Characterization of a quadruple glycoprotein-deleted pseudorabies virus mutant for use as a biologically safe live virus vaccine

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Herpesvirus envelope glycoproteins play important roles in mediating infection initiation and also represent major immunogens. We recently showed that a pseudorabies virus (PrV) mutant lacking the essential glycoprotein gD (gp50), after phenotypic complementation by propagation on genetically engineered PrV gD-expressing cell lines was able to infect primary target cells and spread exclusively by means of direct cell-to-cell transmission. Virions released from non-complementing cells that lacked gD were not infectious because of a defect in penetration and so free infectious virions did not arise after infection of animals by phenotypically complemented gD-negative PrV. This formed the basis for the development of novel non-spreading live herpesvirus vaccines. However, the gD-negative PrV mutant still retained a residual level of virulence, which prevented its use as vaccine, and the need to propagate the gD-negative PrV mutant on trans-complementing cell lines resulted in the appearance of wild-type revertants, rescued by the resident gene in the cell line. To overcome these problems we isolated a PrV mutant designated PrV(376) that, in addition to gD, also lacked the non-essential glycoproteins gG, gI and gE. PrV(376), because of the lack of gD, was also dependent on gD-expressing cells for productive replication. Non-complementing cells infected by phenotypically gD-complemented PrV(376) produced non-infectious particles lacking glycoproteins gD and gE as shown by immunoelectron microscopy. Owing to the absence of any homologous sequences between the viral genome and the viral genes resident in the complementing cell line, rescue by homologous recombination was impossible. In cell culture, plaques of PrV(376) were significantly smaller than those of either wild-type, or gD- or gE-deleted mutants, indicating an additive or synergistic effect of the combined deletion on viral cell-to-cell spread capability. Intranasal or intramuscular infection of pigs with phenotypically gD-complemented PrV(376) showed a complete attenuation of viral virulence, with an expected lack of shedding of infectious virus. The PrV(376)-vaccinated pigs exhibited a significant level of protection against challenge infection, measured by survival and weight loss. In summary, PrV(376) represents a novel type of herpesvirus vaccine that combines innocuity, efficacy and biological safety.

Introduction

Herpesvirus envelope glycoproteins are involved in several important steps during virus infection, such as attachment of free virions to target cells and entry of the nucleocapsid by fusion between the virion envelope and the cellular cytoplasmic membrane (for a review see Spear, 1993). In addition, glycoproteins have been described as major immunogens of herpesviruses. In pseudorabies virus (PrV), a member of the neurotropic alphaherpesviruses, seven glycoproteins have been described so far that are all homologues of glycoproteins identified in the closely related herpes simplex virus (HSV) (reviewed in Mettenleiter, 1991). Four of them, gC (gIII), gE (gI), gI (gp63) and gG (gX) have been found to be dispensable for viral replication, at least in cell culture. In contrast glycoproteins gB (gII), gD (gp50) and gH are essential components of the virion. (At the 1993 International Herpesvirus Workshop, researchers working on alphaherpesviruses agreed upon a common nomenclature for alphaherpesvirus glycoproteins based on the designations of homologous glycoproteins in HSV. This new nomenclature is used throughout this manuscript.)

Virus particles lacking any one of these essential glycoproteins are blocked at the level of penetration (Rauh & Mettenleiter, 1991; Peeters et al., 1992a, b). Whereas it had been shown in HSV that all glycoproteins necessary for virus entry also play essential roles in direct viral cell-to-cell spread (Spear, 1993), this is not true for PrV gD. After phenotypic complementation by propagation on PrV gD-expressing cell lines, gD-
negative PrV is able to infect primary target cells and spread directly from cell to cell. This demonstrates that PrV gD is needed for penetration but is dispensable for direct cell-to-cell transmission (Rauh & Mettenleiter, 1991; Peeters et al., 1992a) which indicates that penetration and cell-to-cell spread have different requirements. This results in a phenotype unique for herpesvirus; once primary infection has occurred, gD-negative PrV is able to spread exclusively by direct cell-to-cell transmission in cell culture and in the animal. Since gD-negative PrV virions released from non-complementing cells are not infectious, free infectious virus cannot be detected after infection of animals with phenotypically complemented gD-negative PrV (Heffner et al., 1993; Peeters et al., 1993). This finding provides the basis for the development of a new type of herpesvirus vaccine that, after primary infection, is biologically contained within the vaccinated animal and therefore does not involve the hazard of uncontrolled spread between animals and persistence of infectious vaccine virus in vaccinated herds.

