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Biologically safe, non-transmissible pseudorabies virus vector vaccine protects pigs against both Aujeszky's disease and classical swine fever

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Envelope glycoprotein D (gD) of pseudorabies virus (PRV) is essential for penetration but is not required for cell-to-cell spread. When animals are inoculated with a phenotypically complemented PRV gD mutant, the virus is able to spread locally by means of direct cell-to-cell transmission, but progeny virions released by infected cells are non-infectious because they lack gD. Therefore, the virus cannot be transmitted from inoculated animals to other animals. This property makes a PRV gD mutant an attractive candidate as a safe vaccine vector. To examine whether a self-restricted, non-transmissible PRV mutant can be used as a biologically safe vaccine vector, a gD/gE-negative PRV recombinant virus which expresses envelope glycoprotein E2 of classical swine fever virus was constructed. Vaccination of pigs showed that the recombinant virus was able to protect pigs against both Aujeszky's disease and classical swine fever.

Vaccination is one of the most effective prophylactic measures to protect humans and animals against infectious diseases. Of the different types of viral vaccines available, live attenuated vaccines are often preferred over inactivated or subunit ones because live vaccines are able to induce a long-lasting humoral as well as a cellular immune response. However, live vaccines may suffer from several drawbacks, which are mainly related to safety (Kimman, 1992). Live attenuated genetically engineered deletion mutant vaccines and vaccine vectors are generally quite safe. However, when they are transmitted from vaccinated to non-vaccinated individuals, they may pose a threat to immunocompromised hosts or other species. Candidate genes for incorporation into viral vaccine vectors often code for structural virion proteins that are highly immunogenic (Hilleman, 1994; Yilma, 1994). Because such proteins are often involved in virus–cell interactions, their incorporation may alter the pathogenicity, tissue-tropism and host-specificity of the vector (Dong *et al.*, 1992). Furthermore, foreign genes and their associated phenotypes may be transferred, by means of homologous recombination, from the attenuated vector virus to a virulent wild-type virus.

The considerations mentioned above argue for the development of live vaccines and vaccine vectors that are self-restricted, i.e. which cannot be disseminated by the vaccinee. Examples of such vaccines are avipox viruses, which may be used as live vector vaccines that do not yield infectious progeny when used for mammalian species (Baxby & Paoletti, 1992). More recently, a more general approach has been developed which makes use of phenotypically complemented mutants, i.e. mutants which are defective in an essential gene and which are grown on complementing cells that provide the missing viral gene product *in trans* (Nguyen *et al.*, 1992; Farrell *et al.*, 1994).

We and others have constructed pseudorabies virus (PRV; the causative agent of Aujeszky's disease in pigs) mutants which are defective for gD, a viral envelope glycoprotein that is essential for penetration but which is not required for cell-to-cell spread (Peeters *et al.*, 1992; Rauh & Mettenleiter, 1992). When cells or animals are inoculated with phenotypically complemented PRV gD mutants, the virus is able to spread by means of direct cell-to-cell transmission, but progeny virions released by infected cells are non-infectious (Peeters *et al.*, 1993; Heffner *et al.*, 1993). Furthermore, it has been shown that PRV gD mutants can be used as a vaccine to protect pigs against Aujeszky's disease (Heffner *et al.*, 1993; Peeters *et al.*, 1994, 1995; Mettenleiter *et al.*, 1994). This finding suggests that PRV gD mutants may also be used as biologically safe, non-transmissible vaccine vectors for the expression of antigens of unrelated viruses.

