP64 (Monsma et al., 1996). The chimeric protein is transported to the cell membrane and is taken up in the viral envelope during budding. This system has also been combined with bacmid technology (Kaba et al., 2003). The recombinant budded virus (BV) particles and lysates of cells infected with a display vector have been shown to evoke protective immune responses (Kaba et al., 2005; Tami et al., 2004; Yoshida et al., 2003). Baculovirus vectors that express foreign genes in fusion with polyhedrin along with wild-type polyhedrin allow for incorporation of antigens into baculovirus occlusion bodies (Je et al., 2003). These occlusion bodies are stable and easy to purify and can be used directly for immunization (Wilson et al., 2005).

C. Adaptations for Secreted Proteins

Expression of surface (glyco)proteins that go through the export pathway is in general more difficult than expression of soluble cytoplasmic proteins and results in much lower yields (van Oers et al., 2001). To increase the production level, surface proteins are often expressed as secreted proteins by removing hydrophobic transmembrane regions (TMR) that serve to anchor the protein to cell membranes. Removing these domains by recombinant DNA technology leads to secreted proteins which can then be purified from the culture
medium. However, some caution is needed because this approach may affect folding and vaccine efficacy.

Not all proteins present at the surface under native conditions are automatically transported to the cell surface when expressed in insect cells, such as the p67 surface protein of the bovine parasite *Theileria parva* (Nene *et al.*, 1995). When the original signal peptide was replaced with an insect analogue, such as the honeybee mellitin signal peptide (Tessier *et al.*, 1991), p67 was properly routed to the cell surface (Kaba *et al.*, 2004a). A similar routing of p67 to the export pathway could be obtained by fusion to GP64 in a surface display vector (Kaba *et al.*, 2002), where the GP64 signal peptide directed the protein to the cell surface.

Membrane and secreted proteins pass through the endoplasmic reticulum (ER) and the Golgi apparatus on their way to the cell surface and may become glycosylated during this process. The abundant baculovirus protein chitinase is also transported to the ER and accumulates there due to a KDEL retention sequence (Saville *et al.*, 2004; Thomas *et al.*, 1998). Chitinase is expressed in the late phase of baculovirus infection and is involved in the dissolution of the insect chitinous cuticle to enhance the spread of viral occlusion bodies (Hawtin *et al.*, 1997). Deletion of chitinase from the baculovirus vector resulted in higher levels of secreted recombinant protein (Possee *et al.*, 1999) possibly because chitinase “clogs up” the protein translocation machinery and competes with recombinant secretory proteins. The FlashBac system described earlier lacks this chitinase gene. Another baculovirus protein, v-cathepsin also accumulates in the ER and is activated on cell death by proteolytic cleavage (Hom *et al.*, 2002). Processing of pro-v-cathepsin into active cathepsin is also triggered by chaotropic agents, such as sodium dodecyl sulfate, and this may result in proteolysis of recombinant proteins during extraction and purification (Hom and Volkman, 1998). A bacmid vector that lacked both chitinase and v-cathepsin (AcBacΔCC) improved the stability of a secreted recombinant protein, thereby increasing the yield of full-length protein molecules (Kaba *et al.*, 2004a).

Folding of complicated transmembrane glycoproteins can be improved by coexpression of molecular chaperones. The serotonin transporter (SERT) protein is a brain glycoprotein with 12 predicted transmembrane domains. Coexpression of the chaperones calnexin and, to a lesser extent, of immunoglobulin heavy chain-binding protein (BiP) or calreticulin increased the yield of functional SERT threefold. The foldase ERp57 did not have this effect (Tate *et al.*, 1999). Calreticulin and calnexin were also shown to increase the level of active lipoprotein lipase when coexpressed
in insect cells, and to stimulate dimerization of the recombinant protein (Zhang et al., 2003). Expression of calnexin and calreticulin in a stable transgenic insect cell line, which was than infected with a recombinant baculovirus, resulted in a lower ratio of secreted versus intracellular recombinant protein than when cells were coinfected with two baculoviruses, one carrying the gene of interest and the other a chaperone (Kato et al., 2005). This result suggests that chaperone expression levels should be of the same order as recombinant protein levels.

D. Baculovirus Vectors with Mammalian Promoters

Another special adaptation is the incorporation of mammalian promoters in baculovirus vectors to drive foreign gene expression. Baculovirus vectors with mammalian promoters (BacMam™ viruses) have the potential to serve as gene delivery vectors in gene therapy (Huser and Hofmann, 2003; Kost and Condreay, 2002) and have also been tested for vaccination purposes (Abe et al., 2003; Aoki et al., 1999; Facciabene et al., 2004; Poomputsa et al., 2003). In this case, a mammalian promoter or a viral promoter active in mammalian cells, such as the human cytomegalovirus (HCMV) IE1 promoter, drives intracellular expression of the antigen. Exposure on the cell surface via the major histocompatibility complex (MHC) activates the cellular immune system and in this respect, these types of vaccines resemble DNA vaccines. BacMam™ vectors are produced in insect cells and are replication incompetent in mammalian cells (Table II). A further advantage is that multiple genes can be inserted simultaneously into the baculovirus genome allowing for multivalent vaccines. Expression of multiple proteins is an advantage of the baculovirus expression system over other systems, especially adenovirus vectors, where the maximal increase in genome size is more limited due to packaging restrictions.

E. Adaptations for Vector Genome Stability

For manufacturing subunit vaccines, large-scale production units will be needed, for instance for the production of malaria or the annual influenza subunit vaccines. Baculovirus–insect cell systems have been scaled-up to large-scale cultures in either fermentors (bioreactors) or cellbag devices (WAVE reactors). Insect cell bioreactors up to 2000 L have been reported. The bioprocess technology behind this large scale production has been reviewed by others (Hunt, 2005; Ikonomou et al., 2003; Vlak et al., 1996). A problem repeatedly encountered when
expressing recombinant proteins with baculovirus vectors is a drop in expression levels with increasing virus passage (reviewed in Krell, 1996). This so-called “passage effect” is intrinsic to baculovirus replication in cell culture, but is less critical for small laboratory-scale protein production when the number of virus passages is low (<10). It is a significant problem though for large-scale industrial production of vaccines in insect cell bioreactors (Van Lier et al., 1996) and prevents the use of continuous bioreactors. The major causes of loss of recombinant protein expression are (1) mutations in the FP25K gene, reducing the activity of the polyhedrin promoter (Harrison et al., 1996), (2) the generation of defective interfering particles (DIs) which replicate at the expense of the full-length recombinant virus (Kool et al., 1991; Pijlman et al., 2001; Wickham et al., 1991), (3) the intracellular accumulation of concatenated viral sequences, for example, non-hr (homologous repeat) origins of DNA replication which interfere with replication of full-length genomes (Lee and Krell, 1994; Pijlman et al., 2002), and (4) spontaneous deletion of the heterologous gene from the baculovirus vector. The latter aspect is especially seen in bacmid-derived vectors, which are extremely sensitive to spontaneous removal of the expression cassette, a large piece of DNA which is not under selection (Pijlman et al., 2003a). To prevent the amplification of DIs, baculovirus vectors must be used at low multiplicities of infection (MOI) (de Gooijer et al., 1992; Wickham et al., 1991) and it is now common practice to keep the number of viral passages to a minimum and establish low-passage virus banks as seed stocks for production purposes.

In recent years, several approaches have been used to improve the stability of the baculovirus genome. The accumulation of non-hr-containing sequences can easily be prevented by removing this sequence from the baculovirus backbone (Pijlman et al., 2002). Reducing the distance between origins of replication in the bacmid system by insertion of an extra hr sequence within the expression cassette also resulted in prolonged foreign gene expression in a test bioreactor (Pijlman et al., 2004). In the FlashBac system, all destabilizing bacterial-derived sequences are removed on recombination with the transfer vector. To prevent loss of the foreign gene cassette, a bicistronic vector was developed that contained the foreign gene and the baculovirus essential gene GP64 on a single bicistronic transcriptional unit linked by an internal ribosome entry site (IRES). GP64 was deleted from its original locus. In this bicistronic vector, loss of the foreign gene would automatically result in loss of expression of the essential gene, which is needed for the generation of complete virus particles as well as for DIs. GFP expression levels were kept at a high level for at least
20 passages with this vector providing dominant selection for GP64 (Pijlman et al., 2006). This system awaits testing for expression of proteins of medical importance. By combining several of the methods described in this section, it is likely that genome stability will be further improved.