Of the envelope glycoproteins of PrV, gC and gD have been described as major immunogens (Ben-Porat et al., 1986; Marchioli et al., 1987). Potent complement-independent neutralizing monoclonal antibodies (MAbs) against both glycoproteins have been isolated (Hampel et al., 1984; Warthen & Warthen, 1984). In addition, gC has been shown to represent a major target for cell-mediated immunity (Zuckermann et al., 1990). The non-essential glycoprotein gE is thought to be one of the prominent virulence-determining factors of PrV (Lomniczi et al., 1984; Mettenleiter et al., 1987; Kimman et al., 1992). It is found in a non-covalently linked complex with gI that probably represents the biologically active entity (Zuckermann et al., 1988). Molecular analyses have shown a lack of gE in several live attenuated PrV vaccine strains (Mettenleiter et al., 1985). This deficiency has been attributed to selection for a gE-negative phenotype during the passage of wild-type PrV in chicken-derived cells, a procedure commonly used for the attenuation of virulent virus (Mettenleiter et al., 1988). Since PrV field strains generally express gE, the presence or absence of anti-gE antibodies is being used to differentiate vaccinated and infected pigs, which is an important prerequisite for economical eradication of the disease (van Oirschot et al., 1986). Similar assays are based on the presence of antibodies directed against either the non-essential non-structural gG or the non-essential structural gC, which are missing in several genetically engineered PrV vaccine strains (for a review see Wittmann & Rziha, 1989).

Recently it has been shown that a phenotypically complemented gD-negative PrV mutant exhibited a significantly decreased virulence compared to wild-type PrV after intranasal infection of pigs (Heffner et al., 1993). Under these experimental conditions, infection by wild-type PrV led to severe neurological and respiratory disorders whereas the gD-negative PrV mutant did not induce any neurological symptoms. However, the occurrence of respiratory distress in the animals was indicative of a residual virulence (Heffner et al., 1993). Surprisingly, despite the absence of antibodies against gD, animals were protected to a significant level against a stringent challenge infection after vaccination by phenotypically complemented gD-negative PrV (Heffner et al., 1993). These data have shown that gD is not absolutely required for induction of a good immune response and that live vaccines based on gD-negative PrV mutants that are no longer capable of spreading between animals or of establishing themselves in the field might constitute a novel type of herpesvirus live vaccine.

To reduce the residual virulence of the gD-negative PrV mutant we attempted to delete, in addition, genes encoding the virulence-associated gE and gI, as well as the gene for gG and replace them with a β-galactosidase reporter gene (Mettenleiter & Rauh, 1990). This virus mutant was expected to have an even more reduced virulence; it would not contain sequences homologous to the viral genes that are resident in the gD-expressing cell line necessary for propagation of the mutant and would therefore eliminate the problem of rescue; and the mutant would also be compatible with differentiating antibody tests based on either gE or gG. We describe here the isolation of a gD-, gE, gG- and gI-deleted PrV mutant designated PrV(376). This mutant could be propagated in vitro in gD-expressing cell lines without any noticeable levels of rescue. Infection of pigs with phenotypically gD-complemented PrV(376) showed a complete absence of symptoms. After a challenge infection these animals were protected against PrV to a significant degree. Our results show for the first time the feasibility of combining innocuity, efficacy and a lack of infectious virus production after primary infection in one vaccine strain. These results represent an important step toward the construction of new safe non-spreading herpesvirus vaccines.