To test this, we inserted the gene encoding envelope glycoprotein E2 (previously called E1; Wensvoort *et al.*, 1988, 1990) of classical swine fever virus (CSFV; also called hog cholera virus) into the genome of the PRV gD/gE double-

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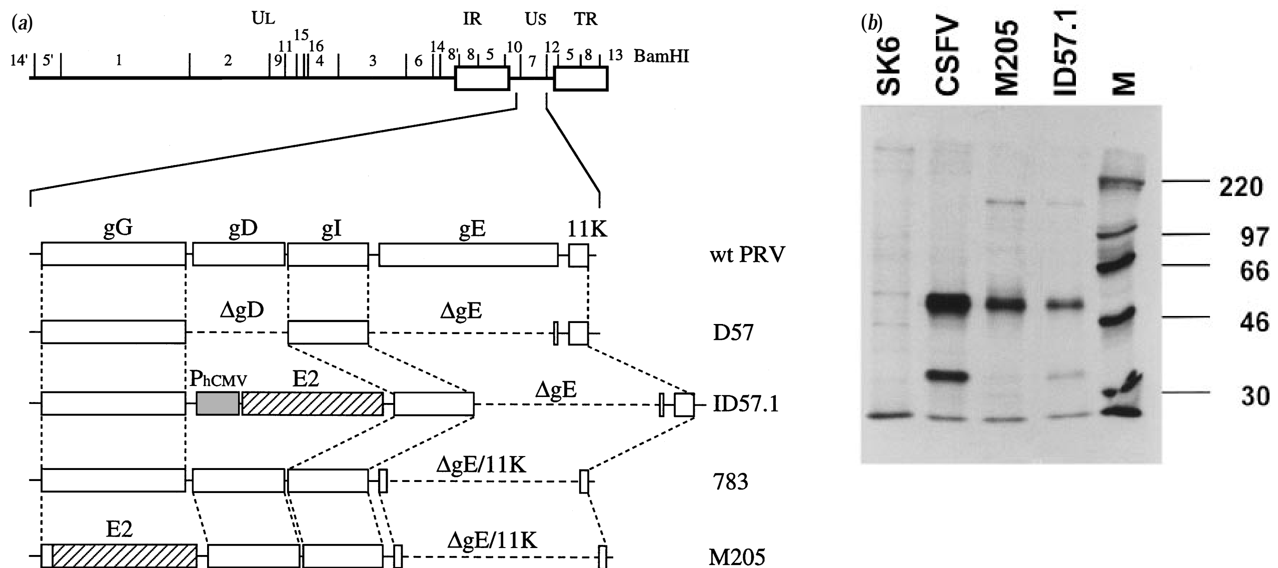

Fig. 1. (*a*) Physical map of the PRV genome (top line). Open rectangles represent the internal and terminal inverted repeats (IR and TR), which divide the genome into a unique long (UL) region and a unique short (US) region. The positions and numbering of the restriction fragments generated by *Bam*HI are shown. The lower part shows an enlargement of part of the US region and the positions in wild-type (wt) PRV of the gG, gD, gI, gE and 11K genes. Strain ID57.1, a derivative of strain D57 (Mulder *et al.*, 1996), expresses the CSFV E2 gene under control of the hCMV promoter in the gD locus. Strain M205 (van Zijl *et al.*, 1991), a derivative of vaccine strain 783 (Moormann *et al.*, 1990*a*), expresses the CSFV E2 gene under control of the gG promoter in the gG locus. (*b*) Autoradiograph of an SDS-polyacrylamide gel showing the E2 proteins (51–54 kDa) immunoprecipitated from lysates of cells infected with strains M205 and ID57.1. CSFV-infected cells and mock-infected SK-6 cells served as controls. The 31 kDa E1 protein (previously called E3) of CSFV forms a complex with E2 and is co-precipitated with E2 in CSFV-infected cells (Wensvoort *et al.*, 1990). The sizes of the molecular mass markers (M) are given in kilodaltons. Labelling of cells, immunoprecipitation and SDS–PAGE was essentially as described by van Zijl *et al.* (1991).

deletion mutant D57 (Mulder *et al.*, 1996). It has previously been shown that E2 is highly immunogenic and that it can be used for the protection of pigs against classical swine fever (van Zijl *et al.*, 1991; Hulst *et al.*, 1993). In this study we compared the vaccine properties of the newly constructed PRV strain (designated ID57.1) with those of PRV strain M205 (van Zijl *et al.*, 1991) (Fig. 1*a*). The latter recombinant, a derivative of the PRV vaccine strain 783 (Moormann *et al.*, 1990*a*) engineered to express the CSFV E2 protein, has previously been shown to be able to protect pigs against both Aujeszky's disease and classical swine fever (van Zijl *et al.*, 1991).

