IMMUNE AND PROTECTIVE ABILITIES OF UBIQUITINATED AND NON-UBIQUITINATED PSEUDORABIES VIRUS GLYCOPROTEINS

R. GRAVIER[1], D. DORY[1*], F. RODRIGUEZ[2], S. BOUGEARD[3], V. BEVEN[1], R. CARIOLET[1], A. JESTIN[1]

[1]Viral Genetics and Biosafety Unit, French Food Safety Agency, BP-53, Fr-22440, Ploufragan, France; [2]Centre de Recerca en Sanitat Animal, Barcelona; Spain; [3]Epidemiology and Quality Assurance in Pig Production Research Unit, French Food Safety Agency, Ploufragan, France

Received September 19, 2006; accepted February 23, 2007

Summary. – Plasmids encoding ubiquitinated (ubi-) or non-ubiquitinated (non-ubi-) glycoproteins of Pseudorabies virus (PRV) were used for vaccination of pigs. We found that the fusion of ubiquitin to viral glycoproteins increased their degradation in proteasomes *in vitro*, in which ubiquitin plays a key role. In the animals immunized with the plasmids encoding PRV ubi-glycoproteins and then challenged with PRV, we detected a slightly decreased cellular immune response on days 13 and 19 after immunization and a reduced nasal excretion of infectious virus on day 2 after the challenge. Afterwards, no effect of the ubiquitination of the glycoproteins on humoral or cellular immunity and on excretion of infectious virus was observed. Similarly, no effect of the ubiquitination on protective abilities of PRV glycoproteins was found.

Key words: DNA vaccine; glycoproteins; protection; Pseudorabies virus; ubiquitination

Introduction

PRV (the species *Suid herpesvirus 1*, the genus *Varicellovirus*) is a neurotropic virus containing a linear double-stranded DNA that codes for 11 glycoproteins (Mettenleiter, 2000). This infectious agent causes Aujeszky's disease in pigs manifested by nervous disorders, respiratory distress, and marked weight loss, mortality in young piglets and abortion in pregnant sows. Vaccination of pigs is still mandatory in many countries due the economic consequences of this disease. Recently, a DNA vaccine, an alternative to the conventional vaccine, was successfully used to protect pigs against PRV infections. A combination of plasmids encoding PRV glycoproteins B (gB), C (gC) and D (gD) (Dory *et al*., 2005a; Fischer *et al*., 2003; Hong *et al*., 2002) that are involved in essential steps of viral infection was employed (Mettenleiter, 2000). Several B cell epitopes detected on PRV gB and gC and T cell epitopes detected at least on PRV gC induce humoral and cytotoxic responses in pigs (van Rooij *et al*., 1998, 2000; Ober *et al*., 1998, 2000; Zaripov *et al*., 1998, 1999). However, PRV-gD induces the highest level of neutralizing antibodies and a weak cytotoxic T cell response (van Rooij *et al*., 2000). Various procedures were used to enhance the efficiency of DNA vaccination, as co-injection of a plasmid encoding porcine granulocyte-macrophage colony-stimulating factor, unmethylated cytosine-guanosine containing oligodeoxynucleotides or dimethyldioctadecyl ammonium bromide. These vaccines produced increased humoral and cellular response as well as clinical protection (Dufour *et al*., 2000; Dory *et al*., 2005a; van Rooij *et al*., 2002). A new generation of non-replicating plasmids derived from Sindbis virus was found to be highly efficient in inducing protective immunity against PRV in pigs (Dory *et*

*Corresponding author. E-mail: d.dory@ploufragan.afssa.fr; fax: +33296-016283.

Abbreviations: CTL = cytotoxic T lymphocyte; gB, gC, and gD = glycoprotein B, C, and D, respectively; HRP = horseradish peroxidase; i.m. = intramuscular; i.n. = intransally; IFN-γ = interferon γ; LCMV = Lymphocytic choriomeningitis virus; MHC = major histocompatibility complex; MOI = multiplicity of infection; MRD = mean relative daily weight gain; PBMC = peripheral blood mononuclear cells; PRV = Pseudorabies virus; ubi- = ubiquitinated; non-ubi- = non-ubiquitinated; VN = virus neutralization

al., 2005b). In spite of much smaller amount of injected plasmids (25 times less than conventional DNA plasmid), the level of protection was similar. Nevertheless, even with this progress, the viral excretion in immunized animals remained high, indicating the need for further improvement of DNA vaccines. The improvement strategy consists of fusing ubiquitin to proteins used for vaccination. Degradation of fused proteins in proteasomes enhances their presentation to the major histocompatibility complex (MHC), which could improve the cell-mediated immune response. This is especially important for PRV vaccination, because the cell-mediated immunity is involved in the protection against this virus (van Rooij *et al*., 2004; Zuckermann, 2000).

To date, the targeting of an antigen to the proteasome pathway by fusion with ubiquitin has been tested in mice and rabbits with discordant results. Although some studies failed to detect enhanced cytotoxic T lymphocyte (CTL) activity (Fu *et al*., 1998; Vidalin *et al*., 1999), others were successful (Delogu *et al*., 2000; Konishi *et al*., 2003; Leachman *et al*., 2002; Liu *et al*., 2001; Ramakrishna *et al*., 2004). For example, the fusion of ubiquitin to the nucleoprotein (NP) of Lymphocytic choriomeningitis virus (LCMV) increased the NP degradation that correlated with a reduction of virus titer in the spleen of vaccinated animals after virus challenge (Rodriguez *et al*., 1997).

The ubiquitin-proteasome-dependent proteolytic pathway is involved in the degradation of proteins that control vital processes and in the MHC class-I-restricted antigen presentation of many viral and non-viral proteins (Eggers *et al*., 1995; Hershko *et al*., 2000; Michalek *et al*., 1993). In most cases, the whole process of proteasomal targeting starts with the attachment of one ubiquitin moiety through its C-terminal Gly^{76} to an e-amino group of any Lys in the targeted protein (Johnson *et al*., 1995). The binding of the first ubiquitin moiety often results in the attachment of other ubiquitin moieties creating a multi-ubiquitin chain in which the Gly^{76} of the new ubiquitin added to the complex binds to the Lys^{48} of the last ubiquitin (Chau *et al*., 1989). This reversible ATP-dependent, covalent attachment of multiple ubiquitin molecules targets proteins to the proteasome that degrades proteins into peptides, which are usually used for amino acid recycling. Interferon-γ (IFN-γ) is known to activate the synthesis of a proteasome activator, PA28 and several other proteasome immunosubunits, thereby changing the specificity and affinity of enzymes of the proteasome (immunoproteasome), improving the degradation of viral proteins and generation of the peptides used in MHC class-I assembly and presentation to T lymphocytes (Kruger *et al*., 2003).

Two different ways of fusing ubiquitin to proteins have already been used (Dantuma *et al*., 2000). The first approach (N-End rule) utilizes the ability of ubiquitin to bind to N-terminal residues and internal Lys with high affinity (Varshavsky, 1996).This bond allows the ubiquitin cleavage from the fused protein leaving a destabilized N-terminal residue on the protein that facilitates its degradation. The second approach known as ubiquitin fusion degradation strategy prevents ubiquitin cleavage, thereby stabilizing the mono-ubiquitinated fusion product and facilitating its entry into the classical ubiquitin pathway (Johnson *et al*., 1995). In this approach, the C-terminal Gly^{76} of ubiquitin is replaced with an Ala or Val that inhibits rapid cleavage of ubiquitin at the Gly-X-X sequence by a specific enzyme.