III. Viral Subunits Expressed in the Baculovirus System

Since its recognition as a production system for subunit vaccines (Vlak and Keus, 1990), the baculovirus–insect cell expression system has been used extensively for the expression of candidate vaccine antigens. A comprehensive overview of the viral antigens from viruses of vertebrates that have been expressed in this system is provided in Table III. Only those antigens that were tested for their ability to induce protective immune responses are included. In addition, many viral antigens have successfully been expressed in insect cells for the development of diagnostics, and to perform structural and functional studies, but these studies are excluded from this chapter. Various viral antigens ranging from capsid and envelope proteins to nonstructural proteins have been chosen for the development of subunit vaccines. These viral antigens can be divided into those that are expressed as single or oligomeric protein subunits, and those that self-assemble into VLPs. Different approaches to vaccination are described in the examples later, with special attention paid to influenza subunit vaccines.

A. Viral Envelope Proteins

Envelope proteins are synthesized as single or oligomeric subunits. The expressed envelope proteins are often functionally active and have been reported to oligomerize, an indication that they are correctly folded (Crawford et al., 1999). Commonly, viral envelope glycoproteins are glycosylated in insect cells. Examples of baculovirus-produced subunit vaccine candidates (Table III) are the fusion proteins and hemagglutinins of paramyxoviruses, such as Newcastle disease virus, and the E proteins of Flaviviridae, including West Nile virus, dengue viruses, and CSFV. Two commercially available veterinary subunit vaccines against classical swine fever (BAYOVAC CSF E2™ and PORCILIS PESTI™) are based on the CSFV E2 glycoprotein produced in insect cells (Ahrens et al., 2000; Bouma et al., 1999, 2000; Depner et al., 2001; van Aarle, 2003). The E2 envelope glycoprotein of CSFV was expressed as a secreted protein by removing the TMR and this resulted in a
| Virus family or genus               | Abbreviation | Host         | Antigen(s) | Neutralizing antibodies | T cells/ cytokines | Protection host/model | References                      |
|-----------------------------------|--------------|--------------|------------|-------------------------|-------------------|-----------------------|--------------------------------|
| **Asfarviridae**                  |              |              |            |                         |                   |                       |                                 |
| African swine fever virus         | ASFV         | Pigs         | p22, p30, p54, p72 | Yes – No               | –                 | No                    | Neilan *et al.*, 2004         |
|                                   |              |              | p30–p54 fusion | Yes – Yes               | –                 | Yes                   | Barderas *et al.*, 2001       |
|                                   |              |              | HA         | Yes – Yes               | –                 | Yes                   | Ruiz-Gonzalvo *et al.*, 1996  |
| **Arenaviridae**                  |              |              |            |                         |                   |                       |                                 |
| Lymphocytic choriomeningitis virus| LCMV         | Humans, rodents | GP, NP     | –                       | T cells           | Yes                   | Bachmann *et al.*, 1994       |
| **Arteriviridae**                 |              |              |            |                         |                   |                       |                                 |
| Porcine reproductive and respiratory syndrome virus | PRRSV | Pigs | 3, 5 (7) | Yes – Yes | – | Yes | Plana Duran *et al.*, 1997 |
| **Birnaviridae**                  |              |              |            |                         |                   |                       |                                 |
| Infectious pancreatic necrosis virus | IPNV       | Fish         | Structural proteins (VLP<sup>d</sup>) | – – Partial | – | – | Shivappa *et al.*, 2005 |
| Infectious bursal disease virus   | IBDV         | Birds        | VP2 (VLP)  | Yes – Yes               | –                 | Yes                   | Pitcovski *et al.*, 1996; Wang *et al.*, 2000 |
|                                   |              |              | VP2 (VLP), VPX, PP | Yes – Yes | – | Yes | Martinez-Torrecoquadrada et al., 2003 |
|                                   |              |              | VP2 + VP3 + VP4 (chimeric) | Yes – Yes | – | Yes | Snyder *et al.*, 1994 |
|                                   |              |              | VP2 + VP3 + VP4 | Yes – Yes | – | Yes | Vakharia *et al.*, 1994 |
|                                   |              |              | VP3        | No – No                 | –                 | No                    | Pitcovski *et al.*, 1999      |
| Yellowtail ascites virus          | YAV          | Fish         | VP2, VP3, NS | Yes – Yes               | –                 | Yes                   | Sato *et al.*, 2000           |
| Family          | Genus               | Species       | Target Organisms                  | Antigenicity/Reactivity | Serological Markers | References                        |
|-----------------|---------------------|---------------|-----------------------------------|-------------------------|---------------------|-----------------------------------|
| **Bunyaviridae** |                     |               |                                   |                         |                     |                                   |
| *La Crosse virus* | LACV               | Humans        | G1                                | Yes                     | –                   | Yes                               | Pekosz *et al.*, 1995            |
| *Hantaan virus*   | HTNV               | Humans, rodents | G1, G2, NP                        | Yes                     | –                   | Yes                               | Schmaljohn *et al.*, 1990        |
| *Rift Valley fever virus* | RVFV             | Humans, ruminants | G1, G2                            | Yes                     | –                   | Yes                               | Schmaljohn *et al.*, 1989        |
| **Caliciviridae** |                     |               |                                   |                         |                     |                                   |
| *Hepatitis E virus* | HEV               | Humans        | Capsid (VLP)                      | Yes                     | –                   | Yes                               | Li *et al.*, 2001, 2004           |
| *Norwalk virus, Genogroup I* | NWV               | Humans        | Capsid (VLP)                      | Antibodies              | –                   | –                                 | Ball *et al.*, 1996, 1998, 1999; Guerrero *et al.*, 2001 |
| *Norwalk virus, Genogroup II* | NWV             | Humans        | Capsid (VLP)                      | Antibodies              | Yes                 | –                                 | Nicollier-Jamot *et al.*, 2004    |
| **Circoviridae** |                     |               |                                   |                         |                     |                                   |
| *Chicken anaemia virus* | CAV               | Birds         | VP1, VP2                          | Yes                     | –                   | Yes                               | Koch *et al.*, 1995              |
| *Porcine circovirus 2* | PCV2              | Pigs          | ORF2                              | Yes                     | –                   | Yes                               | Blanchard *et al.*, 2003         |
| **Coronaviridae** |                     |               |                                   |                         |                     |                                   |
| *Avian infectious bronchitis virus* | IBV              | Chicken       | S1                                | Yes                     | –                   | Partial                          | Cavanagh, 2003; Song *et al.*, 1998 |
| *Feline infectious peritonitis virus* | FIPV           | Cats          | N                                 | No                      | Yes                 | Yes                               | Hohdatsu *et al.*, 2003           |
| *SARS corona virus* | SARS              | Humans        | Spike GP                          | Yes                     | –                   | Yes                               | Bisht *et al.*, 2005             |
| *Transmissible gastroenteritis virus* | TGEV              | Pigs          | S + N + M                         | Yes                     | Yes                 | Partial                          | Sestak *et al.*, 1999            |
| **Deltavirus**   |                     |               |                                   |                         |                     |                                   |
| *Hepatitis deltavirus* | –                  | Humans/ rodents | HD Ag                             | No                      | –                   | –                                 | Karayiannis *et al.*, 1993       |
|                  |                     |               | HD Ag p24, p27                    | Antibodies              | –                   | No                                | Fiedler and Roggendorf, 2001     |