The E2-coding sequence of CSFV was inserted into the gD locus of PRV strain D57 under the transcriptional control of the human cytomegalovirus (hCMV) immediate early promoter. The E2 gene was derived from a cDNA clone of CSFV strain Brescia (Moormann *et al.*, 1990*b*). The gene was cloned as a *Dsa*I–*Eco*RV fragment (nucleotides 2337–3804; *Dsa*I site filled in with the Klenow fragment of *E. coli* DNA polymerase I) between the blunted (Klenow) *Eco*RI site and the *Eco*RV site of plasmid pEVhis13, which contains the hCMV promoter, an ATG start codon and translational stop codons in all three reading frames (Peeters *et al.*, 1992). In the resulting plasmid, the E2 gene is fused in-frame with the start codon of pEVhis13. The putative E2 gene product is predicted to contain seven N-terminal amino acids encoded by the vector. These amino acids are not incorporated in the mature protein since E2 contains a signal sequence. An *Hpa*I–*Pst*I fragment that contained the hCMV promoter, the E2 gene and the translational stop codons was subsequently cloned in the *Eco*RV site, between the PRV gG and gI genes, in transfer plasmid pBP53 (Mulder *et al.*, 1996). A plasmid in which the E2 gene was cloned in the same transcriptional orientation as the gG and gI genes was isolated and designated pBP53E2. Plasmid pBP53E2 was digested with *Pvu*II and *Pst*I and co-transfected with *Eco*RI-digested viral DNA of PRV strain D57 (which contains a single *Eco*R1 site at the gD deletion position) in gD-expressing VII/50 cells (Mulder *et al.*, 1996) by using lipofectin. Individual plaques were transferred to microtitre plates containing VII/50 cells and tested for the expression of E2 by immunological staining using E2-specific monoclonal antibodies (Wensvoort *et al.*, 1988, 1990). Several recombinant viruses that expressed E2 were identified, plaque-purified on VII/50 cells and their genomic structure examined by restriction enzyme digestion followed by Southern blot analysis (data not shown). One recombinant which had the expected genomic structure was designated ID57.1 (Fig. 1*a*). Expression of E2 by strains ID57.1 and M205 was analysed by radio-immunoprecipitation as described by van Zijl *et al.* (1991). The E2 proteins precipitated from lysates of ID57.1- or M205-infected cells were similar in size (51–54 kDa) to the native E2 protein precipitated from cells infected with CSFV (Fig. 1*b*). The amount of E2 expressed