**Methods**

**Viruses and cells.** All viral mutants are based on the Kaplan strain of PrV (Kaplan & Vatter, 1959). Mutants lacking gE, PrV(1311), or gG, PrV(1112), because of the insertion of a β-galactosidase expression cassette into the respective glycoprotein gene have been described previously (Mettenleiter & Rauh, 1990), as has the gD-negative mutant, PrV(133), which also lacks gG because of an unexpected recombination event (Rauh & Mettenleiter, 1991). Viruses were propagated on the African green monkey-derived Vero cell line or on the genetically engineered PrV gD-expressing bovine kidney cell line MT50-3 (Rauh & Mettenleiter, 1991). Plaque tests were performed on Vero cells. To screen for rescued virus in the gD-negative PrV stocks,
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Fig. 1. Construction of PrV(376). (a) BamHI restriction map of the viral genome. Open rectangles represent the inverted repeat regions that separate the genomic DNA into a unique long and unique short portion. (b) The part of the unique short region that includes genes encoding the protein kinase (PK; van Zijl et al., 1990), gG (Rea et al., 1985), gD, gI and gE (Petrovskis et al., 1986a, b), as well as proteins of 11K (Petrovskis & Post, 1987) and 28K (van Zijl et al., 1990) is shown enlarged. Relevant restriction sites are indicated, as is the extent of the deletion introduced into PrV(376). (c) Genomic profile of PrV(376) within this region. In (d) the viral gD–gI expression unit in the complementing cell line MT50-3 is shown.

Electron microscopy. Immunoelectron microscopy of purified PrV virions was performed as described by Rauh et al. (1991).

Animal experiments. Six-week-old specific pathogen-free (SPF) pigs were housed in containment facilities. Animals were given commercial food and water ad libitum. Seven animals were infected intranasally with $10^7$ TCID$_{50}$ of phenotypically complemented PrV(376). A second group of seven pigs received the same dose intramuscularly. Five animals were left untreated as controls. Three weeks after vaccination all pigs were challenged intranasally with $10^7$ TCID$_{50}$ of PrV strain 75V19 and monitored for clinical signs, body temperature, weight gain and virus excretion.

Results

Construction of quadruple glycoprotein-deleted mutant PrV(376)

Plasmid TT-264 comprises an approximately 300 bp SalI–BamHI fragment encompassing the promoter and the first seven codons of the gG gene fused to codon eight of the Escherichia coli lacZ gene encoding β-galactosidase. This resulted in the inactivation of the BamHI site at the 5' end of the lacZ insert (Mettenleiter & Rauh, 1990). At the 3' end of the insert, a unique BamHI site remained that was used for insertion of an approximately 1.4 kb genomic viral SalI–BamHI fragment (see Fig. 1) encompassing the 3' end of the gE gene, the 11K protein gene and the 5' part of the 28K protein gene. To achieve...
this, plasmid TT-264 was cleaved with BamHI and the 1.4 kb SphI-BamHI fragment was inserted by firstly ligating the sticky BamHI end of this fragment to the BamHI site at the 3' end of the lacZ gene. Then the free ends were trimmed by treatment with the Klenow fragment of DNA polymerase and T4 polymerase and blunt-end ligated into the plasmid. This led to restoration of both BamHI sites, at the 3' end of the lacZ gene and at the 3' end of the SphI-BamHI insert (Fig. 1). Correct orientation was verified by restriction enzyme digestion and the resulting plasmid was designated TT-376.

To isolate a virus mutant lacking gG, gD, gI and gE, plasmid TT-376 was cotransfected with wild-type PrV DNA into gD-expressing MT50-3 cells (Rauh & Mettenleiter, 1991). After complete c.p.e. became apparent, cells and supernatants were harvested and plated in serial dilutions onto MT50-3 cells under plaque assay conditions. Two days after infection progeny viruses were screened for mutant phenotypes by Bluo-Gal agarose overlay (Mettenleiter & Rauh, 1990). Blue-staining plaques were picked and plaque-purified three times. One representative virus mutant, named PrV(376), was analysed in detail.