(continues)
| Virus family or genus | Abbreviation | Host | Antigen(s) | Neutralizing antibodies | T cells/cytokines | Protection host/model | References |
|-----------------------|--------------|------|------------|------------------------|------------------|-----------------------|------------|
| **Filoviridae**       |              |      |            |                        |                  |                       |            |
| Ebola virus           | EBOV         | Humans | GP         | Yes                    | T cells          | Partial               | Mellquist-Riemenschneider et al., 2003 |
| Marburg virus         | MBGV         | Humans | GP         | Yes                    | –                | Yes                   | Hevey et al., 1997 |
| **Flaviviridae**      |              |      |            |                        |                  |                       |            |
| Bovine viral diarrhea virus | BVDV      | Cows  | E2         | Yes                    | –                | Yes                   | Bolin and Ridpath, 1996 |
| Classical swine fever virus | CSFV     | Pigs  | E2         | Yes                    | –                | Yes                   | Ahrens et al., 2000; Bouma et al., 1999; Hulst et al., 1993 |
| Dengue 2 virus        | DEN 2        | Humans | E          | Yes                    | –                | –                     | Kelly et al., 2000 |
|                       |              |       | E          | Yes                    | –                | Partial               | Delenda et al., 1994; Velzing et al., 1999 |
|                       |              |       | E          | No                     | –                | Partial               | Feighny et al., 1994 |
|                       |              |       | NS1        | Yes                    | –                | Partial               | Qu et al., 1993 |
| Dengue 4 virus        | DEN 4        | Humans | Cocktail   | Yes                    | –                | Yes                   | Zhang et al., 1988 |
|                       |              |       | Cocktail, E | Yes                    | –                | Partial               | Eckels et al., 1994 |
| Dengue virus 2 + 3    | DEN2/3       | Humans | E protein hybrid | Yes | T cells | – | Bielefeldt-Ohmann et al., 1997 |
| Japanese encephalitis virus | JEV     |       | prME, E, NS1 | Yes | – | Yes/No | Yang et al., 2005 |
| West Nile virus       | WNV          | Humans/birds | prME (VLP) | Yes | – | Yes | McCown et al., 1990 |
| Hepatitis C virus     | HCV          | Humans | E1 + E2 (VLP) | Yes | T cells/cytokines | Yes | Jeong et al., 2004 |
| Virus Family                  | Species | Hosts | Antigen(s) | Immune Response | Detection | Ref.                     |
|------------------------------|---------|-------|------------|-----------------|-----------|-------------------------|
| **St Louis encephalitis virus** | SLEV    | Humans | prME       | Yes             | –         | Venugopal et al., 1995  |
| **Tick-borne encephalitis virus** | TBEV    | Humans | E, C       | –               | T cells/cytokines | Gomez et al., 2003 |
| **Yellow fever virus**       | YFV     | Humans | E, E + NS1 | Yes             | –         | Despres et al., 1991    |
| **Hepadnaviridae**           | HBV     | Humans | HBsAg      | Antibodies      | –         | Attanasio et al., 1991  |
| **Herpesviridae**            |         |       |            |                 |           |                         |
| **Bovine herpesvirus 1**     | BHV-1   | Cows  | gIII, gIV  | Yes             | –         | Okazaki et al., 1994, van Drunen Little-van den Hurk et al., 1991, 1993 |
| **Canine herpesvirus**       | CHV     | Dogs  | gC, gB, gD, gH, gL | Yes/No | T cells | Xuan et al., 1996, Packiarajah et al., 1998 |
| **Equine herpesvirus 1**     | EHV-1   | Horses | gB, gC, gD | Yes/No | T cells | Yes/No | Stokes et al., 1996a, Walley et al., 1995, Foote et al., 2005, Ruitenber et al., 2000, Stokes et al., 1997, Stokes et al., 1996b |
| **Feline herpes virus 1**    | FHV-1   | Cats  | gD, gH     | Yes             | –         | Maeda et al., 1996     |
| **Guinea pig cytomegalovirus** | GPCMV   | Rodents | gB         | Yes             | –         | Schleiss et al., 2004  |
| **Herpes simplex virus 1**   | HSV-1   | Humans | gD         | Yes             | T cells   | Yes | Krishna et al., 1989 |
|                              |         |       | gE         | Yes             | –         | Lin et al., 2004        |
|                              |         |       | gB-gI cocktail | Yes     | –         | Ghiasi et al., 1996    |
|                              |         |       | gD         | Yes             | –         | Ghiasi et al., 1991    |

(continues)
| Virus family or genus            | Abbreviation | Host   | Antigen(s)³ | Neutralizing antibodies² | T cells/cytokines | Protection host/model | References                      |
|---------------------------------|--------------|--------|-------------|--------------------------|------------------|----------------------|--------------------------------|
| Human cytomegalovirus           | HCMV         | Humans | gB          | Yes                      | –                | –                    | Ghiasi et al., 1999           |
|                                 | gE           | Yes    | gD, gG, gK  | –                        | Cytokines        | –                    | Ghiasi et al., 1995           |
|                                 | gK           | No     | –           | –                        | –                | ADE⁷                 | Ghiasi et al., 2000           |
|                                 | gL           | No     | –           | –                        | –                | No                   | Ghiasi et al., 1994           |
| Phocid herpes virus 1           | PhHV-1       | Seals  | gB          | Yes                      | –                | Yes                  | Harder and Osterhaus, 1997    |
| Pseudorabies virus              | PrV          | Pigs   | gII         | Yes                      | –                | Yes                  | Xuan et al., 1995             |
|                                 | gIII         | Yes    | –           | –                        | –                | –                    | Inumaru and Yamada, 1991      |
| Orthomyxoviridae                |              |        |             |                          |                  |                      |                                |
| Equine influenza virus          | H3N8         | Horses | H3          | No                       | –                | Partial              | Olsen et al., 1997            |
| Human influenza A               | H1N1         | Humans | H1 (proteosomes) | Yes                   | –                | Yes                  | Jones et al., 2003            |
|                                 | H2N2         |        | M2          | Yes                      | –                | Yes                  | Slepushkin et al., 1995       |
|                                 | H3N2         |        | H3 + M1 (VLP) | Yes                    | –                | Yes                  | Galarza et al., 2005b         |
|                                 | H3N2         |        | H3          | Yes                      | Yes              | Yes                  | Powers et al., 1995, 1997     |
|                                 | H3N2         |        | H3          | Yes                      | –                | –                    | Treanor et al., 1996          |
|                                 | H3N2         |        | H3, N2      | Yes                      | –                | Yes                  | Brett and Johansson, 2005;    |
|                                 |              |        |             |                          |                  |                      | Johansson, 1999               |
| Virus Family     | Virus Name                    | Hosts  | Surface Antigen | Fusion/Neuraminidase Activity | Notes                                      |
|-----------------|-------------------------------|--------|----------------|-----------------------------|-------------------------------------------|
| Avian influenza virus | H3N2                          | N2     | Yes            | –                           | Yes                                       |
|                  | H6N2                          | N2     | Yes            | –                           | Yes                                       |
|                  | Multiple                      | H1, H3 | Yes            | –                           | –                                         |
|                  | Avian influenza virus         | H5N1   | Birds          | H5                          | No/–                                      |
|                  |                               | H5     | In humans      | –                           | –                                         |
|                  | Multiple                      | H5, H7 | Yes            | Yes                         | –                                         |
| Papovaviridae    | Bovine papillomavirus         | BPV    | Cows           | L1                          | Yes                                       |
|                  | Cottontail rabbit papillomavirus | CRPV   | Rodents        | L1 (VLP)                    | Yes                                       |
|                  |                               |        |                | L1, L1 + L2 (VLP)           | Yes                                       |
|                  | Human papillomavirus          | HPV-16 | Humans         | L1 (VLP)                    | Yes                                       |
|                  | 16                            |        |                | L1 + L2 + E7 (VLP)          | Yes                                       |
| Paramyxoviridae  | Bovine parainfluenza virus    | BPIV-3 | Cattle         | HN                          | Yes                                       |
|                  | Bovine respiratory syncytial virus | BRSV   | Cattle         | F                           | Yes                                       |
|                  | Human parainfluenza virus     | HPIV-3 | Humans         | F                           | Low                                       |

