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Wam Mulder, Jma Pol, E Gruys, L Jacobs, Mcm de Jong, et al.. Pseudorabies virus infections in pigs. Role of viral proteins in virulence, pathogenesis and transmission. *Veterinary Research*, 1997, 28 (1), pp.1-17. hal-00902454

HAL Id: hal-00902454

<https://hal.science/hal-00902454>

Submitted on 11 May 2020

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Pseudorabies virus infections in pigs. Role of viral proteins in virulence, pathogenesis and transmission

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(Received 2 May 1996; accepted 23 July 1996)

Summary — This paper reviews new findings on the biological functions of pseudorabies virus (PRV) proteins. It focuses on the role of PRV proteins in the pathogenicity, immunogenicity and transmission of PRV vaccine strains in pigs. Furthermore, it evaluates potential risks that are connected with the use of PRV vector strains. Special emphasis is placed upon the spread of genetically engineered vaccine strains within pigs or between pigs.

pseudorabies virus protein / pseudorabies virus vector / virulence / pathogenesis / transmission

Résumé — **Rôle des protéines du virus de la maladie d'Aujeszky dans la virulence, la pathogénèse et la transmission chez le porc.** Cet article est une revue de nouveaux résultats concernant les fonctions biologiques des protéines du virus de la maladie d'Aujeszky. Le rôle de ces protéines est étudié dans le pouvoir pathogène, la réponse immunitaire et la transmission de souches vaccinales entre porcs. De plus les risques liés à l'utilisation de souches de virus de maladie d'Aujeszky comme vecteurs sont évalués, et particulièrement le risque de dissémination de souches de virus de maladie d'Aujeszky génétiquement modifiées chez le porc et entre porcs.

protéine du virus de la maladie d'Aujeszky / vecteur de maladie d'Aujeszky / virulence / pouvoir pathogène / transmission

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INTRODUCTION: PRV INFECTION AND VACCINATION

Pseudorabies virus (PRV) (synonyms: Aujeszky's disease virus and suid herpesvirus type 1) is a member of the subfamily *alpha-herpesvirinae*, which also includes the human herpes simplex virus (HSV) types 1 and 2, varicella-zoster virus, bovine herpesvirus type 1, and equine herpesvirus type 1 (Roizman and Baines, 1991; Roizman, 1992). PRV is a highly neurotropic virus that causes neurological disorders in pigs, which are the natural host, as well as a wide range of domestic and wild animals (Gustafson, 1986; Pensaert and Kluge, 1989). The infection is usually fatal to susceptible animals, and only pigs and horses may survive infection (Kimman et al, 1991). Clinical signs of Aujeszky's disease in pigs vary widely, from subclinical symptoms to death. The outcome of the disease depends upon the age of the pigs, the level of passive or active immunity, the virulence of the virus strain, and the dose of virus

causing the infection (reviewed in Gustafson, 1986; Pensaert and Kluge, 1989; Wittmann and Rziha, 1989). Natural infections mainly occur by the respiratory route. Young pigs may develop neurological signs such as vomiting, scratching, trembling, ataxia, paralysis and convulsions, and they may die of severe encephalomyelitis. When the central nervous system (CNS) is infected, PRV generally goes latent (Rziha et al, 1986). Older pigs usually survive the infection, but may develop fever and respiratory signs, for example, sneezing and pneumonia, and they may grow more slowly. In addition, infection of the respiratory tract usually causes the draining lymph nodes in the oropharynx region to become infected. A low number of cell-associated and cell-free virus can be found in viremic pigs (Wittmann et al, 1980; Nauwynck and Pensaert, 1995). PRV-infected mononuclear blood cells may damage internal organs, and may cause reproductive disorders in pregnant sows (Pensaert et al, 1991; Nauwynck and Pensaert, 1992).

Pigs are the sole reservoir of PRV and the only source of virus transmission. PRV is prevalent in most parts of the world and causes severe economic losses in the swine industry. Because the presence of the disease can lead to trade barriers being set up between countries (Van Oirschot, 1994), campaigns have been mounted to eradicate PRV from swine populations with or without the use of vaccines (reviewed in Stegeman, 1995). Several types of PRV vaccines are available, namely conventionally or genetically engineered live attenuated vaccines, killed whole virus vaccines, and subunit vaccines (Wittmann, 1991; Kimman, 1992a; Pensaert et al, 1992; Kimman et al, 1995). However, in general, live vaccines are more effective than dead vaccines and the efficacy of live vaccines can be further enhanced by adjuvants (Pensaert et al, 1992). The protective efficacy of PRV vaccines also depends on the virus strain, the quantity of virus, and the vaccination scheme (Kimman, 1992b).