To enhance the DNA vaccine potential we generated plasmids encoding PRV glycoproteins fused to ubiquitin. A mixture of three plasmids encoding ubi- or non-ubi-glycoproteins was administered to pigs followed by a challenge with infectious PRV. C-terminal Gly^{76} of ubiquitin was changed to Ala and fused to the N-terminus of PRV gB, gC and gD. The assumed enhanced degradation of ubi-glycoproteins was verified *in vitro*. Cellular and humoral immune responses as well as nasal excretion of infectious virus in vaccinated pigs were examined as well.

Materials and Methods

Viruses and cells. Porcine PK15 cells and monkey Vero cells were grown in Eagle's MEM (Cambrex) containing 10% FCS. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood using Ficoll-Hypaque (Amersham Biosciences), washed 3 times with PBS, and incubated in RPMI 1640 (Invitrogen) containing 10% FCS and 15 μg/ml gentamycine (Invitrogen). The PRV NIA3 strain was kindly provided by J.C. Audonnet, Merial, Lyon, France and propagated in PK15 cells. When 80% of the infected cells showed CPE, the cultures were frozen at -80°C. After two cycles of freeze-thawing, the virus-containing medium was centrifuged (1,800 × *g*, 30 mins, 4°C) and the supernatant was stored at -80°C.

Virus titration. Samples of nasal fluid were mixed with 2 ml of Eagle's MEM. Monolayers of PK15 cells were incubated with serial 10-fold dilutions of nasal samples and CPE was read after 5 days of incubation at 37°C. Virus titers were calculated according to Kärber (1931) and Vannier *et al*. 1991).

Construction of plasmids. pcDNA3 plasmids encoding non-ubi-gB, -gC, and -gD of PRV (designated gB-pcDNA3, gC-pcDNA3, and gD-pcDNA3) were constructed, characterized (Dufour and De Boisseson, 2003), and produced by Plasmid Factory (Germany). A mixture of these three plasmids (designated PRV-pcDNA3) was used for vaccination. The mouse ubiquitin sequence was cloned between the *Hind*III and *Eco*RI sites of pcDNA3 under a Human cytomegalovirus promoter. The ubiquitin C-terminal Gly^{76} was replaced with Ala to avoid cleavage and nucleotides were added to the C-terminus of ubiquitin to allow a frameshift. The plasmids encoding PRV ubi-gB, ubi-gC, and ubi-gD and ubiquitin alone were generated and designated ubi-gB-pcDNA3, ubi-gC-pcDNA3, ubi-gD-pcDNA3 and ubi-pcDNA3, respectively. A mixture of the

three plasmids encoding the PRV ubi-glycoproteins was designated ubi-PRV-pcDNA3 and used for vaccination. Each plasmid was transfected to *Escherichia coli* Top10 strain, amplified and purified using the EndoFree Plasmid Mega Kit (Qiagen).

Animals. Fourteen-week-old SPF pigs were divided into groups of 8 animals with a mean weight of 47 kg. Pigs were housed and treated in accordance with the Veterinary office regulations.

Immunization and challenge. Pigs received a single intramuscular (i.m.) injection of 100.8 pmoles of a mixture of 3 plasmids encoding ubi- or non-ubi-glycoproteins, respectively, in the neck. Particularly, the PRV-pcDNA3 group of pigs received a mixture of 183.2 µg of gB-pcDNA3, 155 µg of gC-pcDNA3, and 149.6 µg of gD-pcDNA3. The ubi-PRV-pcDNA3 group of pigs received a mixture of 187.5 µg of ubi-gB-pcDNA3, 159.3 µg of ubi-gC-pcDNA3, and 153.9 µg of ubi-gD-pcDNA3. Control groups received 375.6 µg of ubi-pcDNA3 and 362.4 µg of empty pcDNA3 plasmid, respectively Three weeks later, the immunized pigs were challenged intranasally (i.n.) with $10^{5.5}$ $TCID_{50}$ of PRV.

Western blot analysis. PK15 or Vero cells were transfected with 2 µg of each plasmid using lipofectamine as transfection reagent (Invitrogen). After 2 days, 300 µl of a lysis buffer containing a 50x protease inhibitor cocktail was added to the transfected cells. The obtained lysate (50 µg of proteins) was separated by PAGE (12% gel) and blotted to a Hybond-electrochemiluminiscent nitrocellulose membrane (Amersham). The blot was incubated in 5% dry milk in Tris-buffered saline containing 0.2% Tween-20. The glycoproteins were detected with hybridoma supernatants (diluted 1:25) containing mouse monoclonal antibodies against PRV glycoproteins gB, gC, and gD, respectively, and then incubated with horseradish peroxidase (HRP) -labeled sheep anti-mouse IgG. The bound IgG was detected by Supersignal® West Dura Extended Duration Substrate (Pierce).

Immunoperoxidase staining. Cells transfected with plasmids were incubated with a hyperimmune pig PRV antiserum (diluted 1:200) or the sera collected from pigs 12 and 19 days after vaccination with PRV-pcDNA3 or ubi-PRV-pcDNA3, and 4 days after the challenge. The bound IgGs were visualized with HRP-labeled rabbit anti-swine IgG (diluted 1:1,000) (Sigma) and 3-amino-9-ethylcarbazole as substrate (Serotec). In some experiments, a proteasomal inhibitor, MG132 (Calbiochem) was added to the cell-culture medium to a final concentration of 5 µmol/l.

Indirect ELISA of serum IgG1, IgG2 and total IgG antibodies was performed as described (Dory *et al*., 2005a,b). Briefly, Maxisorb 96-well plates (Nunc) were coated with PRV glycoproteins (kindly provided by J.C. Audonnet, Merial, Lyon, France) and incubated with serial threefold dilutions of tested sera. The bound IgGs were detected with a mouse anti-porcine IgG1, IgG2 or total IgG (Serotec), a HRP-labeled rabbit anti-mouse IgG (Jackson Laboratories), and tetramethyl benzidine (Pierce). Titers of IgG1, IgG2 and total IgG antibodies expressed in log values were defined as the highest serum dilutions giving A_{450} higher than the 3-fold A_{450} of a serum from non-vaccinated and non-challenged pig.

Virus neutralization (VN) test. Heat-inactivated sera were serially diluted 2-fold and 50 µl of each dilution was incubated with 100 $TCID_{50}$ of PRV in a volume of 50 µl in 96-well plates for 1 hr at 37°C. Then, 2.25 x 10^4 PK15 cells in 150 µl was added to each well and incubated for 5 days at 37°C. VN titers expressed in log values were defined as the highest serum dilutions inhibiting CPE in 50% of the wells.