**Characterization of PrV(376)**

To test for correct integration of the mutated fragment into the viral genome, DNA was isolated from purified wild-type PrV or PrV(376) and cleaved with BamHI, double-digested with BamHI and SphI or cleaved with KpnI (Fig. 2). Fragments were separated in a 0.8% agarose gel and visualized under u.v. light after ethidium bromide staining (Fig. 2a), or blotted onto nitrocellulose and hybridized with either viral BamHI fragment 7 (Fig. 2b), BamHI fragment 10 (Fig. 2c), or a lacZ-specific probe (Fig. 2d). It is evident that the wild-type BamHI fragment 10 is missing in DNA from PrV(376) (Fig. 2a, lane 2) because of the fusion of this fragment to lacZ.
sequences and the concomitant loss of BamHI fragment 17, as well as both BamHI sites separating BamHI fragments 10 and 7 in the wild-type PrV DNA (see Fig. 1). This resulted in PrV(376) DNA in a fusion fragment composed of BamHI 10 and BamHI 7, which migrated slightly more slowly than wild-type BamHI 7, and the disappearance of the BamHI 7 fragment present in wild-type DNA (Fig. 2a, lanes 1 and 2). Hybridization with fragment BamHI 7 shows that in PrV(376) only a 1-4 kb BamHI fragment remained from the approximately 6-9 kb wild-type BamHI fragment 7 (Fig. 2b, lane 2). This 1-4 kb fragment can be seen in wild-type PrV DNA after double digestion with BamHI and SphI (Fig. 2b, lane 3). Hybridization with fragment BamHI 10 (Fig. 2c) highlights the absence of a wild-type sized BamHI fragment 10 in PrV(376) DNA, and the appearance of an approximately 7 kb fragment. This fragment encompasses BamHI 10 fused to lacZ, as can be seen after hybridization with a lacZ specific probe (Fig. 2d). Since BamHI 10 contains sequences derived from the inverted repeat regions, hybridization with BamHI 12 is also observed (Fig. 2c, lanes 1 and 2). Patterns obtained after double digestion with BamHI and SphI also confirmed the correct integration of the mutated fragment into the viral genome. After cleavage with KpnI, a large fusion fragment consisting of fragments KpnI I and J was observed in PrV(376) DNA (Fig. 2a to d, lanes 5 compared with lanes 6) due to the deletion of both KpnI sites within the gD gene (see Fig. 1). In summary, DNA analysis showed that a deletion of genomic sequences encompassing the gG (Rea et al., 1985), gD and gI genes, as well as part of the eE gene (Petrovskis et al., 1986a, b) had been introduced into the genome of mutant PrV(376), concomitant with the insertion of a β-galactosidase expression unit.

**PrV(376) lacks glycoproteins gD, gE and gG**

To ascertain the absence of the corresponding gene products in cells infected by PrV(376), immunoprecipitations of 35S-labelled proteins from lysates or supernatants of non-complementing cells infected with either wild-type PrV or phenotypically gD-complemented PrV(376) were performed (Fig. 3). Precipitations with MAbs against gE, gD and gG showed the presence of gE, gD and gG in wild-type PrV-infected cells (Fig. 3, lanes 1 and 4) or culture supernatant (Fig. 3, lane 5), and their absence in PrV(376)-infected cells (Fig. 3, lanes 7, 10 and 11). Although gC was present in both wild-type PrV-infected cells and PrV(376)-infected cells, significantly less gC was precipitated from PrV(376)-infected cells (Fig. 3, lane 9) compared with wild-type PrV-infected cells (Fig. 3, lane 3). This is especially evident after a longer exposure of the autoradiograph film (Fig. 3b). Precipitation of gB was similar from cells infected by either virus, thereby serving as an internal control (Fig. 3, lanes 2 and 8). The results from precipitations using a negative control MAb are also shown (Fig. 3, lanes 6 and 12). These results indicate that PrV(376) did not express gE, gD and gG. Reagents to test for the presence of gI were not available. In addition, gC appears to be underexpressed in PrV(376) compared to wild-type PrV, whereas expression of gB was similar in both viruses.

**Cells infected by PrV(376) produce non-infectious virions lacking gD and gE**

To test whether non-complementing cells infected by phenotypically complemented PrV(376) produce virions and to assay directly for the presence of glycoproteins in...
the virus envelope, purified virions were analysed by immunoelectron microscopy. As shown in Fig. 4, wild-type PrV particles, as expected, exhibited reactivity with MAbs directed against gB, gC, gD and gE. In contrast, PrV(376) particles produced by non-complementing cells did not react with either the anti-gD or the anti-gE MAbs. This shows that PrV(376)-infected non-complementing cells produced virus particles with a wild-type morphology that, however, lacked gD, and consequently infectivity, as well as lacking gE. Reactivity with both the anti-gB and anti-gC MAbs was similar in wild-type and PrV(376) virions. Thus, despite the lower intracellular level of gC in PrV(376)-infected cells found after immunoprecipitation, immunoelectron microscopy did not detect a similarly lowered level of gC in PrV(376) particles.