(continues)
TABLE III (continued)

| Virus family or genus         | Abbreviation | Host          | Antigen(s) | Neutralizing antibodies | T cells/ cytokines | Protection host/model | References                                      |
|------------------------------|--------------|---------------|------------|-------------------------|--------------------|-----------------------|------------------------------------------------|
| **Human respiratory syncytial virus** | HRSV         | Humans        | HN-F fusion | Yes                     | –                  | Yes                   | Brideau et al., 1993                             |
|                              |              |               | HN, F, HN-F | Yes                     | –                  | Yes                   | Lehman et al., 1993                              |
|                              |              |               | F (+HPIV-HN) | Yes                     | –                  | Yes                   | Du et al., 1994; Homa et al., 1993               |
|                              |              |               | FG fusion   | Low                     | –                  | Partial               | Connors et al., 1992                            |
|                              |              |               | FG fusion   | Yes                     | –                  | Yes                   | Brideau et al., 1989; Oien et al., 1993; Wathen et al., 1991 |
| **Newcastle disease virus**  | NDV          | Birds         | F           | –                       | –                  | Yes                   | Mori et al., 1994                               |
|                              |              |               | HN          | Yes                     | –                  | Yes                   | Nagy et al., 1991                               |
| **Peste-des-petits-ruminants virus** | PPRV        | Ruminants     | HN          | Yes                     | Yes                | –                     | Sinnathamby et al., 2001a                        |
| **Rinderpest virus**         | RPV          | Cows          | F, H        | Yes                     | –                  | No                    | Bassiri et al., 1993                            |
|                              |              |               | H           | –                       | Yes                | –                     | Sinnathamby et al., 2001b                       |
| **Rubella virus**            | RV           | Humans        | E2, C       | –                       | –                  | No                    | Cusi et al., 1995                               |
| **Parvoviridae**             |              |               |             |                         |                    |                       |                                                 |
| **B19 virus**                | B19V         | Humans        | VP1, VP2 (VLP) | Yes               | –                  | –                     | Kajigaya et al., 1991                           |
| **Canine parvovirus**        | CPV          | Dogs          | VP2 (VLP)   | Yes                     | –                  | Yes                   | Lopez de Turiso et al., 1992; Saliki et al., 1992 |
| **Duck parvovirus**          | DPV          | Birds         | VP1, VP2 (VLP) | Yes               | –                  | –                     | Le Gall-Recule et al., 1996                     |
| **Mink enteritis parvovirus**| MEV          | Mink          | VP2 (VLP)   | Yes                     | –                  | Yes                   | Christensen et al., 1994                        |
| **Porcine parvovirus**       | PPV          | Pigs          | VP2 (VLP)   | Antibodies              | –                  | –                     | Martinez et al., 1992                           |
| **Picornaviridae** | | | | | | |
|---|---|---|---|---|---|---|
| **Foot-and-mouth disease virus** | FMDV | Cattle | Epitopes fused to GP64 P1–2A + part P2 polyprotein | Yes | – | Yes | Tami *et al.*, 2004 |
| **Hepatitis A virus** | HAV | Humans | polypeptide | Yes | – | – | Partial | Grubman *et al.*, 1993 |
| **Polyomaviridae** | | | | | | |
| **Simian virus 40** | SV40 | Primates | Large T Antibodies | Yes | No | Yes | Shearer *et al.*, 1993 |
| **Reoviridae** | | | | | | |
| **African horse sickness virus** | AHSV | Horses | VP2 (VLP) | Yes | – | Yes | Roy and Sutton, 1998 |
| **Bovine rotavirus** | BoRV | Cows | VP2 + VP4 + VP6 + VP7 (VLP) | Yes | – | Yes | Conner *et al.*, 1996a,b |
| **Human rotavirus** | HRV | Humans | VP2 + VP4 + VP6 + VP7 (VLP) | Yes | – | Yes | Conner *et al.*, 1996a |
| **Simian rotavirus** | SiRV | Primates | VP2 + VP4 + VP6 + VP7 (VLP) | Yes | – | Yes | Conner *et al.*, 1996a,b |

(continues)
| Virus family or genus | Abbreviation | Host          | Antigen(s) | Neutralizing antibodies | T cells/ cytokines | Protection host/model | References                           |
|----------------------|--------------|---------------|------------|-------------------------|-------------------|---------------------|-------------------------------------|
| **Retroviridae**     |              |               |            |                         |                   |                     |                                     |
| *Feline immunodeficiency virus* |            |               |            |                         |                   |                     |                                     |
| FIV                  | Cats         | gp120         | Yes        | –                       | –                 | Partial             | Leutenegger et al., 1998           |
| gp41 MEPR^{\text{f/}} PERV^{\text{g}} p15E fusion |               | Yes          | –           | –                      | –                 | Luo et al., 2006                |
| gp41 + V3 loop       | Yes          | –             | T cells    | –                       | –                 | Luo et al., 1992            |
| gp55 (VLP)           | Boost        | –             | –          | –                       | –                 | Jaffray et al., 2004         |
| gp55–gp120 (VLP)     | Yes          | T cells       | –          | –                       | –                 | Arico et al., 2005           |
| gp120                | Antibodies   | –             | –          | –                       | –                 | Buonaguro et al., 2002; Tobin et al., 1997 |
| gp160                | Partial      | –             | –          | –                       | –                 | Keefe et al., 1994           |
| No                   | No           | No CTL        | –          | No CTL                  | –                 | Perales et al., 1995         |
| Boost^{g}            | Boost        | CTL           | –          | –                       | –                 | Doe et al., 1994             |
|                       |              |               |            |                         |                   | Bristow et al., 1994        |
|                       |              |               |            |                         |                   | Akerblom et al., 1993       |
|                       |              |               |            |                         |                   | Gorse et al., 1994; Graham et al., 1993; Lubeck et al., 1994; Montefiori et al., 1992 |
|                       |              |               |            |                         |                   | Cooney et al., 1993         |
|                       |              |               |            |                         |                   | Lundholm et al., 1994       |
| HIV-1                | Humans       | p24           | –          | T cells                 | –                 | Fyfe et al., 1993           |
| gp41                 | Boost        | –             | –          | –                       | –                 | Gorse et al., 1993; Graham et al., 1993; Lubeck et al., 1994; Montefiori et al., 1992 |
| gp160                | Partial      | –             | –          | –                       | –                 | Cooney et al., 1993         |
| Antibodies           | T cells      | –             | –          | –                       | –                 | Lundholm et al., 1994       |
| Virus Family                  | Virus                        | Species         | Antibodies Reacting to gp160/41 | Boost          | Neutralizing | Notes                                                                 |
|------------------------------|------------------------------|-----------------|---------------------------------|----------------|--------------|----------------------------------------------------------------------|
| Rhabdoviridae                | Rabies virus                 | Mammals         | Yes                             | –              | No           | Prehaud et al., 1989, Fu et al., 1993, Drings et al., 1999          |
|                              | RABV                         | Mammals         | G                               | Yes            | Yes          | Yes                                                                  |
|                              |                               |                 |                                  | Yes            | Yes          |                                                                      |
|                              | MOKV                         | Mammals         | G                               | Antibodies     | –            | Yes                                                                  |
|                              |                               |                 |                                  |                |              |                                                                      |

- Dashes in the table mean not analysed in this study.
- Only those antigens are included that were tested in immunization experiments.
- If not known whether neutralizing indicated as “antibodies.”
- VLP, virus-like particle.
- ADE, antibody-dependent enhancement by nonneutralizing antibodies (resulting in chronic infections).
- MEPR, membrane-proximal region.
- PERV, porcine endogenous retrovirus.
- Boost, boost with baculovirus-produced recombinant protein, prime form other origin.
threefold increase in expression levels, and allowed for purification of E2 from the culture medium (Hulst et al., 1993). In a similar way, the related Bovine diarrhea virus (BVDV) E2 protein was expressed in insect cells (Bolin and Ridpath, 1996). Recent research showed that the BVDV E2 protein needs to be glycosylated to be effectively secreted from baculovirus-infected cells (Pande et al., 2005) and that the glycosylated protein was able to block BVDV infection better in an in vitro assay. Whether the glycosylated E2 protein also performs better as a vaccine is not known. The spike glycoprotein of the SARS coronavirus is one of the most recently expressed proteins in insect cells and protected mice against intranasal SARS infection (Bisht et al., 2005).