Assay of IFN-γ and interleukin 4 (IL-4) mRNA. PBMC were isolated from the blood of pigs collected before vaccination, 8 and 19 days after vaccination, and 2 and 7 days after the challenge. PBMC were stimulated *in vitro* for 16 hrs with PRV at a multiplicity of infection (MOI) of 10 and used for total RNA extraction as described (Dory *et al*., 2005a,b). IFN-γ and IL-4 mRNA expressions were determined by a quantitative real-time PCR described previously (Dory *et al*., 2005a,b). Cytokine mRNA and ß-actin mRNA threshold cycle (Ct) values were determined for each sample. The cytokine Ct was normalized using an internal ß-actin reference (ΔCt = cytokine Ct – ß-actin Ct). Relative amounts of each cytokine mRNA (cytokine mRNA in PRV-stimulated cells/cytokine mRNA in non-stimulated cells) were determined using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

Clinical parameters. The mortality rate, body temperature and clinical signs were monitored daily. The animals were checked for absent, mild, and severe nervous symptoms. Some animals had to be euthanized to prevent unnecessary suffering. Mean relative daily weight gain during seven days post challenge (MRDG7) was determined; ΔG7 corresponded to the difference in MRDG7 between immunized and non-immunized animals (Stellmann *et al*., 1989). Only animals surviving at least 7 days after the challenge were weighed.

Statistical analysis. The obtained data were analyzed using the non-parametric Mann-Whitney test (Mann and Whitney, 1947) included in the Systat 9 software. This test was suitable for data representing small sets with unpaired numbers. The size of each animal group was influenced heavily by the mortality and this fact was considered to ensure statistical validity of the analysis. The following comparisons were performed: (i) PRV-pcDNA3 vs empty pcDNA3, ubi-PRV-pcDNA3 vs empty pcDNA3, or ubi-pcDNA3 vs empty pcDNA3 to evaluate the effect of immunization with ubi-glycoproteins and non-ubi-glycoproteins, and ubiquitin alone vs. control; (ii) ubi-PRV-pcDNA3 vs PRV-pcDNA3 to evaluate the effect of immunization with ubi-glycoproteins vs. non-ubi-glycoproteins. All results were expressed as mean ± SD.

Results

Characterization of the plasmids in vitro and in vivo

The mouse ubiquitin gene was used for the construction of plasmids encoding ubi-glycoproteins, because it showed a 100% homology with the porcine ubiquitin. Initially, the gene encoding mouse ubiquitin was cloned into pcDNA3. The genes for PRV glycoproteins gB, gC and gD, respectively, were cloned in frame with the ubiquitin gene and the plasmids ubi-gB-pcDNA3, ubi-gC-pcDNA3, and ubi-gD-pcDNA3, respectively, were generated. After transfection of PK15 cells with the plasmids encoding PRV ubi-glycoproteins and non-ubi-glycoproteins, gB (one band of 125 K) and gC (two bands of 74 K and 92 K) were detected by Western blot analysis, while slight and no bands were

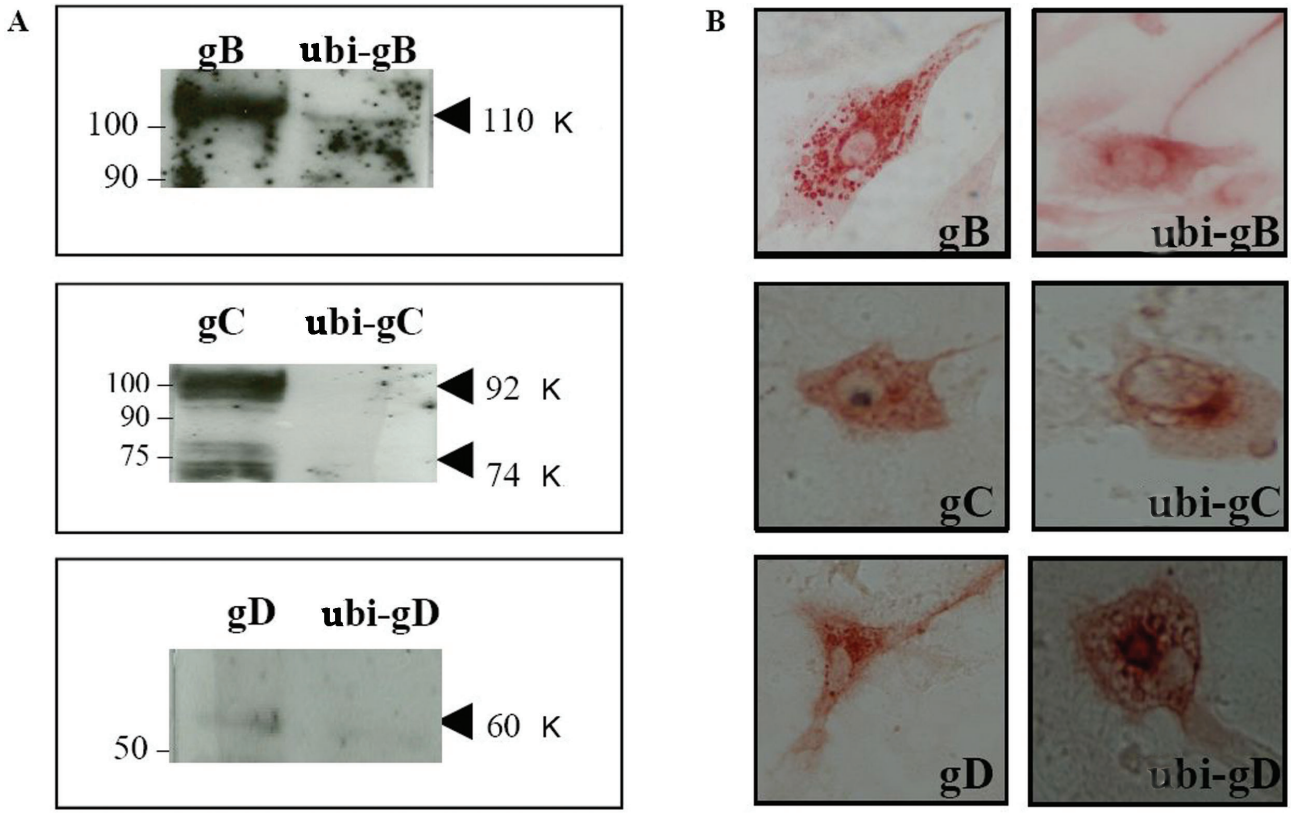

Fig. 1

Western blot analysis of PK15 cells transfected with plasmids encoding PRV ubi- and non-ubi-glycoproteins (A) and immunoperoxidase staining of Vero cells transfected with plasmids encoding PRV ubi-and non-ubi-glycoproteins (B)

Table 1. Antibodies detected by the immunoperoxidase staining in the sera of pigs vaccinated with plasmids encoding PRV ubi- and non-ubi-glycoproteins

Glycoprotein detected	Serum of pigs vaccinated with PRV-pcDNA3			Serum of pigs vaccinated with ubi-PRV-pcDNA3			Hyperimmune serum	Negative serum
	Day post vaccination							
	12	19	25[a]	12	19	25[a]		
gB	+	++	++	++	+++	+++	+++++	–
ubi-gB	+	++	++	+	++	++	++++	–
gC	–	–	–	–	–	–	+	–
ubi-gC	–	–	–	–	–	–	+	–
gD	–	–	–	–	–	–	++	–
ubi-gD	–	–	–	–	–	–	++	–

Vero cells were transfected with the plasmids encoding PRV ubi- and non-ubi-glycoproteins. The intensity of staining is expressed as the average from 3 experiments. [a]Day 25 post vaccination corresponds to day 4 post challenge.

detected for ubi-gB and ubi-gC, respectively (Fig. 1A) (Whealy *et al*., 1990; Robbins *et al*., 1986). The band of 60 K detected for gD was very slight, while none was detected for ubi-gD (Rauh and Mettenleiter, 1991).