Conventionally attenuated live vaccine strains, however, contain several characterized and uncharacterized mutations that may reduce the immunogenicity. Furthermore, the possibility cannot be excluded that these strains may revert to virulence. Consequently, several groups have developed genetically engineered vaccine strains with defined deletions in the genes encoding glycoproteins E (gE), C (gC), or G (gG) (Kit et al, 1987; Marchioli et al, 1987; Moormann et al, 1990). To enhance their safety, thymidine

kinase (TK), which supports viral DNA replication, has been deleted from these virus strains.

In most eradication campaigns, gE-negative vaccine strains are used in conjunction with a serological test that specifically detects antibodies against gE. The use of these so-called marker vaccines makes it possible to identify pigs infected with wild-type virus within vaccinated populations (Van Oirschot et al, 1988, 1990; Kit, 1990).

However, there is still the concern that live PRV vaccines may still be virulent, that they may be able to go latent, and that they may be transmitted to unvaccinated pigs. Subsequent recombination with related vaccine strains or wild-type PRV strains could lead to recombinant PRV strains that go wild. Furthermore, there remains a need for more potent vaccines that induce complete protection (Pensaert et al, 1992). This review summarizes recent findings on the role of PRV proteins in virulence, pathogenesis, immunogenicity and transmission.

VIRAL GENOME AND ENCODED PROTEINS

The genome of PRV is a linear double-stranded DNA molecule of approximately 140 kb (fig 1). It is divided in a unique long sequence of 95 kb and a unique short region of 9 kb, which is flanked by inverted repeat sequences of 15 kb.

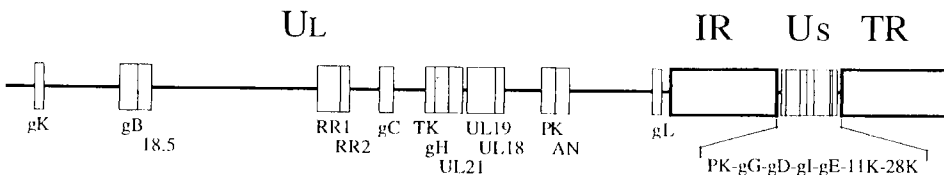


Fig 1. Map of the PRV genome showing the location of the PRV genes that are discussed in this review. The genome consists of a unique long (UL) and a unique short region (US). The regions are separated by inverted repeats, the so-called internal repeat (IR) and the terminal repeat (TR). Indicated are the genes encoding the glycoproteins B (gB), C (gC), D (gD), E (gE), G (gG), H (gH), I (gI), K (gK) and L (gL); the ribonucleotide reductase subunits RR1 and RR2, thymidine kinase (TK), alkaline nuclease (AN), and the US3- and UL13-encoded protein kinases (PK); the UL18, UL19, UL21 gene products, the infected cell protein (ICp) 18:5, and the 11K and 28K proteins. For a more complete map of the PRV genome, the reader is referred to Mettenleiter (1994a) and Gielkins and Peeters (1994).

The viral genome encodes at least 70 proteins, and the nucleotide sequence of more than 40 genes has been obtained (TC Mettenleiter, personal communication). The gene arrangement in PRV is highly collinear with that of herpes simplex virus type 1 (HSV-1), the prototype alphaherpesvirus. And because the amino acid sequences of many PRV and HSV-1 proteins are homologous, these proteins may have identical or similar biological functions in both viruses. Much progress has been made in the structural and functional analysis of the viral glycoproteins and other gene products involved in virulence (Mettenleiter, 1991, 1994a; De Wind, 1992; Kimman et al, 1992d; De Wind et al, 1994; Gielkens and Peeters, 1994).

FUNCTIONS OF PRV PROTEINS IN VIRULENCE AND PATHOGENESIS

The PRV proteins that determine virulence of the virus can be roughly categorized into three groups: 1) envelope glycoproteins that mediate virus entry and virus spread in the host; 2) virus-encoded enzymes involved in DNA metabolism or phosphorylation; and 3) proteins involved in virion assembly.

Envelope glycoproteins

The entry of PRV into host cells is mediated by glycoprotein spikes that project from the surface of the virus particles. These are involved in several important steps during the infection, such as attachment to the host cell, fusion with the cellular membrane, and entry of the nucleocapsid (reviewed in Spear, 1993a). They also mediate viral spread from infected to uninfected cells. Viral spread in the host can occur in two ways, either by virus particles being released from infected cells and the subsequent infection of uninfected cells, or by direct cell-to-cell transmission. Although the exact mechanism for the latter is unknown, it has been suggested that PRV-induced cell-cell fusion requires the expression of viral glycoproteins in the mem-

branes of infected cells (Rauh and Mettenleiter, 1991; Peeters et al, 1992a,b; Spear, 1993b).