Influenza presents a serious risk for both human and animal health. The single-stranded RNA of the influenza virus changes quickly through an accumulation of mutations and frequent recombination events, requiring annual vaccine updates (Palese, 2004). The most threatening recent example is the outbreak of avian influenza of the H5N1 serotype which has killed birds and humans in the Far East since 2003 (WHO) and which caused the first human casualties outside this area in East Turkey in January 2006. The big fear is that such an avian virus will change into a virus that can be transmitted directly from man to man, which may then lead to an influenza outbreak of pandemic dimensions (Palese, 2004). The most widely used influenza vaccines, e.g., Fluzone (Sanofi Pasteur) and Fluvirin (Chiron), consist of chemically inactivated split virus or purified virus subunits. These vaccines have several disadvantages which have recently been reviewed (Cox, 2005; Cox et al., 2004), including reduced efficacy in the elderly, where vaccination does reduce mortality rates but is not very effective in preventing disease. In addition, an enormous number of eggs are needed each year (one egg per dose) which will very likely lead to a shortage of vaccine in the event of a pandemic; some strains grow poorly in eggs requiring coinfections with other strains or genetic adaptations (e.g., H5N1) (Horimoto et al., 2006); and these vaccines can cause strong allergic reactions in some individuals. Live, attenuated influenza vaccines have the advantage of inducing secretory and systemic immunity and are applied intranasally, preventing virus replication in the respiratory tracts (Cox et al., 2004). However, all of these vaccines still need to be grown in chicken embryos, which are ironically also the target for a potentially pandemic virus like H5N1.

To overcome these drawbacks, various cell-based vaccines for influenza are under development as well as recombinant protein vaccines. Clinical trials of vaccines based on influenza virus produced in mammalian cell cultures, such as Madin Darby canine kidney (MDCK)
cells, have been described (Brands et al., 1999; Percheson et al., 1999) and trials with influenza vaccines produced in the human retina cell line Per.C6\textsuperscript{®} (Pau et al., 2001) are ongoing. These products still require inactivation of the influenza virus which may reduce immunogenicity as seen for inactivated vaccines. In response to human casualties of H5 and H7 influenza viruses in Asia in the late 1990s, the immunogenicity and safety of baculovirus recombinant H5 and H7 hemagglutinin (HA) proteins was tested in chickens and resulted in 100% protection against disease symptoms (Crawford et al., 1999). The immunogenicity of the baculovirus-derived H5 vaccine was subsequently evaluated in over 200 healthy human adults. The vaccine was well tolerated and provided neutralizing antibody responses equivalent to those observed in convalescent sera in \( \sim 50\% \) of the individuals after two doses (Treanor et al., 2001). A clinical trial with baculovirus-produced recombinant H3 antigens in 127 adult volunteers showed protective neutralizing antibody levels and a reduction in influenza rates in the following epidemic season compared to a placebo group (Powers et al., 1995). This HA-based vaccine induced both B and T memory cells (Powers et al., 1997). A clinical study of 399 individuals with an average age of 70 years was completed in 2003–2004 with an experimental vaccine (FluBl\textregistered k, Protein Sciences corporation) containing the same three HA antigen variants as present in the licensed inactivated vaccine of that flu season (Treanor et al., 2006). Compared to the licensed vaccine, the recombinant vaccine produced higher antibody titers against the H3 strain, the strain responsible for the majority of influenza deaths each year (Cox, 2005). This result suggests that this vaccine can be especially useful for reduction of the annual number of influenza-related deaths in the elderly, where H3 antibody titers induced by conventional vaccines are too low to be protective. Phase III trials in healthy adults have been completed and showed a 100% protective efficacy even against H3N2 influenza viruses (Manon Cox, personal communication) (http://www.proteinsciences.com/, Jan 2006). Preparation of a recombinant influenza virus vaccine cocktail for the coming flu season may take about 3 months to complete from the moment the new vaccine composition is announced by the World Health Organization (WHO).

The inactivated conventional vaccine and the trivalent recombinant HA-based vaccine under development are based on antibody responses against the HA surface protein and require annual modifications to the vaccine due to antigenic drift of the influenza virus. A baculovirus recombinant vaccine with both HA and neuraminidase (NA) subunits resulted in a bivalent seroconversion with antibodies against both HA
and NA (Johansson, 1999). The efficacy of an H3N2 vaccine based on both HA and NA produced with a recombinant baculovirus was analyzed in a murine model and compared with a conventional killed and a live-attenuated vaccine preparation and an HA single-subunit vaccine (Brett and Johansson, 2005). The NA in the baculovirus-derived vaccine was much more immunogenic than in the conventional vaccines. The advantage of inducing an immune response to both surface proteins is illustrated by the fact that the recombinant vaccine containing both HA and NA did not only prevent infection with homotypic and closely related viruses, but also showed a strong reduction in pulmonary virus titers in infections with a more distantly related virus (H3N2 A/Panama/2007/99 versus A/Fujian/411/2002), in contrast to a vaccine based on HA only. These results suggest that a vaccine containing intact NA tolerates more antigenic drift, thereby reducing the chance of virus escaping the immune system during the flu season.

B. Virus-like Particles

Viral capsid proteins produced in insect cells often self-assemble into VLPs. The advantage of VLPs is that they resemble the natural virus but are not infectious because they lack genetic material. VLPs are also an excellent tool for study of virus structure. VLPs can easily be purified by extraction, centrifugation, or precipitation (Brown et al., 1991) and often give strong immune reactions even in the absence of adjuvants due to their particulate nature. In addition, humoral, cell-mediated, and mucosal immune responses have been reported (Roy, 1996). An example of a vaccine consisting of recombinant VLPs produced with a baculovirus vector is a patented Canine parvovirus vaccine (Lopez de Turiso et al., 1992; Valdes et al., 1999). Sometimes the expression of more than one viral coat protein is needed to make immunogenic VLPs, either due to the complexity of the capsid structure (Bluetongue virus: BTV, Reoviridae) or presence of crucial epitopes on several coat proteins. Multicomponent VLPs can be produced by using vectors with multiple promoters or by coinfections with several baculovirus vectors that each encode one or more viral proteins. One difficulty in making complex VLPs is to achieve appropriate expression levels of each protein present in the viral capsid.

The capsid protein of Hepatitis E virus (Caliciviridae) forms VLPs and these VLPs induce both systemic and mucosal immunity after oral administration in a mouse model. They also protect cynomolgus monkeys when challenged with HEV against infection and hepatitis (Li et al., 2001, 2004). Infectious bursal disease (IBDV, Birnaviridae) of
birds can also be prevented by vaccination with single component VLPs (Martinez-Torrecuadrada et al., 2003; Wang et al., 2000). The major capsid protein L1 of Papovaviridae forms VLPs and has been shown to protect cottontail rabbits against Cottontail rabbit papillomavirus. Combinations of the HPV-16 L1 and L2 capsid proteins and the oncogenic protein E7 protected against tumor formation in a mouse model (Greenstone et al., 1998). Multivalent VLP preparations containing BTV (Reoviridae) VP2 subunits of various serotypes were made by coinfections of several baculovirus vectors and induced long-lasting protection in sheep (Roy et al., 1994). Vaccine candidates in the form of VLPs with up to four different VPs have also been successfully produced for BTV (Pearson and Roy, 1993; van Dijk, 1993) as well as for several other Reoviridae (Conner et al., 1996a,b). Immunization of mice with an influenza VLP containing the two matrix proteins M1 and M2, and the surface proteins HA and NA showed almost complete protection against an H3N2 virus via both intramuscular and intranasal immunization routes (Galarza et al., 2005a).