Intracellular localization of PRV ubi-glycoproteins and non-ubi-glycoproteins produced by corresponding plasmids was done by the immunoperoxidase staining of transfected Vero cells. gB was mainly found in intracellular vesicles

(Fig. 1B), whereas gD was found in defined intracellular structures close to the nucleus and in the membranes of transfected cells. However, the intracellular staining of gC-transfected cells was more discrete. On the other hand, most of the ubi-glycoproteins encoded by ubi-gB-pcDNA3, ubi-gC-pcDNA3, and ubi-gD-pcDNA3 accumulated in intracytoplasmic structures of transfected cells (Fig. 1B). These seemed larger in the presence of the proteasomal inhibitor MG132 (data not shown), suggesting that a partial degradation of the ubi-glycoproteins occurred also in the absence of MG132. Taken together, the Western blot analysis as well as the immunoperoxidase staining indicated that the ubiquitination of PRV glycoproteins resulted in partial degradation of gB, gC and gD in transfected cells.

The antisera produced in pigs after immunization with the mixtures of 3 plasmids encoding ubi- and non-ubi-glycoproteins, respectively, were tested by immunoperoxidase staining. Vero cells transfected with gB-pcDNA3 and ubi-gB-pcDNA3, respectively, were incubated with pig sera collected 12 and 19 days after immunization (Table 1). Interestingly, the cells transfected with gB-pcDNA3 were more intensively stained with the ubi-PRV-pcDNA3 antiserum than those transfected with ubi-gB-pcDNA3. These results could be explained by a higher affinity of the antibodies against ubi-PRV-pcDNA3 due to the better presentation of the antigen. Ubi- and non-ubi-gC- and gD-pcDNA- transfected cells were not recognized by the antisera from animals immunized with PRV ubi- and non-ubi-glycoproteins, respectively. The hyperimmune serum against PRV showed only a weak staining.

IgG1, IgG2 and total IgG serum antibodies

The T_H1-like immune response induces B cells to produce IgG2 antibodies. On the other hand, IgG1 antibodies are markers of theT_H2-like immune response that is important for the development of the cytotoxic response.

Serum samples from the pigs immunized with plasmids encoding PRV ubi- and non-ubi-glycoproteins, respectively, were assayed for PRV antibodies by ELISA. The sera of non-immunized animals had no antibodies against PRV. In the sera of pigs immunized with control plasmids ubi-pcDNA3 or pcDNA3 (empty), PRV-specific IgG1 and IgG2 antibodies were first detected on day 12 after the challenge (Fig. 2A and B). Similar levels of anti-PRV IgG1 antibodies were detected in pigs injected with either ubi-PRV-pcDNA3

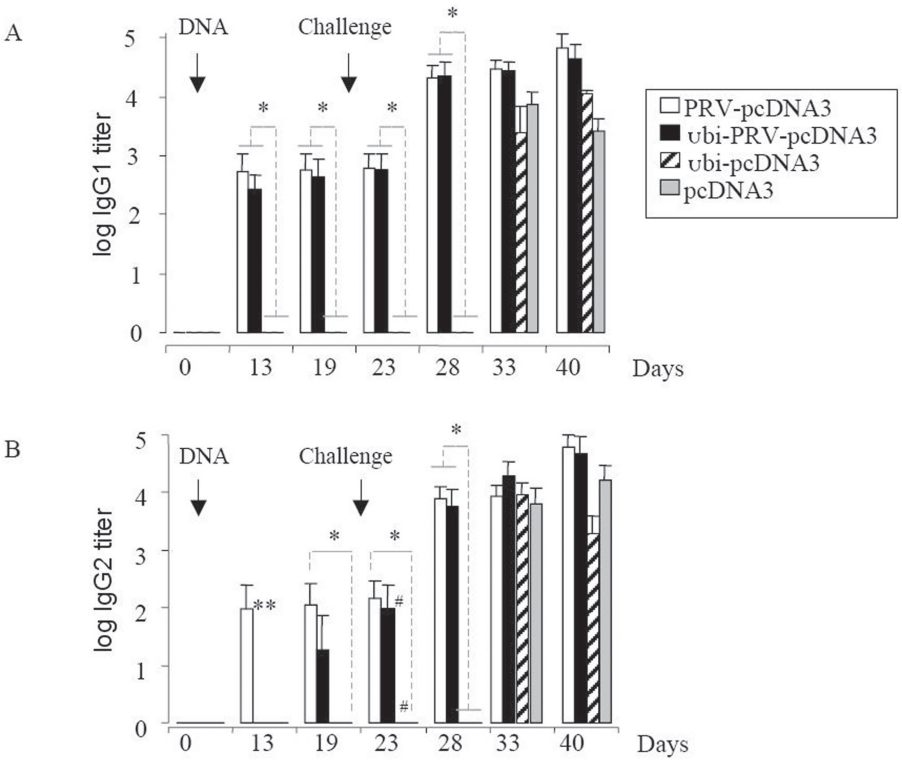

Fig. 2

Elisa titers of PRV IgG1 (A) and IgG2 (B) antibodies of the sera of pigs vaccinated with plasmids encoding PRV ubi-and non-ubi-glycoproteins and challenged with PRV

*p <0.05; **p = 0.064; #p = 0.10.

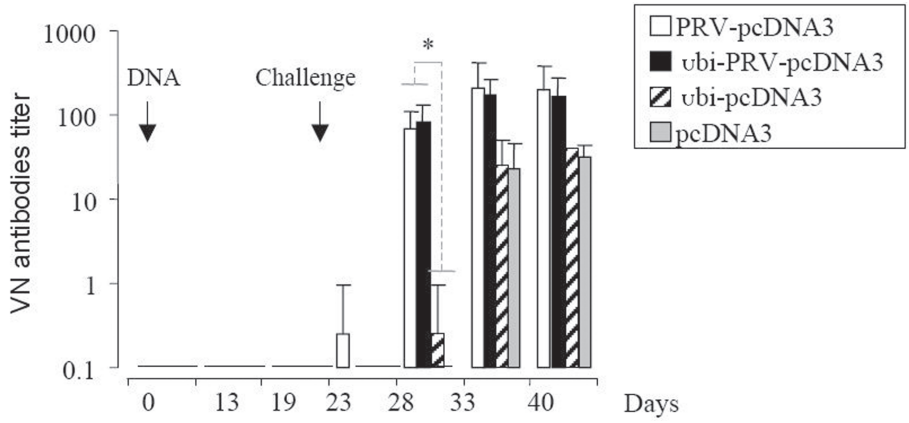

Fig. 3

VN antibodies in the sera of pigs vaccinated with plasmids encoding PRV ubi-and non-ubi-glycoproteins and challenged with PRV

*p <0.05.

or PRV-pcDNA3 on day 13 after immunization (Fig. 2A). The mean IgG1 titer remained stable (around 1.5 x 10^2) until day 2 after the challenge, when it increased to a plateau (1.5 x 10^4) that remained stable until the end of the experiment (Fig. 2A). This revealed that the PRV-specific IgG1 humoral immune response was not affected by the fusion of glycoproteins to ubiquitin. Similar results were obtained for total IgG (data not shown).