**Plaque size of mutant PrV(376)**

Despite its essential nature for infectivity of free virions, it had been established that PrV gD is not essential for direct cell-to-cell spread of the virus and consequent plaque formation (Peeters et al., 1992a; Rauh & Mettenleiter, 1991). Although not essential, gE appears to modulate the efficiency of direct cell-to-cell transfer of the virus (Zsak et al., 1992). We therefore analysed whether the concomitant absence of gD, gE and gI had a synergistic effect on virus spread. To this end MDBK cells were infected with gG-negative PrV(1112), gE-negative PrV(1311), gD-negative PrV(133) and the quadruple mutant PrV(376), which all contained a β-galactosidase expression cassette. Three days after infection, plaques were stained with Bluo-Gal and analysed under the microscope. As shown in Fig. 5, compared to the gG-negative mutant which produces wild-type-like plaques (Mettenleiter et al., 1990; Thomsen et al., 1987a), lack of gE slightly decreases plaque size. However, this decrease is much less dramatic than that observed by others using a different PrV strain (Jacobs et al., 1993a). Deletion of gD had a more pronounced effect. PrV(376) exhibited the smallest plaques which indicates an additive or synergistic effect of these deletions on direct viral cell-to-cell spread. Since a β-galactosidase-expressing gI-negative PrV mutant was not available, the effect of gI deletion could not be tested in this experiment. However, it has previously been shown that gI-negative PrV behaves like gE-negative PrV in cell culture and that a double deletion of gE and gI did not alter the observed phenotype (Zuckermann et al., 1988).

**Rescue frequency of PrV(376) on MT50-3 cells**

A major concern in the use of virus mutants deleted in essential genes that have to be propagated on complementing cell lines is the possibility of reversion. Driven by
homologous flanking sequences present in both the viral genome and the virus-derived genes in the cellular genome, recombination can occur, leading to the restoration of a wild-type phenotype to the mutant virus. PrV(376) was constructed in such a way that its genome lacked any homology to the gD-gI expression unit in the MT50-3 cell line used for trans-complementation. In several experiments, we did not observe reversion of the gD-negative genotype to gD-positive wild-type viruses, but reversion did occur when a PrV mutant was used that lacked gD but still retained homologous sequences on both sides of the mutation (data not shown).

**PrV(376) represents a safe and efficacious vaccine**

Originally we used a PrV mutant that only lacked gD for efficacy tests in pigs (Heffner et al., 1993). Although a good level of protection from challenge infection was induced, this virus mutant still retained residual virulence, resulting in the appearance of respiratory symptoms. In PrV(376) the additional deletion of gE, which has been shown to contribute to viral virulence (Mettenleiter et al., 1987; Kimman et al., 1992; Jacobs et al., 1993b), was expected to attenuate the virus further. However, deletion of four glycoproteins could seriously impede induction of a protective immune response.

Indeed, gD has been shown to represent a major PrV immunogen (Marchioli et al., 1987) and gE has also been proposed to play an important role in immunity (Fuchs et al., 1990), although vaccine strains deleted in gE induce good protective immune responses (reviewed in Wittmann & Rziha, 1989). To test for the efficacy of PrV(376) as a vaccine, seven 6-week-old piglets were infected intranasally and a similar group of seven animals were infected intramuscularly with 10⁵ TCID₅₀ of phenotypically gD-complemented PrV(376). Viral titres had been determined on complementing MT50-3 cells. The animals were monitored daily for clinical signs of infection, changes in body temperature, and in the rate of weight gain, and virus excretion. After vaccination with PrV(376) no clinical signs or fever were observed, weight gain was normal and excretion of vaccine virus did not occur (data not shown). This demonstrated that simultaneous loss of glycoproteins gD, gE, gG and gI led to a complete attenuation of the virus for 6-week-old pigs.