The rationale for using VLPs as vaccine candidates is obvious for nonenveloped viruses, because in these viruses the capsid proteins are directly exposed to the immune system. However, they may also be useful for displaying epitopes of enveloped viruses. HIV is an enveloped virus and in this case VLPs have been produced based on gp55 (gag) to which immunogenic segments of the envelope protein gp120 were coupled (Arico et al., 2005; Buonaguro et al., 2002; Tobin et al., 1997). An extension of these chimeric VLP-based vaccines is to use VLPs of one virus to display epitopes of heterologous proteins that do not form VLPs by themselves. Examples of such systems are Human parvovirus B19 VLPs which carry linear epitopes in fusion with the viral VP2 protein. This system was used to display epitopes of Murine hepatitis virus A59 (MHV; Coronaviridae) and Herpes simplex virus (HSV; Herpesviridae) (Brown et al., 1994). Such chimeric VLPs protected mice against a lethal challenge with MHV of HSV. Epitope-presenting chimeric VLPs have also been developed based on Mouse papillomavirus (Tegerstedt et al., 2005) and Flock house virus VLPs (Scodeller et al., 1995).

C. Inclusion of Recombinant Cytokines in the Vaccine

Mono- and oligomeric protein subunits are often less potent and need to be formulated carefully before administration to extend their half-life and to present them in a proper form to the immune system, for instance by uptake by antigen-presenting cells (APCs)
(Dertzbaugh, 1998; Schijns, 2003). Adjuvant possibilities for human application are very limited because of safety considerations and this may limit the application of monomeric subunit vaccines in humans. VLPs on the other hand have been shown to induce protection even without the addition of adjuvants (Li et al., 2004; Roy, 1996). An alternative way to modulate the immune response is by the addition of recombinant cytokines as vaccine adjuvants. Cytokines can either be added separately to the vaccine or may be included in VLPs. This approach may not only modulate the magnitude but also the type of immune response (Lofthouse et al., 1996). By carefully choosing which cytokine is added the immune response can be driven in a certain direction. Interferon gamma (IFN-γ) may be added to stimulate macrophages, while addition of interleukin-12 (IL-12) promotes cell-mediated adaptive immunity (Abbas and Lichtman, 2005). IL-12 can be efficiently produced with baculovirus vectors as functional dimers that shift the immunogenic balance to Th1 cells in bovine calves (Takehara et al., 2002). IL-12 added to influenza VLPs enhances antibody responses but in this case VLPs alone already result in 100% protection (Galarza et al., 2005a). Immune reactions to helminths involve Th2 responses. Interleukin-4 (IL-4) drives the immune response to differentiation of Th2 cells and to the production of helminth-specific IgE antibodies (Abbas and Lichtman, 2005). Addition of IL-4 may therefore be helpful for vaccines against helminths (Lofthouse et al., 1996). For baculovirus-derived products the addition of cytokines has not been fully exploited, but it is commonly used for DNA vaccines. The addition of costimulators, such as B7 or CD40, to baculovirus-produced vaccines has not been reported.

D. Baculoviruses as DNA Vaccines

Baculoviral vectors with mammalian promoters driving the expression of viral genes have been used in a limited number of vaccine trials. A candidate Pseudorabies virus vaccine expressing its glycoprotein B from a recombinant baculovirus vector with a mammalian promoter resulted in seroconversion in immunized mice (Aoki et al., 1999). Intramuscular injection with baculovirus BVs expressing the E2 glycoprotein of Hepatitis C virus controlled by the CMV immediate-early promoter-enhancer provided specific humoral and cellular responses (Facciabene et al., 2004). Similar results were obtained with the carcino-embryonic antigen (CEA) indicating that these types of vaccines can also be effective against tumors. The addition of the Vesicular stomatis
virus (VSV) G protein to the baculovirus envelope increased immunogenicity in this experiment, possibly by enhancement of virus fusion. BacMam vectors have also been used to produce mutated, attenuated influenza virus in mammalian cells by delivery of an altered NS1 gene (Poomputsa et al., 2003). Surprisingly, a baculovirus vector with the influenza hemagglutinin gene (H1) controlled by the chicken beta-actin promoter gave a similar level of protection as a wild-type baculovirus against a lethal influenza challenge in intranasally immunized mice (Abe et al., 2003). The authors ascribe this to the induction of a strong innate immune response by the baculovirus, protecting the mice from a subsequent lethal challenge with influenza virus.

A possible drawback to the use of baculoviruses directly for vaccination and possibly also for surface display and polyhedra-incorporation vectors is the accumulation of anti-baculovirus antibodies upon repeated vaccination, resulting in rapid inactivation of subsequent vaccines of the same type. Pigs for instance have been shown to produce high levels of baculovirus-neutralizing antibodies after injection of baculovirus BVs (Tuboly et al., 1993). A solution to this problem could be to design multivalent vaccines, since baculoviruses can take up a large amount of foreign DNA. Another problem with the use of baculovirus particles as vaccines is rapid degradation by the complement system which also affects gene therapy applications. Pseudotyping of BVs with the VSV glycoprotein instead of GP64 resulted in a reduction in complement inactivation (Tani et al., 2003). Incorporation of human decay-accelerating factor (DAF), a complement-regulatory protein, in the BV envelope has been shown to protect baculovirus gene therapy vectors against complement-mediated inactivation (Huser et al., 2001). This strategy could also be applied for vaccine purposes.

### E. Combinations of Vaccine Strategies

HIV VLPs containing only gp55 (gag) have been used to boost immunization induced by a gp55 DNA vaccine (Jaffray et al., 2004). In this case, a capsid protein of an enveloped virus is a reasonable subunit for vaccination because DNA vaccines are expressed intracellularly and fragments of the resulting proteins are presented by MHC complexes to induce cellular immune responses. In this way the natural situation of intracellular expression of viral genes is mimicked. T cell responses, especially the action of cytotoxic T lymphocytes (CTL) are crucial in defense against HIV. HIV combination vaccines where adenovirus, vaccinia (HIVAC-1e), or vesicular stomatitis virus vectors were used for immunization in combination with a boost with a protein
subunit (gp160, gp41) have been tested in phase I trials in humans (Cooney et al., 1993; Graham et al., 1993; Lubeck et al., 1994; Luo et al., 2006; Perales et al., 1995; Zheng, 1999). Viral carriers also express HIV antigens inside cells, and these antigens are displayed either by specialized APCs or other cells to the immune system. The aim of this regimen with a DNA/carrier vaccine and a protein boost regimen is therefore to induce both neutralizing antibodies and T cell responses.

F. Viral Marker Vaccines and Differential Diagnosis Technology

Nonvaccination policies exist for many animal diseases because of the risk that vaccinated animals may be protected against disease but may still be carriers of the virus. In cases of outbreaks, ring vaccination is sometimes applied, but large-scale vaccination is generally not allowed. A prerequisite for the broader use of animal vaccines is the development of marker vaccines that enable differentiation between vaccinated and infected animals. This is especially important for endemic diseases in animals where monitoring is of crucial importance to avoid spread of the virus to nonendemic regions or to other host species such as humans or wild animals. Marker vaccines also have good prospects for eradication of animal diseases in general (van Aarle, 2003). For such purposes, marker vaccines do not have to give 100% herd immunity, because reducing susceptibility and transmission can be sufficient to have a major effect in controlling animal disease (Henderson, 2005). Marker vaccines need to be accompanied by specific diagnostic tests that are commonly based on determining serum titers of a viral component that is absent in the vaccine but present in the pathogen, to which antibodies can be raised.

The baculovirus expression system has proven to be useful in producing not only the protein subunits for marker vaccines but also the recombinant polypeptides for these diagnostic tests. The commercially available CSFV vaccine based on the E2 glycoprotein is a marker vaccine because only antibodies against E2 are generated. An enzyme-linked immunosorbent assay (ELISA) test for serum antibodies against the other immunogenic surface protein E_RNS can be used to discriminate immunized animals from virus carriers (Langedijk et al., 2001; van Aarle, 2003). Another possibility is to use only some of the epitopes of an antigen for immunization and others for the diagnostic analysis, as demonstrated for CSFV (van Rijn et al., 1999). The coexistence of a subunit vaccine and a discriminative diagnostic test enabled registration of CSFV vaccines in Europe. Marker vaccines will also be very useful for immunization of poultry against avian
influenza (Crawford et al., 1999), where monitoring for the presence of virus is essential. Animals immunized with HA or HA/NA vaccines could be screened for antibodies against the viral matrix proteins. Similar assays have been developed for other subunit vaccines, such as one that discriminates between VSV-infected and immunized animals, where the marker vaccine is based on the glycoprotein and the assay on the nucleocapsid protein produced in insect larvae (Ahmad et al., 1993).