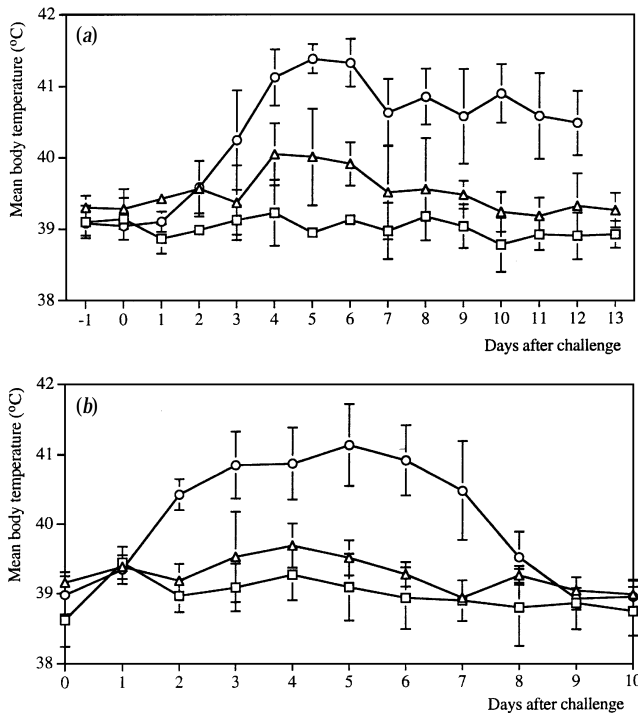

Fig. 2. (*a*) Mean body temperature (±SD) after challenge-inoculation with 100 LD_{50} CSFV of pigs vaccinated with strains ID57.1 (△) or M205 (□), and unvaccinated control pigs (○). (*b*) Mean body temperature (±SD) after challenge-inoculation with 10^5 p.f.u. of PRV strain NIA-3 of pigs vaccinated with strains ID57.1 (△) or M205 (□), and unvaccinated control pigs (○).

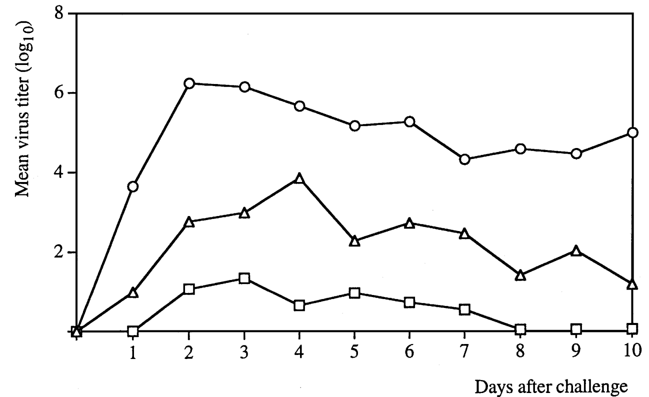

Fig. 3. Time-course of shedding of challenge virus in oropharyngeal fluid after challenge-inoculation of pigs vaccinated with strains ID57.1 (△) or M205 (□), and unvaccinated control pigs (○), with 10^5 p.f.u. of the virulent PRV strain NIA-3. Oropharyngeal fluid samples were collected and their virus content determined as described previously (Peeters *et al*., 1994).

by cells infected with ID57.1 was determined by using an E2-specific ELISA (Wensvoort *et al*., 1990) and was found to be approximately 80% relative to the amount expressed by cells infected with M205.

Two groups of five 10-week-old specific-pathogen-free pigs were inoculated intramuscularly at days 1 and 28 with 2×10^7 p.f.u. of either strain ID57.1 or strain M205. Two groups of five pigs each served as controls (see below). After inoculation, no signs of disease were observed. The animals in both inoculated groups developed antibodies against E2 when tested in an E2-specific complex-trapping blocking assay (Wensvoort *et al*., 1988) at day 42 after the first inoculation (> 90% specific blocking). All animals in the control group remained seronegative for antibody to E2 (< 10% specific blocking). The CSFV virus-neutralizing antibody titre was measured by using a neutralizing peroxidase-linked assay as described by Terpstra *et al*. (1984). The mean virus-neutralizing antibody titre (log_{10}) in serum from animals inoculated with strain ID57.1 was 2·7, whereas that in serum from animals inoculated with strain M205 was 2·9.

At day 44 after the first inoculation, the animals inoculated with strains ID57.1 or M205, and the animals of one of the control groups, were challenge-inoculated intranasally with 100 LD_{50} CSFV strain Brescia 456610 (Wensvoort *et al*., 1988). All pigs from the control group developed fever (Fig. 2*a*) and became seriously ill, as indicated by lack of appetite, vomiting, dullness and blue ears. The animals were almost constantly lying down and from day 7 after challenge onwards, one pig was unable to walk. The animals were euthanized at day 12 after challenge. In contrast, pigs which had been inoculated with either strain ID57.1 or strain M205 did not show any sign of disease, although the animals which had been vaccinated with strain ID57.1 showed a mild and transient increase in body temperature after challenge inoculation (Fig. 2*a*). Neutralizing antibodies against PRV were measured at days 28 and 74 after the first inoculation as described by De Leeuw & van Oirschot (1985). Mean virus-neutralizing titres ±SD (log_{10}) of pigs inoculated with strain ID57.1 were $1{\cdot}20 \pm 0{\cdot}19$ at day 28 and $2{\cdot}25 \pm 0{\cdot}25$ at day 74, whereas those of pigs inoculated with strain M205 were $2{\cdot}16 \pm 0{\cdot}15$ at day 28 and $3{\cdot}54 \pm 0{\cdot}07$ at day 72, respectively.