Because PRV glycoproteins show similarities with their respective glycoproteins in other herpesviruses, it was agreed at the *18th International Herpesvirus Workshop* (Pittsburgh, Pennsylvania, July 1993) that the nomenclature for the envelope glycoproteins of all herpesviruses should follow the alphabetic designation of HSV-1 glycoproteins. Ten PRV glycoproteins have been identified thus far: gB, gC, gD, gE, gG, gH, gI, gK, gL and gN (Mettenleiter, 1994a; Jöns et al, 1995). Four of these (gC, gE, gI and gG) are not necessary for virus replication in tissue culture, and are therefore considered nonessential. However, none of the field virus strains isolated from pigs lack gI or gC (Kit, 1990), gE (Van Oirschot, 1989) or gG (Marchioli et al, 1987), which suggests that they are probably essential for the virus to survive in the pig populations. In contrast, glycoproteins gB, gD, gH and probably gL are essential for the infectivity of the virus, since virus particles lacking one of these glycoproteins cannot penetrate host cells (Rauh and Mettenleiter, 1991; Peeters et al, 1992a,b).

Glycoprotein C (gC) mediates the attachment of PRV, HSV and BHV-1 to host cells (Spear, 1993a). This glycoprotein contains a heparin-binding domain that is involved in the primary attachment of PRV to heparan sulphate proteoglycans on the cell surface (Sawitzky et al, 1990; Mettenleiter, 1990). This low affinity binding is heparin-sensitive. Stable attachment of both PRV and HSV to host cells is subsequently mediated by the binding of gD to an unidentified cellular receptor and results in a heparin-resistant binding (Johnson and Ligas, 1988; Karger and Mettenleiter, 1993). Cells expressing gD encoded by HSV-1, HSV-2, PRV and BHV-1 can be resistant to viral infection (Spear, 1993a). For HSV, it has been shown that the virus can bind, but cannot penetrate cells expressing gD (Campadelli-Fiume et al, 1988; Johnson and Spear, 1989). Surprisingly, however, gC is not essential for virus infectivity, indicating either that other viral glycoproteins also recognize hep-

aran sulphate or that there exists a pathway for virus attachment that does not require gC. After attachment, the virion envelope and the cytoplasmic membrane fuse, although a principal fusion protein has not yet been identified. The current opinion is that several glycoproteins act together to cause this fusion (Mettenleiter, 1994b). Glycoproteins B and H (and probably gL), which mediate membrane fusion, are essential for free virions to be infective (Rauh and Mettenleiter, 1991; Peeters et al, 1992a,b; Klupp et al, 1994). Recently, Klupp et al (1994) showed that gH and gL form a noncovalently linked complex that functions as an entity. The glycoproteins B and H are present in the membrane of infected cells and are also required for direct cell-to-cell transmission of the virus in tissue culture. It has been suggested that they induce a transient fusion of infected and uninfected cells, allowing virus particles to spread (Peeters et al, 1993).

Interestingly, and in contrast to gD of HSV and BHV-1, gD of PRV is not necessary for cell-to-cell transmission. After gD is phenotypically complemented by propagating gD mutants on cell lines that express gD, gD-negative PRV is able to infect primary target cells and spread directly from cell-to-cell both *in vitro* and *in vivo* in mice (Babic et al, 1993; Heffner et al, 1993; Peeters et al, 1993). Moreover, we recently found that phenotypically complemented gD-negative PRV can also spread transneuronally, *ie.* axonal spread across synapses into and within the CNS of pigs (Mulder et al, 1996). Immunohistochemical examination of infected pigs showed that PRV gD mutants had infected second- and third-order neurons in the olfactory bulb, brain stem, and medulla oblongata. Thus, not only in rodents, but also in pigs, the natural host of PRV, the transneuronal spread of the virus can occur independent of gD. Although it is not known how herpesviruses spread through neurons and across synapses, Card et al (1993) suggested that transneuronal transfer of PRV requires the fusion of viral envelopes with the synaptic membranes, which leads to the release of virions in the synaptic cleft and subsequent

reinfection of postsynaptic neurons. Our finding that PRV can spread across neurons without the presence of gD suggests that the mechanism of neuronal transfer may resemble the cell-to-cell spread of PRV in tissue culture. We speculate that the nature of the cell surface may determine whether gD is required for penetration or not. It has been reported that whereas apical attachment of HSV-1 to polarized Madin-Darby canine kidney cells depends on gC, but basal attachment does not (Sears et al, 1991). Similarly, it may be that gD is only required for PRV to enter the cell at its apex, but not required for lateral or basal entry. Different proteoglycans present on the cell surface may be responsible for different interactions with the virus (Kjellen and Lindahl, 1991). As mentioned above, heparan sulphate proteoglycan has been shown to play a major role in the gC-mediated attachment of HSV-1 and PRV to cells (Spear, 1993a).