In contrast, the PRV IgG2 antibody production was apparently different for ubi-PRV-pcDNA3 and PRV-pcDNA3. The group of pigs vaccinated with PRV-pcDNA3 produced some amount of IgG2 antibodies, whereas no or low level was detected in the ubi-PRV-pcDNA3 group on days 13 and 19 after immunization (Fig. 2B). In fact, 3 and 4 out of 8 pigs produced IgG2 antibodies in the PRV-pcDNA3 group on days 13 and 19, respectively (data not shown), whereas none and 1 out of 8 pigs produced IgG2 antibodies on the same days in the ubi-PRV-pcDNA3 group. The probability of error in stating that the PRV-pcDNA3 group produced a higher amount of IgG2 antibodies than the ubi-PRV-pcDNA3 group was 6.4% ($p = 0.064$) and 10% ($p = 0.10$) on days 13 and 19, respectively. Just the PRV-pcDNA3 group produced a statistically significant ($p < 0.05$) higher amount of IgG2 antibodies than the pcDNA3 (empty) group, whereas no statistically significant differences were observed between the ubi-PRV-pcDNA3 and the control ubi-pcDNA3 groups.

We detected a delay in the production of IgG2 antibodies in the ubi-PRV-pcDNA3 group in comparison with the PRV-pcDNA3 group until day 23. Two days after the challenge, the production of IgG2 antibodies in both groups was about the same (Fig. 2B). The PRV-pcDNA3 group showed a statistically significant ($p < 0.05$) higher production of IgG2 antibodies than the pcDNA3 group. The ubi-PRV-pcDNA3 group produced only a slightly higher amount of IgG2 antibodies than the control ubi-pcDNA3 group, but the difference was not significant. Twelve days after the-challenge, the production of IgG2 antibodies was similar in all the challenged groups (Fig. 2B).

Altogether, these results indicated that the PRV-specific IgG1 response was not affected by the fusion of PRV glycoproteins to ubiquitin. The PRV-specific IgG-2 response was slightly affected by ubiquitination of the glycoproteins, but the probability of error in stating that the IgG2 antibody productions in the ubi-PRV-pcDNA group was lower than that in the PRV-pcDNA3 group on days 13 and 19 after immunization was 6.4 and 10%, respectively. The detected differences between the two groups started to decline soon after the challenge.

VN antibodies

No VN antibodies were detected in pigs vaccinated with plasmids encoding PRV ubi- and non-ubi-glycoproteins before and after vaccination and 2 days after the challenge (Fig. 3). VN antibodies were detected in the groups of pigs vaccinated with ubi-PRV-pcDNA or PRV-pcDNA3 on day 7 after the challenge (Fig. 3), demonstrating a successful priming by DNA vaccination. VN antibodies in pigs vaccinated with control ubi-pcDNA3 or empty pcDNA3 plasmids were first detected on day 12 after the challenge. In the groups vaccinated with ubi-PRV-pcDNA3 or PRV-pcDNA3 a peak titer was obtained on day 33. Summing up, the production of PRV-specific VN antibodies was not affected by the fusion of PRV glycoproteins to ubiquitin.

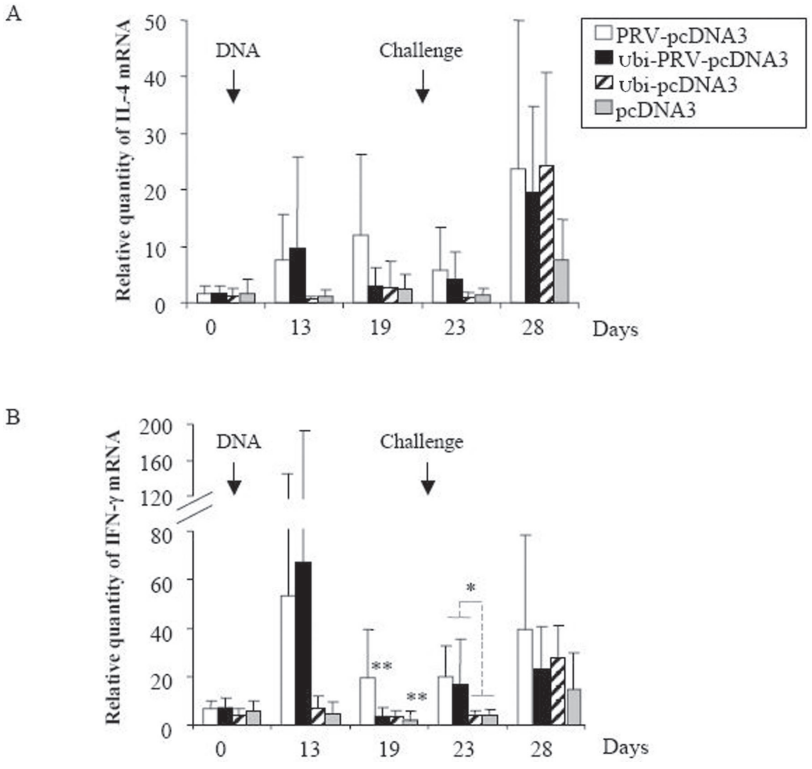

Fig. 4

Expression of IL-4 (A) and IFN-γ (B) mRNAs in PRV-stimulated PBMC originating from pigs vaccinated with plasmids encoding PRV ubi- and non-ubi-glycoproteins and challenged with PRV

The mean relative quantity of cytokine mRNA ± SD is shown. *p <0.05; **p = 0.059).

IFN-γ and IL-4 mRNA expression in PRV-stimulated PBMC

IFN-γ has several immunoregulatory roles and effector functions involved in T_H1- responses and IL-4 plays a key role in T_H2-responses (Finkelman *et al*., 1988, Wood and Seow, 1996). mRNAs of both cytokines were determined to assess humoral and cellular immune responses. Relative quantities of IFN-γ and IL-4 mRNAs were determined in PBMC after their *in vitro* stimulation with virulent PRV (Dory *et al*., 2005a,b). Before the DNA vaccination, low background levels of IFN- γ and IL-4 mRNAs were detected in all groups of animals (Fig. 4A and B).

The amount of IL-4 mRNA remained low throughout the study in the pigs injected with control ubi-pcDNA3 or pcDNA3 (empty) even after the challenge. No difference in IL-4 mRNA production was observed between the PRV-pcDNA3 and ubi-PRV-pcDNA3 groups (Fig. 4A). This indicated that the PRV-specific T_H2-like proliferation was unaffected by the fusion of PRV glycoproteins to ubiquitin, at least as determined by our techniques.