IV. BACULOVIRUS-PRODUCED VACCINES AGAINST PROTOZOAN PARASITES AND HELMINTHS

Parasites of the genera Plasmodium, Theileria, and Babesia are protozoan blood parasites causing malaria, theileriosis, and babesiosis. These parasites have a complex life cycle and are transmitted by either mosquito or tick vectors. Candidate subunit vaccines against these parasites can be roughly separated into preblood (preerythrocyte or prelymphocyte) stage vaccines, blood stage vaccines, transmission-blocking vaccines, and multistage vaccines. An overview of parasite subunits expressed in the baculovirus insect cell system for vaccine purposes is given in Table IV.

A. Plasmodium

A comprehensive record of all subunit and recombinant carrier vaccines under development for human malaria is maintained by WHO (Reed, 2005). Most of these vaccines are still in a preclinical stage but several Plasmodium falciparum vaccines are in phase I and phase II trials in malaria endemic countries. Plasmodium is transmitted in the form of sporozoites by Anopheles mosquitoes. A vaccine candidate based on the circumsporozoite protein (CSP) is aimed at blocking these sporozoites. Both B and T cell responses appear to be essential for protective immunity based on the CSP protein. CSP has been produced in insect cells but was minimally immunogenic when tested in 20 volunteers (Herrington et al., 1992). Alternative approaches to experimental CSP vaccines, which facilitate T cell responses, are in phase II trials (Ballou et al., 2004). These vaccines include CSP displayed on HBsAg VLP particles, modified vaccinia Ankara virus as recombinant carrier, or DNA vaccines.

The merozoite is the extracellular, erythrocyte-invasive form of the Plasmodium parasite. Merozoite surface proteins are promising
| Pathogen            | Antigen                      | Trial                      | Immunologic response                                      | References                      |
|---------------------|------------------------------|----------------------------|-----------------------------------------------------------|---------------------------------|
| *Protozoa*          |                              |                            |                                                           |                                 |
| *Babesia rodhaini*  | P26 surface protein          | Immunization of rats       | 40–100% protection                                         | Igarashi *et al.*, 2000          |
| *Plasmodium berghei*| Ookinete surface protein 21  | Injection in mice          | Antibodies, oocyst formation blocked in *Anopheles stephensi* mosquitoes | Matsuoka *et al.*, 1996          |
| *Plasmodium cynomolgi* | Merozoite surface protein (MSP)-1 | Challenge in primates | Protection                                                 | Perera *et al.*, 1998            |
| *Plasmodium falciparum* | Circumsporozoite protein (CSP) | Human safety and immunity trial | No response to native CSP | Herrington *et al.*, 1992        |
| *Theileria parva*   | Sporozoite surface protein p67 | Challenge in cattle        | 50% protection                                             | Nene *et al.*, 1995             |
|                     | Sporozoite surface protein P67 (GFp fusion, surface display) | Challenge in cattle | Upto 80% protection                                         | Kaba *et al.*, 2004b, 2005       |
| *Theileria sergenti* | P32                         | Challenge in cattle        | Protection                                                 | Onuma *et al.*, 1997             |
| *Trypanosoma cruzi* | TolT                         | Mice immunization/ *in vitro* inhibition by CD4 cells | 50–60% reduction of parasite numbers in infected macrophages | Quanquin *et al.*, 1999          |
| *Helminths*         |                              |                            |                                                           |                                 |
| *Fasciola hepatica* | Procathepsin L3              | Challenge in rats          | 50% protection                                             | Dalton *et al.*, 2003            |
| *Ostertagia ostertagi* | Metalloprotease 1           | Challenge in cattle        | No protection                                              | De Maere *et al.*, 2005a         |
|                     | Aspartyl-protease inhibitor  | Challenge in cattle        | No protection                                              | De Maere *et al.*, 2005b         |
| *Schistosoma mansoni* | Calpain (Sm-p80)             | Challenge in mice          | 29–39% reduction in worms                                  | Hota-Mitchell *et al.*, 1997     |
vaccine candidates due to their accessibility for antibodies and their expected role in erythrocyte invasion. The major merozoite surface protein (MSP-1) is an important prebloodstage candidate vaccine with homology to epidermal growth factor (EGF) and antibodies directed against this protein block erythrocyte invasion (Holder and Blackman, 1994). *Plasmodium cynomolgi* functions as a model system for the highly similar *Plasmodium vivax* in humans. An active, C-terminally processed form of *P. cynomolgi* MSP-1, was produced in insect cells and protected primates in a challenge experiment (Perera et al., 1998). The C-terminally mature MSP-1 of *P. falciparum* was also successfully expressed in insect cells and used for ultrastructural studies (Chitarra et al., 1999; Pizarro et al., 2003), but has not been tested in human trials. Meanwhile, *E. coli*-expressed MSP-1 has entered phase II clinical trials (Ballou et al., 2004).

*Plasmodium* parasites in the ookinete stage are taken up by mosquitoes and antigens specific for this stage can function as transmission-blocking subunit vaccines. The major ookinete surface antigen Pbs21 (P28) of *Plasmodium berghei* was expressed in *B. mori* larvae, preserving conformational B cell epitopes which were lost upon expression in *E. coli*. The recombinant Pbs21 antigen produced in insect cells blocked oocyte formation in *Anopheles* mosquitoes fed on immunized mice (Matsuoka et al., 1996). The immunogenicity of the recombinant protein was strongly reduced when the protein was expressed as a secreted protein by removing its glycosylphosphatidylinositol (GPI) anchor signal (Martinez et al., 2000). This protein provides a good example of loss of immunogenicity by expressing a membrane protein in a secreted form. Subunit vaccines based on the *Plasmodium*-induced erythrocyte membrane protein 1 (EMP-1) are aimed at blocking vertical transmission from mother to child (maternal malaria) via the placenta. This transmission involves the sequestration of *P. falciparum*-infected erythrocytes through EMP-1, which binds to chondroitin sulphate A in the placenta. EMP-1 expressed in the baculovirus system induces inhibitory antibodies which react with both homologous and heterologous EMP-1 proteins (Costa et al., 2003).

**B. Theileria and Babesia**

*T. parva* is the causative agent of East Coast fever, a deadly cattle disease endemic in large parts of Africa. Immunization with recombinant sporozoite surface protein p67 is aimed at blocking invasion of lymphocytes by this parasite. P67 is an example of a protein that was not easy to express in a native form in insect cells as well as in many
other systems. In insect cells, it was expressed at low levels and in contrast to expectations was not present on the cell surface. Similar to *E. coli*-expressed p67, it did not react with a monoclonal antibody against native p67, indicating that the folding of the protein was not correct (Nene *et al.*, 1995). Several adaptations were therefore made to the expression system. Expression of p67 coupled to the honeybee mellitin signal instead of the original signal peptide resulted in correct routing of this protein to the cell surface, but the folding was still not optimal (Kaba *et al.*, 2004a). Fusion of p67 to the C terminus of GFP drastically increased expression levels and resulted in recognition by the conformation-sensitive monoclonal antibody (Kaba *et al.*, 2002). A similar effect was also seen when parts of this protein were fused to the baculovirus GP64 glycoprotein in a surface display vector, which led to expression on the cell surface and on baculovirus BVs (Kaba *et al.*, 2003). The recombinant GFP-p67 protein and the C terminal half of p67 coupled to GP64 induced high levels of sporozoite-neutralizing serum antibodies and showed up to 80% protection against lethal *T. parva* challenge in a double-blind placebo-controlled experiment (Kaba *et al.*, 2004b, 2005). The next phase will be to evaluate the quality of these experimental vaccines under field conditions in countries in which East Coast fever is endemic.