In contrast to gD, gE is an essential protein in transneuronal spread of PRV. Studies have shown that gE-negative PRV replicates in peripheral tissues, infects first-order neurons, and spreads towards the CNS via both the olfactory and trigeminal routes. However, gE-negative PRV is much less able to infect second- and third-order neurons in the porcine CNS (Jacobs et al, 1993a; Mulder et al, 1994b; Kritas et al, 1994a,b; 1995). Enquist et al (1994) used genetic complementation to demonstrate that gE or gI enable the transneuronal spread of PRV into the visual centres of the rat after the virus enters the first-order neurons in the retina. In addition, gE and gI appear to function during the anterograde transport of PRV, as demonstrated by studying the dissemination of PRV strains in the maxillary nerve and the trigeminal ganglion of intranasally inoculated pigs (Kritas et al, 1995). *In vitro* the gE-gI complex promotes cell-to-cell spread and is involved in release of the virus from cells and in cell fusion (Zsak et al, 1992; Jacobs et al, 1993b; Jacobs, 1994). These findings suggest that the gE-gI complex may promote the neuron-to-neuron transmission of PRV by a mechanism that is similar to cell-to-cell spread in tissue culture. Thus, the gE-gI complex may promote the

release of virus from first-order neurons, or may promote the fusion of synaptically linked neurons. The possibility cannot be excluded, however, that the gE–gI complex has other functions in the transneuronal transfer of PRV, such as promoting the intraneuronal transport of the virus.

Like gE-negative PRV, gI-negative PRV has a reduced transneuronal spread in pigs, but to a lesser extent than gE-negative PRV (Kritas et al, 1994a,b, 1995). This neuronal spread might be restricted because the interaction with gE is disturbed. Results from pig and rodents studies are similar. Both gE and gI proteins are required for the infection of second- and third-order neurons in some circuits of the rat eye and heart, but not in other circuits (Card et al, 1992; Ter Horst et al, 1993; Whealy et al, 1993; Standish et al, 1994).

Virus-encoded enzymes

The UL23-encoded thymidine kinase (TK) and the UL39- and the UL40-encoded ribonucleotide reductase subunits (RR1, RR2) support viral DNA replication. TK and RR function in the salvage and de novo pathways of deoxyribonucleotide synthesis, respectively (Reichard, 1988). Although viral RR and TK are not essential for the virus to grow in dividing cells (De Wind et al, 1993), they are required to produce infectious virus particles in non-dividing cells, such as neurons and resting peripheral blood mononuclear cells (Mulder et al, 1995a). A low number of cell-associated and cell-free PRV can be found in viraemic pigs and may damage internal organs and cause abortion, even in vaccinated animals (Wittmann et al, 1980; Nauwynck and Pensaert, 1992, 1995). It has been reported that lymphoid cells, in addition to tonsils and various neural cells, may harbour latent virus (Wittmann and Rziha, 1989).

We found that replication of PRV in porcine peripheral blood monocytes and lymphocytes depends on the cell type, the viral genotype, and on the state of cellular activation (Mulder et al,

1995a). Moreover, although viral TK and RR activity were required in order for PRV to replicate in vitro in resting peripheral blood lymphocytes and monocytes, the glycoproteins E and G and the US3-encoded protein kinase (PK) were not. And while Con A stimulation of lymphocytes restored the viral TK defect, it did not restore the viral RR defect (Mulder et al, 1995a). In agreement with this, virus mutants lacking RR or TK caused no severe clinical signs of disease in pigs, and the replication of an RR mutant was more severely retarded (100- to 1 000-fold) in the nasal and oropharyngeal tissues than the replication of a TK mutant (De Wind et al, 1993; Kimman et al, 1994). Thus, results of both in vitro and in vivo experiments indicate that viral RR activity is more important for efficient viral DNA synthesis than viral TK activity.