At day 76 after the first inoculation, the animals of the vaccinated groups and the animals of the second control group were challenge-inoculated intranasally with 10^5 p.f.u. of the virulent PRV strain NIA-3. As shown in Fig. 2(*b*), all animals in the non-vaccinated control group developed fever. The animals became seriously ill, showed loss of appetite and vomited. Some animals showed neurological signs such as ataxia. One animal died at day 7 after challenge. In contrast, none of the pigs which had been vaccinated with either strain ID57.1 or M205 showed any sign of disease after challenge inoculation. The animals vaccinated with strain ID57.1 only showed a slight and transient increase in body temperature (Fig. 2*b*). Whereas the animals in the control group lost weight until day 8 after challenge, animals vaccinated with strains ID57.1 or M205 did not (data not shown). Fig. 3 shows that shedding of challenge virus during the first 10 days after PRV challenge-inoculation is reduced by 100- to 1000-fold in animals which had been vaccinated with strain ID57.1 when compared with the non-vaccinated control group. In animals which had been

vaccinated with strain M205 the reduction is approximately 100000-fold.

Our results indicate that pigs can be protected from clinical signs of both Aujeszky's disease and classical swine fever by a non-transmissible gD/gE-negative PRV mutant that expresses E2 of CSFV. Our data indicate that protection against virulent PRV is somewhat better by strain M205 than by strain ID57.1, as judged from the virus-neutralizing antibody titres and the reduction of shedding of challenge virus. Despite a somewhat lower expression of E2 by strain ID57.1 as compared with strain M205, no significant differences are apparent in the induction of an antibody response against CSFV, as judged from the virus-neutralizing antibody titres against CSFV. Nevertheless, pigs inoculated with strain ID57.1 showed a transient increase in body temperature after CSFV challenge, whereas pigs inoculated with strain M205 did not. These results suggest that strain M205, which is able to spread from the inoculation site to other parts of the body, may be able to induce a better local immune response in the nasal mucosa (the site of challenge inoculation) than strain ID57.1, which is only able to replicate at the inoculation site. Furthermore, in addition to the gD phenotype, other differences between strains M205 and ID57.1 such as the gG, 11K and thymidine kinase phenotypes may also be responsible for differences in immunogenicity.

As argued above, widespread use of vaccine vectors is highly dependent on their biological safety. PRV gD mutants are able to replicate within the inoculated animal, thereby evoking a protective immune response, but progeny virions are non-infectious and thus cannot be transmitted from inoculated animals to contact animals. Furthermore, as indicated previously (Peeters *et al*., 1995), transfer of genes present in the gD locus to PRV field strains by means of homologous recombination results in replacement of the gD gene, yielding non-infectious recombinants. In several experiments, we (Peeters *et al*., 1994) and others (Heffner *et al*., 1993) were unable to detect infectious virus in animals inoculated with PRV gD mutants. The emergence of variants with gD-independent infectivity as observed in cell culture by Schmidt *et al*. (1997) is highly unlikely *in vivo* since the virus completes only a limited number of cell passages and eventually will be cleared from the inoculated animal by immunological mechanisms. Therefore, a gD/gE-negative PRV mutant fulfils the most important requirement for a vaccine vector, i.e. biological safety. The deletion of the gE gene not only reduces the virulence of gD-negative PRV (Mulder *et al*., 1996; Peeters *et al*., 1993) but also allows the serological differentiation between animals vaccinated with gD/gE-negative PRV and animals infected with PRV field strains (van Oirschot *et al*., 1988).

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