Three weeks after the single vaccination, a challenge infection with 10⁷ TCID₅₀ of the highly virulent PrV strain 75V19 was performed. Two out of five unvaccinated control animals died at days 5 and 6 after the challenge. Survivors showed extensive growth retardation for more than 11 days (Fig. 6). In contrast, all animals vaccinated with PrV(376) survived. Growth retardation was significantly diminished. Pigs that had been vaccinated intramuscularly started to gain weight at
the same rate as before the challenge at day 5 after the challenge. Animals vaccinated intranasally stagnated for one more day before gaining weight. Both groups regained their prechallenge weight, at days 8 and 9 post-challenge respectively. In contrast, even as late as day 12 after challenge the unvaccinated control group had not yet reached their prechallenge weight. In addition, excretion of challenge virus was much reduced in the vaccinated animals (data not shown). In summary, animals vaccinated either intranasally or intramuscularly with phenotypically gD-complemented PrV(376) were protected to a significant degree from challenge infection.

Discussion

Vaccination against Aujeszky’s disease with attenuated live PrV vaccine strains is widely performed (for a review see Wittmann, 1991). These strains were obtained either by ‘classical’ means, such as adaptation to growth in cells from animals that are not natural hosts or selection for drug resistance or temperature-sensitive phenotypes (reviewed in Wittmann & Rziha, 1989). Recently, genetic engineering methods have been employed and resulted in the licensing by the U.S. Department of Agriculture of the first genetically engineered live virus vaccine (Kit, 1990). A genetically engineered live PrV vaccine (Visser & Lütticken, 1989) has also been approved by the European registration authority. All genetically engineered PrV vaccines in use carry mutations that inactivate the viral thymidine kinase gene, which is an important determinant for neurovirulence. In addition, these strains lack one or more non-essential glycoproteins to enable serological differentiation of the immune response induced by wild-type virus infection from vaccine antibodies (van Oirschot et al., 1986). Based on this serological discrimination between vaccinated and wild-type infected animals, large-scale eradication programmes have been devised.

Although genetically engineered live PrV vaccines have been shown to be efficacious and safe, there remains the possibility of spread between vaccinated and unvaccinated animals, of persistence in the field (Christensen et al., 1992) and of recombination between different vaccine strains (Henderson et al., 1991), which can lead to enhanced virulence as shown after inoculation of sheep (Henderson et al., 1990). Therefore, especially for use as vector vaccines carrying heterologous genes of other viruses or pathogens, an increase in the biological containment of the vaccine virus is needed. Recently, we developed a new concept for vaccination against Aujeszky’s disease using phenotypically complemented gD-deleted PrV mutants (Heffner et al., 1993). PrV gD has been shown to be necessary for virion infectivity, as it is involved in fusion between the virus envelope and the cellular cytoplasmic membrane, which precedes infectious entry of the nucleocapsid into the cytoplasm (Peeters et al., 1992a; Rauh & Mettenleiter, 1991). Therefore virions lacking gD are not infectious. Surprisingly, PrV gD was found to be dispensable for direct viral cell-to-cell spread, both in cell culture and in animals (Babic et al., 1993; Heffner et al., 1993; Peeters et al., 1993). This led to the proposal that animals could be vaccinated with genetically gD-negative PrV mutants that had been phenotypically complemented by propagation on gD-expressing cell lines. These phenotypically complemented virions are capable of infecting primary target cells in the animal like wild-type virions. However, subsequent viral spread can only occur by direct cell-to-cell transmission. Consequently, the presence of free infectious virus was demonstrable neither in organ extracts of infected mice nor in nasal swabs from infected pigs (Heffner et al., 1993). Despite the absence of the major immunogen gD, inoculation with this virus mutant led to the induction of a good protective immunity (Heffner et al., 1993).

However, the gD-negative PrV mutant used had several disadvantages. Although it was attenuated compared to wild-type PrV, residual virulence resulting in the appearance of respiratory symptoms after infection was observed (Heffner et al., 1993). In addition, homologous sequences were still present in the viral genome for recombination with the gD–gI expression unit (Kost et al., 1989) in the complementing cell line. This bears the risk of high-frequency rescue of the mutation in the viral genome by the wild-type genes in the complementing cell line, leading to restoration of the defective gene for gD, which could severely limit the usefulness of this mutant as a vaccine. A third disadvantage was the continuous presence of both gG and gE, whose absence in vaccine strains that are already in use is employed for the differentiation of vaccine antibodies from field virus-induced antibodies. The virus strain presented here appears to overcome all these problems. Deletion of a 5320 bp region from the short unique portion of the viral genome, encompassing the genes encoding gG, gD, gI and most of the gE gene, removed all sequences that were homologous to corresponding sequences in the complementing cell line MT50-3. So far we have not observed restoration of the defect in the viral genome by homologous recombination with the viral genes in the cell line. Although we cannot absolutely rule out the possibility that non-homologous recombination might occur, its frequency would be several orders of magnitude lower than homologous recombination, thus precluding detection of the event in our assays. The result of this putative recombinational event between wild-type PrV and PrV(376) can only be
the transfer of the gG, gD, gI and gE deletion into the wild-type strain and the restoration of expression of these genes in the vaccine strain. This would result in the appearance of a wild-type-like PrV and a vaccine-like PrV strain, which exactly mimics the situation before recombination.