Another virus-encoded enzyme, alkaline nuclease, which is encoded by the UL12 gene, has not been found essential for growth of PRV in tissue culture, although it is important for virulence of PRV in mice (De Wind et al, 1992a, 1994). It has been reported that this alkaline nuclease of HSV plays a role in processing or packaging viral DNA into viral particles (Weller et al, 1990; Shao et al, 1993). Another enzyme encoded by PRV that functions in the repair pathway of nucleic acid metabolism is uracil DNA glycosylase (UL2; Dean and Cheung, 1993; Klupp et al, 1993). How and to what extent this enzyme effects the virulence of PRV, however, is unknown. Finally, the dUTPase (UL50) of HSV-1 is reportedly involved in neurovirulence in mice (Pyles et al, 1992). The corresponding PRV homolog, however, has not been identified yet.

Another group of viral enzymes are the protein kinases (PK), which comprise a large family of enzymes. These PK regulate the protein phosphorylation that plays a major role in signal transduction, regulation of growth and differentiation of cells (Hanks et al, 1988). Both the US3 and UL13 gene encode a serine/threonine kinase that is nonessential for growth of PRV in cell culture (Van Zijl et al, 1990; De Wind et al, 1992a). UL13 mutants grow as efficiently as

wild-type virus in cell culture, and their virulence for mice is not diminished (De Wind et al, 1992b, 1994). In contrast, the growth of US3-encoded PK mutants is retarded compared to wild-type virus and depends on cell type (Kimman et al, 1994; Mulder et al, 1995a). Moreover, US3 mutants are less virulent than UL13 mutants in both mice and pigs (Kimman et al, 1992a; 1994; De Wind et al, 1994). Wagenaar et al (1995) found that a defect in the morphogenesis of US3 mutants is responsible for their reduced replication *in vitro* and their reduced virulence *in vivo*. They found that PK-negative virus particles were not debudded at the outer nuclear membrane, both in epithelial cells of porcine nasal mucosa explants and in cells of the porcine kidney cell line SK6 (Kasza et al, 1971). As a result, US3-mutant virions accumulated in the perinuclear membrane. Little is known about the substrates of the PRV kinases. There is one report showing *in vitro* phosphorylation by US3 PK of a major virion phosphoprotein of 122 kDa (Zhang et al, 1990).

Proteins involved in virion assembly

The capsid of herpesviruses is an icosahedral protein structure that encloses the core containing the viral DNA. Seven capsid proteins have been identified for HSV-1 (encoded by UL18, UL19, UL26, UL26.5, UL35 and UL38) (Rixon, 1993; Haarr and Skulstad, 1994). The sequences encoding two major PRV capsid proteins of 142 and 32 kDa have recently been elucidated (Yamada et al, 1991; Klupp et al, 1992), and are the homologues of genes UL19 and UL18 of HSV-1. In addition, the PRV gene that is the homolog of the HSV-1 UL21 gene has been identified (De Wind et al, 1992b). The PRV UL21 gene encodes a 62 kDa protein, and is probably closely associated with capsids during capsid assembly and plays a role in processing or packaging viral DNA (De Wind et al, 1992b). The function of the 79 kDa PRV protein that is homologous to infected cell protein (ICP)18.5 (UL28) of HSV-1 was studied by Mettenleiter et al (1993). This protein appears to be essential

for virus replication and could play an essential role in the formation of mature virions.

PRV PROTEINS IN IMMUNITY

When infected with a virulent PRV strain, pigs develop an immune response that can completely or almost completely prevent the virus from replicating after the pig becomes reinfected. However, vaccination seldom produces this level of immunity. Most vaccines in fact offer only partial protection against viral replication (Pensaert et al, 1992). In fact, serum antibodies, whether actively or passively acquired, as well as mucosal IgA antibodies appear to induce only partial protection (Kimman et al, 1992e). Moreover, pigs that are fully immune after a first infection do not develop a secondary B cell response upon a second infection, although they do develop a strong secondary T cell response. It is possible that this T cell response may induce the emergence of cytolytic cells that quickly eliminate the challenge virus, and thus preventing a secondary B cell response. However, the lymphoproliferative response probably also triggers antiviral activity, such as the release of interferons and tumour necrosis factor (Pol, 1990; Schijns et al, 1991).

It is known that the envelope glycoproteins B, C and D are major targets for immune responses. Cells that express these glycoproteins can be recognized and killed by various immune mechanisms, such as antibody and complement mediated cell lysis, antibody-dependent cell-mediated cytotoxicity, and cytotoxic T-lymphocytes (reviewed in Chinsakchai and Molitor, 1994).