*Babesia* species are a major cause of parasitemias in cattle and dogs. *Babesia divergens* is the major cause of bovine babesiosis in Europe and the increased incidence of this disease is correlated with an increase in the numbers of ticks (*Ixodus ricinus*) that transmit this parasite. *B. divergens* is also responsible for zoonotics in immunocompromised humans (Zintl *et al.*, 2003). The soluble parasite antigen (SPA) of bovine and canine *Babesia* species has been developed as a vaccine against clinical manifestations in dogs and is produced in mammalian cells infected with *Babesia* (Schetters, 2005). Several other *Babesia* antigens have been expressed in the baculovirus expression system to develop ELISA tests for diagnosis. The baculovirus-expressed *Babesia radhaini* P26 protein was shown to induce protection against the disease in rats (Igarashi *et al.*, 2000).

*Theileria* and Babesia parasites are transmitted by ixodic ticks and in the future candidate antiparasite vaccines may be combined with vaccines directed against the tick vector (Bishop *et al.*, 2004). These vaccines may be tick antigens directly exposed to the host immune system, such as vitellin, the most abundant *B. microplus* egg protein (Tellam *et al.*, 2002) or cement proteins involved in the attachment of the tick to the skin of the host. Concealed antigens can also give good results, such as the *B. microplus* Bm86 gut antigen (Gavac™), which results
in binding of antibodies taken up from immunized animals to a gut transmembrane protein (Willadsen et al., 1995). Ticks are known to immunomodulate their host by secreting specific immunomodulators and transmitted parasites also profit from the reduction in immune response. Tick vaccines may therefore be aimed at reducing the chance of transmission by directly affecting the feeding process, by interacting with immunomodulators, or by reducing tick populations.

C. Trypanosoma and Leishmania

Chagas’ disease in the Americas is caused by Trypanosoma cruzi and is found in humans, dogs, cats, and rodents. T. cruzi is a macrophage-invading protozoan, and complicating factors in the development of vaccines are immune escape and autoimmunity due to molecular mimickry (Girones et al., 2005). Macrophages are also the target for Leishmania parasites, which use complex immune evasion strategies that affect host cell signaling (Olivier et al., 2005). Immunization with the T. cruzi Tol A-like protein (TolT) expressed in insect cells resulted in T cell-dependent antiparasitic activity (Quanquin et al., 1999). For Leishmania several candidate subunit vaccine antigens with protective potential have been identified, including the surface protein gp63 (Coler and Reed, 2005), but these proteins have not been expressed with baculovirus vectors in insect cells.

D. Helminths

Helminths or parasitic worms that are a serious threat to human and animal health worldwide, are divided into the Annelida (segmented worms), Platyhelminthes (flatworms including flukes), and Nematoda (roundworms). These worms have complex life cycles, which often involve more than one host. Because helminths vary widely among populations, vaccines are not competitive so far with chemical broad spectrum antihelminths (Bos and Schetters, 1990). Helminths may also modulate the immune system as exemplified by the filarial nematodes, which are present in 150–200 million humans worldwide and cause river blindness for example. These filarial nematodes are difficult to combat because they modulate T cell responses leading to chronic helminth infections (reviewed by Hoerauf et al., 2005). This T cell modulation is not restricted to the response to filarial larvae but also affects allergies, the response to other pathogens and vaccines through modulation of not only antigen-specific T cells but also APCs, which affects immune responses in general.
There are a few examples of baculovirus-expressed helminth proteins but few of these have been tested in immunization studies (Table IV). However, the baculovirus expression system may be a valuable tool for these parasitic worms as illustrated by the following examples: a baculovirus-derived subunit vaccine against liver fluke (*Fasciola hepatica*) based on procathepsin L3 conferred 50% protection to rats, in contrast to the yeast-expressed protein which did not confer any protection (Reszka *et al.*, 2005). Bilharzia or schistosomiasis is caused by *Schistosoma* spp. (Platyhelminthes), blood parasites of humans in tropical areas. Several *Schistosoma* genes have been expressed in insect cells, but primarily for analysis of enzymatic functions. The large subunit of calpain (SmP80) produced in the baculovirus expression system reduced the worm burden in mice (Hota-Mitchell *et al.*, 1997). Antigens of the tapeworm *Taenia solium* have been expressed with baculovirus vectors for diagnostic purposes (Lee *et al.*, 2005; Levine *et al.*, 2004).

V. CONCLUSIONS AND PROSPECTS

There are many examples in the literature where immunization with recombinant proteins produced in the baculovirus–insect cell expression system conferred good protection against infectious disease. Subunit vaccine development begins with careful identification of the antigen, which is related to whether neutralizing adaptive immune responses are raised against the particular protein in natural infections. Once selected, the open reading frame (ORF) of the antigen is cloned while keeping flanking DNA sequences to a minimum, which can best be achieved with a proof-reading PCR enzyme. The sequence around the ATG translational start site is best modified to that of the polyhedrin or p10 gene in the wild-type baculovirus, with at least an adenosine residue at the –3 position (Chang *et al.*, 1999). Tags may be added to facilitate purification, preferably in such a way that they can subsequently be removed. Tags are not required for VLPs because they can be purified by centrifugation. Transmembrane (glyco) proteins are best produced in a secreted form by removal of TMRs to increase expression levels. However, this may occasionally affect the folding of the protein. An alternative approach is to fuse the immunogenic domains of envelope proteins to GP64. Vectors that lack chitinase and v-cathepsin genes are preferable for expression of envelope proteins. During the preparation of seed stocks, care should be taken to keep the virus passage number low and to use
a multiplicity of infection of <0.1 to minimize the formation of DIs. Careful checking of the purity of recombinant bacmids or plaque purified recombinant viruses by PCR is crucial to avoid loss of recombinant virus in subsequent passages due to empty vectors that out-compete the recombinants.

Only two baculovirus-produced products are approved for veterinary practice, namely, two vaccines against classical swine fever consisting of the E2 surface glycoprotein. With these vaccines in the market (although a nonvaccination policy still exists) the confidence in this type of subunit vaccine will grow as well as the possibilities for registration, thereby increasing the likelihood that more vaccines of this kind will appear in the market. This expectation of an increasing number of products may be expanded to human applications when registration of a trivalent recombinant influenza vaccine, for which phase III clinical trials in humans in the United States have been completed recently, can be achieved. Therapeutic anticancer vaccines, such as a vaccine against prostate cancer, may be more readily accepted, in view of the severe side effects of anticancer drugs and irradiation techniques. The application of baculovirus display or BacMam™ vectors for vaccines against infectious disease may be applied in the future for animal use. Because more foreign proteins are incorporated into the vaccine than just the targeted antigen, such vaccines for human use are likely to be in the more distant future because of safety considerations.

Baculovirus expression systems compete with other cheaper production systems, which are more widely used and which scale-up more easily, such as *E. coli* and yeast (Table I), and once a good protection level is achieved there is no commercial interest to switch to the more expensive insect cell system. For those cases though, where folding or posttranslational modifications are crucial to epitope formation the baculovirus–insect cell system is a versatile expression system and many candidate vaccines have been successfully tested. Alternative methods are provided by the development of DNA vaccine technology and recombinant carrier vaccines based on vaccinia, adenovirus, or BacMam™ vectors (Table II). These methods are more prone to induce cellular immune responses than many protein subunit vaccines. The combination of a primary vaccine with an intracellular delivery system (recombinant carrier vaccines, DNA vaccines, BacMam™ vectors) followed by a boost immunization with recombinant protein subunits appears to be a promising approach, aimed at both cellular and humoral immune responses. This approach can be further strengthened through the addition of recombinant cytokines that drive the
immune response in a specific direction. A major challenge now is to broaden the array of viral vaccines produced in insect cells and to develop effective vaccines against more complex organisms, such as protozoan parasites and multicellular worms, for which the baculovirus expression system also holds promise. With the increasing insight into immunology leading to new methods of vaccine production and delivery, accompanied by a wealth of genomic and proteomic data, many new generation vaccines are expected within the foreseeable future.

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