After vaccination of pigs with the phenotypically gD-complemented quadruple glycoprotein-deleted PrV(376), no adverse side-effects were observed. This indicated that the additional deletion of gE and gl, which have been demonstrated to influence PrV virulence (Mettenleiter et al., 1987; Kimman et al., 1992), further reduced virulence of the gD-negative PrV and led to complete attenuation for 6-week-old pigs. Challenge infection showed that even in the absence of four glycoproteins, PrV(376) was able to induce a protective immune response. In fact, PrV(376) easily fulfilled the criterion of the pig potency test outlined by the European Pharmacopoeia (1991). The calculated relative average daily percentage of weight gain (AG) for the intramuscular and intranasal routes was 2.2 and 1.5 respectively. This is in excess of the AG of 1.2 required by the European Pharmacopoeia test.

The advantages of PrV(376) in terms of biological safety, innocuity and potency make this virus strain a first choice as the basis for development of PrV vector vaccines. Several heterologous proteins have already been expressed in PrV (Thomsen et al., 1987b), including antigens of unrelated viruses (van Zijl et al., 1991; T. C. Mettenleiter, unpublished results). However, widespread use of genetically engineered recombinant PrV vectors is dependent upon their biological safety. The gD-negative PrV mutants have a unique phenotype; they are able to spread within the infected animal by direct cell-to-cell transmission, thereby stimulating a good immune response but they cannot be shed in infectious form since the absence of gD renders these virions uninfected (Heffner et al., 1993; Peeters et al., 1993). This leads to a form of natural biological containment of the virus recombinant within the infected/vaccinated animal. Removal of the vaccinated animals will lead to removal of the virus recombinant from the population. Lack of uncontrolled spread, as well as an inability to establish itself in the field, will also reduce the probability of recombination events between field strains and viral mutants although, as outlined above, it appears highly unlikely that recombination involving PrV(376) could result in the appearance of novel PrV strains with unwanted properties.

There are two other interesting features in PrV(376) that are currently under further investigation. The underexpression of gC within cells infected by PrV(376) compared with wild-type PrV-infected cells might be indicative of the regulation of gC expression by one (or more) of the glycoproteins deleted in PrV(376). We are currently testing single mutants to analyze whether deletion of any specific glycoprotein can account for this phenomenon. Surprisingly, immunoelectron microscopy failed to detect a similarly decreased level of gC in extracellular PrV(376) virions. However, detailed quantitative analyses are necessary to substantiate this finding. Our data seem to indicate that gC is accumulating in PrV(376) virions despite its low level within infected cells.

A second interesting result concerns the cell-to-cell spread capabilities of different glycoprotein mutants compared with PrV(376). A single deletion of gD or gE resulted in only a slight decrease in plaque size, but the combined deletion of gD, gE, gG and gl led to an additive, or even synergistic effect resulting in a dramatic decrease in cell-to-cell spread as indicated by plaque size. Deletion of gG alone did not affect plaque size when compared to wild-type PrV (Mettenleiter et al., 1990). This finding indicates that gD and gE probably affect different stages in the processes responsible for cell-to-cell spread. We are currently analysing this hypothesis in more detail.

In summary PrV(376), lacking glycoproteins gD, gE, gG and gl, represents a safe and efficacious vaccine against Aujeszky's disease. This virus mutant also forms an excellent basis for the development of new PrV vector vaccines for combating various diseases in pigs and possibly other animals.

The expert technical assistance of M. Kaufmann is gratefully acknowledged. This work was supported by grants from the DFG (Me 854) and Intervet International.

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(Received 25 November 1993; Accepted 25 January 1994)