One immune mechanism, the induction of neutralizing antibodies, peaks within 2–3 weeks after infection with PRV (Wittmann et al, 1976; Martin et al, 1986). Antibodies in pigs that are infected by PRV field strains, are directed against gB, gC, gD, gE, gH, gG and gI (Kit, 1990), immediate early (IE) and nucleocapsid proteins (Cheung, 1990; McGinley et al, 1992). Of these glycoproteins, gB, gC and gD appear to be the major targets for neutralizing antibodies, whereas

gG and gE are not (Ben Porat et al, 1986; Zuckermann et al, 1988). Furthermore, gC is a target antigen for cytotoxic T cells in both mice and pigs (Zuckermann et al, 1990). However, it also binds to the complement C3 component from pigs and cows, but not from humans (Heumer et al, 1993), as a result of which the complement is less able to neutralize the virus. This phenomenon is interesting, because the interaction between gC and C3 is specific to certain animal species and not to others, and it may therefore attribute to the host range of alpha-herpesviruses.

The role of PRV glycoproteins B, C and D in inducing protection has been derived from vaccination experiments with purified glycoproteins (Iglesias et al, 1990; Mukamoto et al, 1991), with glycoproteins expressed by adenovirus vector (Eloit et al, 1990), and with vaccinia virus vectors (Riviere et al, 1992; Brockmeier et al, 1993; Mengeling et al, 1994). In addition, their protecting properties were studied using the baculovirus expression system (Xuan-XueNan et al, 1995), and an immune stimulating complex (ISCOM) (Tsudi et al, 1991).

Little is known about how PRV proteins interact with effector cells during the cell-mediated immune response. It has been shown that gB and gC can induce the proliferation of T cells (Kimman et al, 1996a). In HSV, not only the structural proteins, but also the immediate early proteins are target antigens for cytotoxic T lymphocytes and natural killer cells (Martin et al, 1988; Banks et al, 1991; Fitzgerald-Bocarsly et al, 1991). When peripheral blood lymphocytes of PRV-immune miniature pigs of SLA^{d/d} haplotype were antigenically stimulated in vitro, cytotoxic cells that were able to lyse PRV-infected L14 cells were generated. L14 cells are immortalized B-cells of SLA^{d/d} haplotype. The PRV-infected L14 cells were killed in the presence of CD2⁺CD4⁻CD8^{bright}-cells, did not appear to be MHC-restricted, was strongly augmented by in vitro antigenic stimulation. Moreover, killing did not appear to be virus-specific, that is, the target cell line K562 for natural killer cells was also lysed by effector cells of immune pigs.

These results indicate that lymphokine activated killer (Lak) cells kill the PRV-infected L14 cells. The Lak cells appear to recognize different viral proteins. L14 cells that were transfected with and stably expressed gB or gC were killed to the same extent as PRV-infected target cells. In contrast, L14 cells that were transfected with gD or the immediate early protein were not lysed above background values (Kimman et al, 1996b).

Surprisingly, PRV proteins that apparently do not have particular epitopes that trigger the immune system, such as those involved in virus replication or phosphorylation, may still contribute to the immunogenicity of live PRV strains. Kimman et al (1994) and De Wind et al (1993) found that inactivating TK, RR or the US-3-encoded PK affected the immunogenicity of PRV, possibly because these virus mutants replicated less efficiently in vivo. In addition, the effect of inactivating gE and PK or gE and TK appeared to be synergistic (Kimman et al, 1994). They hypothesized that these viral enzymes help induce protective immunity by increasing the viral antigenic mass presented to the immune system. In general terms, PRV strains that replicate strongly, not only appear to be more virulent, but also more immunogenic and protective.

PRV PROTEINS IN TRANSMISSION

There has been some concern voiced about the possible transmission of live PRV strains to unvaccinated animals, and the possibility that these might then recombine with related vaccine or wild-type strains, leading to the spread or survival of recombinant PRV. Recently, in fact, Christensen et al (1992) suggested that certain field isolates of PRV may have been derived from attenuated vaccine strains. When attenuated vaccine strains are administered intramuscularly, virus is generally not excreted through the mucosa or transmitted to susceptible animals (Kimman et al, 1995). However, the possibility cannot be excluded that vaccine strains might, by error or deliberately, be administered intranasally.