In contrast, the profile of IFN-γ mRNA production slightly differed between the ubi-PRV-pcDNA3 and PRV-pcDNA3 groups (Fig. 4B). On day 13 after vaccination, the pigs vaccinated with ubi-PRV-pcDNA3 and PRV-pcDNA3, respectively, produced the same levels of IFN-γ mRNA, although the population was very heterogeneous (Fig. 4B). On day 19 after vaccination, the pigs vaccinated with ubi-PRV-pcDNA3 produced much smaller amounts of IFN-γ mRNA in comparison with those vaccinated with PRV-pcDNA3. The differences between these two groups were not statistically significant ($p = 0.14$) (Fig. 4B). Moreover, the probability of error in stating that the PRV-pcDNA3 group produced more IFN-γ mRNA than the pcDNA3 (empty) group was 5.9% ($p = 0.059$), whereas no differences were observed between the Ubi-PRV-pcDNA3 and Ubi-pcDNA3 groups ($p = 0.916$). Two days after the challenge, the groups vaccinated with ubi-PRV pcDNA3 and PRV-pcDNA3 produced the same amounts of IFN-γ mRNA.

Protective efficacy of DNA vaccines

The immunization with DNA vaccines was tolerated well and no side effects were observed in any of the vaccinated animals. The protective efficacy of DNA vaccine was evaluated by analyzing clinical symptoms after PRV

Table 2. Clinical parameters in DNA-vaccinated pigs challenged with PRV

DNA vaccine	Mortality[a]	Nervous symptoms	Fever days[b]	MRDG7[c]	ΔG7[d]
PRV-pcDNA3	0/8	0/8	2.4 ± 1.5	–0.88 ± 0.39	1.00
ubi-PRV-pcDNA3	0/8	0/8	2.5 ± 1.4	–0.83 ± 0.45	1.05
ubi-pcDNA3	3/8	7/8	4.4 ± 1.5	–1.68 ± 0.34	0.20
pcDNA3	2/8	7/8	5.0 ± 2.1	–1.88 ± 0.61	n.a.
No vaccine, no challenge	n.a.	n.a.	n.a.	+1.75 ± 0.34	3.6.

[a]Dead/total.
[b]Fever days (T >40.5°C) ± SD.
[c]MRDG7 expressed as kg/100 kg/day ± SD.
[d]Difference in MRDG7 between pigs vaccinated with ubi and non-ubi PRV glycoproteins and ubi and non-ubi control plasmids.
n.a. = not applicable.

challenge, such as mortality, body temperature, nervous symptoms, weight loss and excretion of infectious virus (Table 2). Three out of 8 pigs injected with the control ubi-pcDNA3 and 2 out of 8 pigs injected with the control pcDNA3 (empty) plasmid died. One pig injected with ubi-pcDNA3 and 2 pigs injected with pcDNA3 had to be euthanized for ethical reasons. These two groups of pigs showed the same mean fever periods and body weight losses during the first week after the challenge. In contrast, the groups of pigs vaccinated with ubi-PRV-pcDNA3 and PRV-pcDNA3 showed no mortality, no nervous symptoms, reduced mean period of high fever and positive ΔG7 values of 1.05 and 1.00 reflecting a reduction of weight loss.

In all groups of pigs the virus excretion was detected on day 2 after the challenge (Fig. 5). The probability of error in stating that the ubi-PRV-pcDNA3 group excreted less virus than the PRV-pcDNA3 group was 7.8% (p = 0.078). The PRV-pcDNA3 and control pcDNA3 (empty) groups excreted the same amount of virus. All the groups excreted the same amount of virus on day 5 after challenge. Two vaccinated groups, PRV-pcDNA3 and ubi-PRV-pcDNA3 excreted significantly less virus on day 7 after the challenge than the control groups injected with pcDNA3 (empty) and ubi-pcDNA3.

Summing up, the pigs vaccinated with the plasmids encoding PRV ubi- and non-ubi-glycoproteins were equally protected against PRV infection. Nevertheless, two days after the challenge, the small difference between these two groups in the amount of excreted virus was evaluated as insignificant. At later times after the challenge, both vaccinated groups of pigs excreted the same amount of virus.

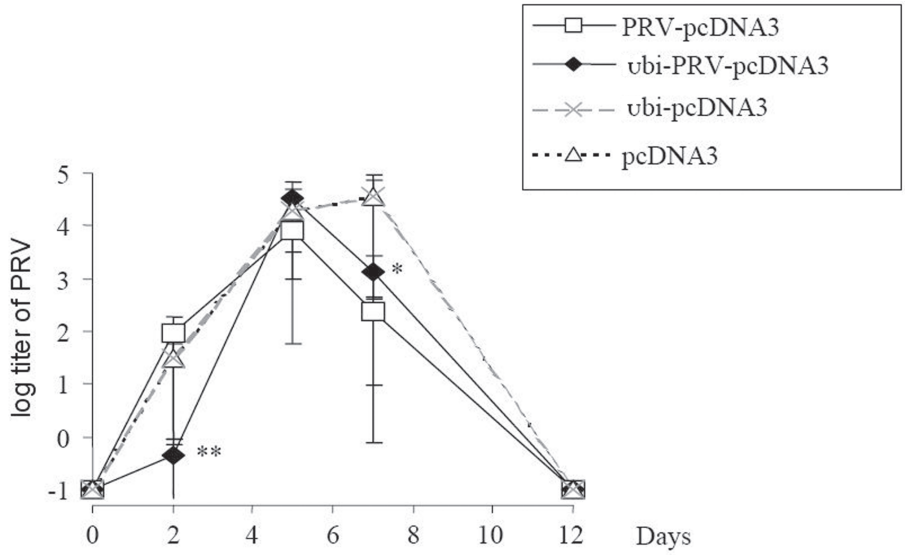

Fig. 5

Virus titers of nasal fluids from DNA-vaccinated pigs before and after PRV challenge

The mean virus titer expressed as log $TCID_{50}$/g nasal fluid ± SD. *p <0.05; **p = 0.078.

Discussion

Several strategies to improve DNA vaccination against PRV infection in pigs have been tested with various degree of success (Dufour *et al*., 2000; van Rooij *et al*., 2002; Dory *et al*., 2005a,b). Despite all positive advances, the vaccine efficacy still needs an improvement, particularly the reduction of virus excretion and the enhancement of CTL response, which are important in protection against PRV infection (Depierreux *et al*., 1997). In order to induce a CTL response, antigens have to undergo an intracellular degradation into small peptides via the ubiquitin-proteasome pathway (Sijts *et al*., 2001). This can be achieved by covalent attachment of the antigen to ubiquitin (Delogu *et al*., 2000; Konishi *et al*., 2003; Leachman *et al*., 2002; Liu *et al*., 2001; Ramakrishna *et al*., 2004) that leads to an enhancement of the protection in some viral and non-viral systems (Rodriguez *et al*., 1997, 1998; Xiang *et al*., 2000).