In order to develop safe PRV live vaccines that are not transmitted to susceptible pigs, certain PRV genes should be deleted. To determine the genes of PRV to which the transmission of the virus should be attributed, we used a small-scale experimental model (De Jong and Kimman, 1994) to analyze PRV mutant strains and PRV vector strains under experimental conditions (Mulder et al, 1995b). The basic reproduction ratio or R , was used to predict the spread of virus from infected animals to contact-exposed animals. In the model, R has a threshold property: when $R > 1$, the infection can spread; when $R < 1$, the infection will disappear (Anderson and May, 1982, 1985; De Jong and Diekmann, 1992). The transmission of PRV strains that lacked gE or TK and the transmission of a PRV (gE⁻, TK⁻, gG⁻) vector strain that expresses E1 of HCV were studied. The value of R for a gE-negative strain was found to be 10.1, and for a TK-negative strain it was 5. The R value for the vector strain expressing E1 was 0.18, which did not differ significantly from the R value of control strain, which did not express E1. Only the R of the gE-negative strain was significantly greater than 1 ($P = 0.0005$).

The R values in populations larger than those used in the experiments will be the same as estimated from these experiments. The supposition that R is independent of the population size follows logically from the mass-action argument (De Jong et al, 1995) and was also shown to be true for PRV in pigs (Bouma et al, 1995). Thus our studies indicated that PRV strains that lack only gE or TK may be transmitted from intranasally inoculated pigs to contact pigs, but a strain that lacks gE, TK and gG appears unable to spread to susceptible contact pigs. However, vaccination reduces transmission of PRV (De Jong and Kimman, 1994). Therefore, PRV strains are less likely to be transmitted in vaccinated populations. These studies show that in order to develop nonspreading PRV strains, not only TK or gE, but both or others should be deleted. Moreover, the results show that a small-scale experimental model can be used to estimate and predict the transmission of PRV mutant strains.

In theory, live PRV vaccines could become transmissible through recombining with related vaccine strains or wild-type PRV. Coinfection of a host with two different herpesvirus strains may result in the generation of recombinant strains (Henderson et al, 1991; Dangler et al, 1993; Glazenburg et al, 1995). The question thus arose as to whether a gE-negative, TK-positive PRV strain could be transmitted in a population which is also infected with wild-type PRV. When pigs were coinoculated with a gE-negative PRV strain and a gE-positive (wild-type) PRV strain to determine whether they competed in transmission (Mulder et al, 1995b), both strains were transmitted to contact pigs and no severe competition was detected. However, less gE-negative virus was excreted in oropharyngeal fluid in coinoculated pigs than in pigs inoculated with only gE-negative virus. Furthermore, co-inoculated pigs excreted gE-positive virus longer than gE-negative virus. This finding could mean that gE-negative viruses will have less chance to survive in pigs as gE-positive viruses.

Although gE⁻/TK⁻ PRV strains do not appear to spread, the risks of transmission of PRV vaccine strains can be further reduced by deleting essential genes or inserting foreign genes in essential PRV genes (for example gD), so that if recombination were to occur, the resulting strains would be nonviable.

PRV AS A VECTOR VACCINE

PRV strains can be used as vectors to express one or more foreign genes of other microorganisms. For example, Van Zijl et al (1991) showed that immunizing pigs with PRV recombinants that expressed the envelope glycoprotein E1 (E1) of hog cholera virus (HCV) protected them against both pseudorabies and hog cholera. So far, different proteins have been expressed by recombinant PRV to: 1) induce immunity against foreign pathogens; 2) monitor virus infection more easily; or 3) demonstrate functional homology between viral proteins (Thomsen et al, 1987; Whealy et al, 1988, 1989; Mettenleiter et al,

1990; Mettenleiter, 1991; Kopp and Mettenleiter, 1992; Sedegah et al, 1992). Like vaccinia virus, which is the best known and most widely studied vector (Bostock, 1990; Moss, 1991), PRV has a large genome (± 140 kb) in which large fragments of foreign DNA can be stably integrated. Non-essential genes can be deleted to generate additional space. Foreign genes have been stably inserted in the TK, gG, gE and gD loci of PRV (Mettenleiter et al, 1990; Van Zijl et al, 1991; Peeters et al, unpublished results).

Inserting foreign genes into live vector vaccines demands extra safety precautions, however, because the expression of foreign genes may alter the biological properties of the vector virus. Three potential risks should always be examined (Kimman, 1992c). First, the tropism of the vector virus for particular cells, tissues or hosts could change as a result of which the virulence of the vector virus for different hosts could change. Second, through homologous or illegitimate recombination, the foreign gene(s) could be transferred from the vector virus to other vaccine or wild-type strains. Third, the transmission properties of the vector virus could change, as a result of which recombinant virus could spread. Little is known about the properties that determine cell or host tropism. Some results have also been published on changes in cell or host tropism in poxvirus vector strains (Taylor et al, 1991), and on genes that determine host tropism for poxviruses (Perkus et al, 1989). Unfortunately, however, it is unknown which genes determine these properties of a given microorganism.

Perhaps the greatest concern about the use of PRV vector vaccines is that a recombinant pathogen goes wild. This could occur if recombination causes the foreign gene to be transferred from the vector vaccine to a virulent field strain. However, the virulence of such a recombinant would not only depend on the foreign gene inserted, but also on the site where it was inserted. If a foreign gene was inserted in a viral gene that contributed to virulence (for example, TK and gE of PRV), recombination with the

field virus would probably result in a less virulent virus. However, if it was inserted in a viral gene that does not contribute to virulence (for example gG of PRV), recombination with the fieldvirus would probably result in a strain that is as virulent or more virulent than the wild-type strain. Recently we constructed just such a worst-case recombinant strain, with the envelope glycoprotein E1 of HCV inserted into the gG locus of PRV, and tested it in pigs. The (gG⁻.E1⁺) vector strain was indeed virulent for pigs, but the incorporation of E1 did not essentially change the pathogenesis of the PRV vector (Mulder et al, 1994a).

Several experimental studies have demonstrated that coinoculated modified live PRV (vaccine) strains could recombine *in vivo* to create virulent recombinant strains (Henderson et al, 1990, 1991; Katz et al, 1990; Dangler et al, 1993). However, part of the increased virulence may have been caused by complementation (Glazenburg et al, 1994; Visser and Rziha, 1994). A prerequisite for a recombinational event between PRV strains is that these viruses have to enter the same cell. However, vaccine viruses are generally administered intramuscularly and replication is restricted to the site of inoculation (Kimman et al, 1995), whereas after natural infection the virus replicates primarily in the nasopharyngeal mucosa, tonsils, regional lymph nodes and lungs. Recombination has been observed only in a worst-case scenario: in mice and pigs *in vivo* recombination of PRV strains only occurred when high doses of two virus strains were coinoculated at the same location. (Glazenburg et al, 1995). Two separately inoculated mutant virus strains still recombined in mice even after a lapse of 2 h (Glazenburg et al, 1994). The frequency of recombination appeared to depend on the capacity of the virus strains to replicate and on the sequence or the genomic location of the genetic markers. Although the possibility of recombination between vaccine and PRV field strains cannot completely be excluded, the risks can be further greatly lessened if (several) virulent genes are deleted from live vaccines, if the coinoculation of PRV vac-

cines with complementing gene deletions is avoided, and if the pig population is thoroughly vaccinated.

PROSPECTS AND NEW DEVELOPMENTS

Many researchers are trying to develop non-transmissible PRV strains or strains that cannot be transmitted after recombination with wild-type strains. Essential PRV genes, such as the essential glycoproteins involved in viral entry and spread, are inactivated by deleting these genes or by inserting a foreign gene. Vaccination with phenotypically complemented gD mutants seems promising because the gD protein has a unique biological property. The gD-negative mutants, however, can still spread in the host via direct cell-to-cell transmission, and therefore probably induce a solid immune response. Moreover, progeny virions that are shed by vaccinated animals are noninfectious because they lack gD, and the vector vaccine strains cannot be transmitted to other susceptible animals. (Heffner et al, 1993; Mettenleiter et al, 1994; Peeters et al, 1994). The gD mutants, however, could still be problematic, because they are able to infect and replicate within the CNS of pigs (Mulder et al, 1996). Nonetheless, by deleting extra viral proteins, such as the gE/gI complex, the transneuronal spread of gD-negative PRV can be prevented. The immunogenicity of gD mutants with additional mutations in gE and/or gI must still be assessed.

Fortunately, area-wide eradication of PRV is feasible (Stegeman et al, 1994; Stegeman, 1995). If it is achieved, the need to vaccinate against PRV will come to an end. However, in border areas where different countries adjoin, vaccination against PRV will probably continue to be necessary for some time in order to control new small outbreaks. These may be caused by importing pigs from other regions where PRV is still prevalent or by the spread of virus through the air. Besides vaccination against PRV, PRV vector strains expressing foreign gene(s) of other

pathogens could be used for vaccination purposes in pigs or other animal species.

CONCLUSION

The deletion of two virulence-determining genes, such as TK and gE, is probably necessary to prevent PRV from spreading. A drawback is that deleting several genes renders PRV strains less replicating, thus less immunogenic. In addition, thorough vaccination of the pig population reduces the transmission of PRV vaccine strains and may prevent them from spreading or to recombining with other PRV strains.

Recombining live PRV (vector) vaccine strains will probably cause no real problems, because it was only demonstrated under worst-case scenarios. Moreover, inserting foreign genes in essential genes of PRV (for example gD, gH, gB) will render recombinant strains non-viable.

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