The aim of the present study was to generate plasmids encoding PRV gB, gC, and gD fused to ubiquitin and evaluate their effect when injected to pigs. As expected, the fusion of ubiquitin to each of the PRV glycoproteins enhanced their intracellular degradation as shown by Western blot analysis. This result probably reflected the partial degradation of ubi-glycoproteins in the proteasome and/or the cleavage of ubiquitin from the glycoprotein, which were observed in 10–20% of cases even after the replacement of the C-terminal Gly^{76} of ubiquitin with Ala (Rodriguez *et al*., 1997). This might explain the expression of ubi-glycoproteins after *in vitro* transfection and the unexpected induction of specific humoral response after *in vivo* administration due to the presence of small amounts of expressed glycoproteins in extracellular milieu. In clear contrast, the entire absence of antibody induction reported for covalent attachment of ubiquitin to the LCMV NP protein correlated with total degradation of the protein *in vitro* (Rodriguez *et al*., 1997). Differences in the degradation of LCMV ubi-NP protein and PRV ubi-glycoproteins might be due to the following factors. (i) Partial impairment of transport of glycoproteins to proteasome and/or their degradation within proteasome due to strong transmembrane signals present in the glyco proteins. (ii) The competition between the attached ubiquitin (proteasomal targeting signal) and other intracellular targeting moieties of glycoproteins, as was shown for the hepatitis B core antigen (Rodriguez F, unpublished data). (iii) Different stability of ubi-glycoproteins depending on the N-terminal amino acid (Asn, Gly, and Gly for gB, gC, and gD, respectively) after ubiquitin cleavage. (iv) The low proportion of potential sites for ubiquitination, namely 2, 0.6 and 1% for gB, gC, and gD, respectively, compared to 10% for LCMV NP (Andersson and Barry, 2004). (v) The amount of injected plasmids. A failure of the ubiquitin targeting to improve vaccination occurred when maximum doses of DNA were used for immunization. Suboptimal doses of DNA actually improved the immune responses induced by the ubiquitin fusion (Andersson and Barry, 2004; Fu *et al*., 1998; Ye *et al*., 2002). (vi) The fact that 3 different PRV glycoproteins were used in combination.

No differences were observed for IgG1 and VN antibodies production between the animals vaccinated with PRV ubi- and non-ubi-glycoproteins, but the ubiquitination of PRV glycoproteins seemed to modify the induced cellular immune response. Moreover, IFN-γ mRNA was produced by PRV-stimulated PBMCs in both groups of animals on day 13 after vaccination. However, on day 19 after vaccination, IFN-γ mRNA was produced only by PRV-stimulated PBMC originating from the animals vaccinated with PRV-pcDNA3 but not with ubi-PRV-pcDNA3. This fact might be related to a shorter stimulation of PBMC due to a shorter half-life of the ubi-glycoproteins compared with non-ubi-glycoproteins. Nevertheless, a clear-cut conclusion about the delayed appearance of IgG2 and IFN-γ could not be drawn. The pigs vaccinated with either DNA vaccine were equally protected against PRV infection. No death, no nervous symptoms, and the same reduction of body weight loss were observed in the PRV-pcDNA3 and the ubi-PRV-pcDNA3 groups. Considering the important issue of nasal excretion of infectious virus, a delay in the excretion was detected in the ubi-PRV-pcDNA3 group. Starting with day 5 after the challenge, both vaccinated groups excreted the same amount of infectious virus.

In conclusion, the PRV ubi-glycoproteins gB, gC and gD used in DNA vaccines seemed to modify cellular but not humoral immune response of pigs. This modification had no impact on clinical protection of the pigs but seemed to delay the excretion of infectious PRV.

Acknowledgements. The authors thank Dr J.C. Audonnet, Merial, Lyon, France for providing PRV glycoproteins and Mr B. Jan, French Food Safety Agency, Ploufragan, France for technical assistance This research was supported by the grant QLRT-1999-459 from the European Community. F.R. was supported by the project AGL2004-07857-C03-01 from the Department of Education and Science, Spain.

References

Andersson HA, Barry MA (2004): Maximizing antigen targeting to the proteasome for gene-based vaccines. *Mol. Therapy* **10**, 432–446.

Chau V, Tobias JW, Bachmair A, Marriott D, Ecker DJ, Gonda DK, Varshavsky A (1989): A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* **243**, 1576–1583.

Dantuma NP, Lindsten K, Glas R, Jellne M, Masucci MG (2000): Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. *Nat. Biotechnol.* **18**, 538–543.

Delogu G, Howard A, Collins FM, Morris SL (2000): DNA vaccination against tuberculosis: expression of a ubiquitin--conjugated tuberculosis protein enhances antimycobacterial immunity. *Infect. Immun.* **68**, 3097–3102.

Dory D, Beven V, Torche AM, Bougeard S, Cariolet R, Jestin A (2005a): CpG motif in ATCGAT hexamer improves DNA-vaccine efficiency against lethal Pseudorabies virus infection in pigs. *Vaccine* **23**, 4532–4540.

Dory D, Torche AM, Beven V, Blanchard P, Loizel C, Cariolet R, Jestin A (2005b): Effective protection of pigs against lethal Pseudorabies virus infection after a single injection of low-dose Sindbis-derived plasmids encoding PrV gB, gC and gD glycoproteins. *Vaccine* **23**, 3483–3491.

Dufour V, Chevallier S, Cariolet R, Somasundaram S, Lefčvre F, Jestin A, Albina E (2000): Induction of porcine cytokine mRNA expression after DNA immunization and pseudorabies virus infection. *J. Interferon Cytokine Res.* **20**, 885–890.

Dufour V, De Boisseson C (2003): Use of a Sindbis virus DNA-based expression vector for induction of protective immunity against pseudorabies virus in pigs. *Vet. Immunol. Immunopathol.* **93**, 125–134.

Eggers M, Boes-Fabian B, Ruppert T, Kloetzel PM, Koszinowski UH (1995): The cleavage preference of the proteasome governs the yield of antigenic peptides. *J. Exp. Med.* **182**, 1865–1870.

Finkelman FD, Katona IM, Urban JF, Hlmes J, Ohara N, Tung AS, Sample JG, Paul WE (1988): IL-4 is required to generate and sustain in vivo IgE responses. *J. Immunol.* **141**, 2335-2341.

Fischer L, Barzu S, Andreoni C, Buisson N, Brun A, Audonnet JC (2003): DNA vaccination of neonate piglets in the face of maternal immunity induces humoral memory and protection against a virulent pseudorabies virus challenge. *Vaccine* **21**, 1732–1741.

Fu TM, Guan L, Friedman A, Ulmer JB, Liu MA, Donnelly JJ (1998): Induction of MHC class I-restricted CTL response by DNA immunization with ubiquitin-influenza virus nucleoprotein fusion antigens. *Vaccine* **16**, 1711–1717.

Hershko A, Ciechanover A, Varshavsky A (2000): Basic Medical Research Award. The ubiquitin system. *Nat. Med.* **6**, 1073–1081.

Hong W, Xiao S, Zhou R, Fang L, He Q, Wu B, Zhou F, Chen H (2002): Protection induced by intramuscular immunization with DNA vaccines of pseudorabies in mice, rabbits and piglets. *Vaccine* **20**, 1205–1214.

Johnson ES, Ma PC, Ota IM, Varshavsky A (1995): A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem.* **270**, 17442–1756.

Kärber G (1931): Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Naunyn-Schmiedebergs. *Arch. Exper. Pathol. Pharmakol.* **162**, 480–483.

Konishi E, Ajiro N, Nukuzuma C, Mason PW, Kurane I (2003): Comparison of protective efficacies of plasmid DNAs encoding Japanese encephalitis virus proteins that induce neutralizing antibody or cytotoxic T lymphocytes in mice. *Vaccine* **21**, 3675–3683.

Kruger E, Kuckelkorn U, Sijts A, Kloetzel PM (2003): The components of the proteasome system and their role in MHC class I antigen processing. *Rev. Physiol. Biochem. Pharmacol.* **148**, 81–104.

Leachman SA, Shylankevich M, Slade MD, Levine D, Sundaram RK, Xiao W, Bryan M, Zelterman D, Tiegelaar RE, Brandsma JL (2002): Ubiquitin-fused and/or multiple early genes from cottontail rabbit papillomavirus as DNA vaccines. *J. Virol.* **76**, 7616–7624.

Liu WJ, Zhao KN, Gao FG, Leggatt GR, Fernando GJ, Frazer IH (2001): Polynucleotide viral vaccines: codon optimisation and ubiquitin conjugation enhances prophylactic and therapeutic efficacy. *Vaccine* **20**, 862–869.

Mann HB, Whitney DR (1947): On a test of whether one of two random variables is stochastically larger than the other. *Ann. Math. Statist.* **18**, 50–60.

Mettenleiter TC (2000): Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis - state of the art, June 1999. *Vet. Res.* **31**, 99–115.

Michalek MT, Grant EP, Gramm C, Goldberg AL, Rock KL (1993): A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. *Nature* **363**, 552–554.

Ober BT, Summerfield A, Mattlinger C, Wiesmuller KH, Jung G, Pfaff E, Saalmuller A, Rziha HJ (1998): Vaccine-induced, pseudorabies virus-specific, extrathymic CD4+CD8+ memory T-helper cells in swine. *J. Virol.* **72**, 4866–4873.

Ober BT, Teufel B, Wiesmuller KH, Jung G, Pfaff E, Saalmuller A, Rziha HJ (2000): The porcine humoral immune response against pseudorabies virus specifically targets attachment sites on glycoprotein gC. *J. Virol.* **74**, 1752–1760.

Ramakrishna L, Anand KK, Mahalingam M, Mohankumar KM, Ramani S, Siddappa NB, Ranga U (2004): Codon optimization and ubiquitin conjugation of human immunodeficiency virus-1 Tat lead to enhanced cell-mediated immune responses. *Vaccine* **22**, 2586–2598.

Rauh I, Mettenleiter TC (1991): Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. *J. Virol.* **65**, 5348–5356.

Robbins AK, Whealy ME, Watson RJ, Enquist LW (1986): Pseudorabies virus gene encoding glycoprotein gIII is not essential for growth in tissue culture. *J. Virol.* **59**, 635–645.

Rodriguez F, Zhang J, Whitton JL (1997): DNA immunization: ubiquitination of a viral protein enhances cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction. *J. Virol.* **71**, 8497–8503.

Rodriguez F, An LL, Harkins S, Zhang J, Yokoyama M, Widera G, Fuller JT, Kincaid C, Campbell IL, Whitton JL (1998): DNA immunization with minigenes: low frequency of memory cytotoxic T lymphocytes and inefficient antiviral protection are rectified by ubiquitination. *J. Virol.* **72**, 5174–5181.

Sijts A, Zaiss D, Kloetzel PM (2001): The role of the ubiquitin-proteasome pathway in MHC class I antigen processing: implications for vaccine design. *Curr. Mol. Med.* **1**, 665–676.

Stellmann C, Vannier P, Chappuis G, Brun A, Dauvergne M, Fargeaud D, Bugand M, Colson X (1989): The potency testing of pseudorabies vaccines in pigs. A proposal for a quantitative criterion and a minimum requirement. *J. Biol. Stand* **17**, 17–27.

van Rooij EMA, Haagmans BL, de Visser YE, de Bruin MGM, Boersma W, Bianchi ATJ (1998): Effect of vaccination route and composition of DNA vaccine on the induction of protective immunity against pseudorabies infection in pigs. *Vet. Immunol. Immunopathol.* **66**, 113–126.

van Rooij EM, Haagmans BL, Glansbeek HL, de Visser YE, de Bruin MG, Boersma W, Bianchi AT (2000): A DNA vaccine coding for glycoprotein B of pseudorabies virus induces cell-mediated immunity in pigs and reduces virus excretion early after infection. *Vet. Immunol. Immunopathol.* **74**, 121–136.

van Rooij EM, Glansbeek HL, Hilgers LA, te Lintelo EG, de Visser YE, Boersma WJ, Haagmans BL, Bianchi AT (2002): Protective antiviral immune responses to pseudorabies virus induced by DNA vaccination using dimethyldioctadecylammonium bromide as an adjuvant. *J. Virol.* **76**, 10540–10545.

van Rooij EM, de Bruin MG, de Visser YE, Middel WG, Boersma WJ, BianchiAT (2004): Vaccine-induced T cell-mediated immunity plays a critical role in early protection against pseudorabies virus (suid herpes virus type 1) infection in pigs. *Vet. Immunol. Immunopathol.* **99**, 113–125.

Vannier P, Hutet E, Bourgueil E, Cariolet R (1991): Level of virulent virus excreted by infected pigs previously vaccinated with different glycoprotein deleted Aujeszky's disease vaccines. *Vet. Microbiol.* **29**, 213–223.

Varshavsky A (1996): The N-end rule: functions, mysteries, uses. *Proc. Natl. Acad. Sci. USA* 93, 12142–12149.

Vidalin O, Tanaka E, Spengler U, Trepo C, Inchauspe G (1999): Targeting of hepatitis C virus core protein for MHC I or MHC II presentation does not enhance induction of immune responses to DNA vaccination. *DNA Cell. Biol.* **18**, 611–621.

Whealy ME, Robbins AK, Enquist LW (1990): The export pathway of the pseudorabies virus gB homolog gII involves oligomer formation in the endoplasmic reticulum and protease processing in the Golgi apparatus. *J. Virol.* **64**, 1946–1955.

Wood PR, Seow HF (1996): T cell cytokines and disease prevention. *Vet. Immunol. Immunopathol.* **54**, 33–34.

Xiang R, Lode HN, Chao TH, Ruehlmann JM, Dolman CS, Rodriguez F, Whitton JL, Overwijk WW, Restifo NP, Reisfeld RA (2000): An autologous oral DNA vaccine protects against murine melanoma. *Proc. Natl. Acad. Sci. USA* **97**, 5492–5497.

Ye M, Morello CS, Spector DH (2002): Strong CD8 T-cell responses following coimmunization with plasmids expressing the dominant pp89 and subdominant M84 antigens of murine cytomegalovirus correlate with long-term protection against subsequent viral challenge. *J. Virol.* **76**, 2100–2112.

Zaripov MM, Morenkov OS, Siklodi B, Barna-Vetro I, Gyongyosi-Horvath A, Fodor I (1998): Glycoprotein B of Aujeszky's disease virus: topographical epitope mapping and epitope-specific antibody response. *Res. Virol.* **149**, 29–41.

Zaripov MM, Morenkov OS, Fodor N, Brown A, Schmatchenko VV, Fodor I (1999): Distribution of B-cell epitopes on the pseudorabies virus glycoprotein B. *J. Gen. Virol.* **80**, 537–5541.

Zuckermann FA (2000): Aujeszky's disease virus: opportunities and challenges. *Vet. Res.* **31**, 121–131.