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Protection of pigs against Aujeszky's disease by DNA vaccination

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Vaccination with DNA constructs encoding viral antigens has been shown to induce antiviral immunity in various model hosts. However, relevant natural virus–host systems have so far been analysed to only a very limited extent. To test the efficacy of DNA vaccination in an economically important large animal, pigs were immunized against Aujeszky's disease, a serious virus infection caused by the alphaherpesvirus pseudorabies virus (PrV), which is characterized by severe central nervous and respiratory symptoms. After vaccination with plasmid vectors containing genes for immunogenic envelope glycoproteins C or D (gC or gD) of PrV under control of the major immediate early promotor of human cytomegalovirus, animals developed serum antibodies which recognized the respective antigen in immunoblot and exhibited neutralizing activity. Animals vaccinated with the gC expression plasmid were fully protected against a lethal challenge with PrV strain 75V19, and showed partial protection against the highly virulent NIA-3 strain. In contrast, protection was not observed after vaccination with the gD plasmid. Three intramuscular or intradermal immunizations with as little as 1 μg of gC plasmid DNA resulted in seroconversion and partial protection against lethal NIA-3 infection. Specific antibodies were detected until at least 9 months after vaccination. In addition, a cellular immune response specific for gC could be demonstrated in proliferation assays of peripheral mononuclear lymphocytes. Our results thus demonstrate the potency of DNA vaccination for protection of large animals against a lethal virus infection.

Introduction

Pseudorabies virus (PrV), a member of the *Alphaherpesvirinae*, is the causative agent of Aujeszky's disease, one of the most serious infectious diseases of pigs. Symptoms and clinical manifestation are dependent on virus strain-specific virulence and age of the animals. They range from severe affliction of the central nervous system, leading to death of the animals, to mild respiratory symptoms with elevated temperature and loss of appetite, resulting in a decrease in weight gain. Economic losses are also caused by stillbirths and abortions in breeding farms. To reduce these financial losses, vaccination of pigs with attenuated live or inactivated vaccines is widely performed. Deletion of genes encoding immunogenic nonessential glycoproteins in vaccine strains allows serological differentiation between infected and vaccinated animals. Whereas vaccination confers protection against disease, it does not prevent infection by a wild-type strain, and both vaccine virus and the superinfecting wild-type strain are able to establish latency (reviewed by Mettenleiter, 1994).

The most potent virus-neutralizing antibodies are directed against glycoproteins C and D (gC and gD) of PrV (Coe & Mengeling, 1990; Eloit *et al.*, 1988; Hampl *et al.*, 1984; Marchioli *et al.*, 1988; Mukamoto *et al.*, 1991; Wathen & Wathen, 1984), and a large part of the neutralizing activity in convalescent sera of swine appears to be directed against gC (Ben-Porat *et al.*, 1986). gC has also been reported as a target for porcine cytotoxic T-cells (Zuckermann *et al.*, 1990). Subunit vaccines consisting of gC or gD, as well as anti-idiotypic anti-gD antibodies have also been shown to elicit protective immunity (Marchioli *et al.*, 1987; Tsuda *et al.*, 1992; Tsuchida *et al.*, 1992). These data indicate that viral gC and gD represent major targets for the host's immune system.

Recently, naked DNA encoding immunogenic proteins of infectious agents has been introduced for vaccination. Injection

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of DNA results in its uptake into cells, expression of the gene and endogenous synthesis of the antigen (Hasset & Whitton, 1996; Ulmer *et al.*, 1993, 1996). The biological safety of these vaccines is high since infectious agent is not required for vaccine production and is not contained in the vaccine. In contrast to inactivated or subunit vaccines, which mainly stimulate humoral immune responses, proteins expressed from DNA constructs can enter the MHC class I- and MHC class II-restricted pathway of antigen processing. This leads to efficient induction of both humoral and cellular immune responses (Ciernik *et al.*, 1996; Manickan *et al.*, 1995; Tang *et al.*, 1992).

So far, the efficacy of DNA vaccination has mainly been tested in experimental model systems, preferentially in mice. Only a small amount of information is available about relevant natural virus–host systems. In cattle, DNA vaccination has been shown to decrease respiratory symptoms induced by bovine herpesvirus 1 (BHV-1; Cox *et al.*, 1993), and chicken have been protected against avian influenza (Fynan *et al.*, 1993; Robinson *et al.*, 1993) and Newcastle disease (Sakaguchi *et al.*, 1996) by vaccination with plasmids encoding haemagglutinin and fusion protein, respectively. Protection against lethal viral challenge of large animal species has not been reported so far. It was, therefore, of interest to analyse whether pigs can be protected from a lethal PrV challenge by immunization with DNA constructs.

Methods

■ **Viruses and cells.** PrV strains Ka (Kaplan & Vatter, 1959), NIA-3 (Baskerville, 1973), 75V19 (Andries *et al.*, 1978) and the gC-negative mutant PrV-17 (Schreurs *et al.*, 1988) were propagated on porcine kidney (PK-15) cells. Titration was performed on bovine kidney (MDBK) cells. For transfections, African green monkey kidney (Vero) cells were used. Cells were grown in Eagle's MEM supplemented with 5% foetal calf serum.

■ **Construction of plasmids.** Genes encoding gC or gD of PrV were cloned into the eukaryotic expression vector pRc/CMV (Invitrogen) using standard procedures (Sambrook *et al.*, 1989). Since the gD and gI genes of PrV are transcribed into a single bicistronic mRNA (Kost *et al.*, 1989), the complete gD/gI transcription unit was used. Resulting plasmids were designated gC-CMV and gDgI-CMV. Plasmids were transformed into *E. coli* DH5α and purified using Qiagen Maxiprep kit (Qiagen). The DNA concentration was estimated after agarose gel electrophoresis by comparison with known standards, and by spectrophotometry. For immunization, DNA was dissolved in pyrogen-free PBS pH 7·6.

■ **Transient expression of PrV glycoproteins.** To verify the identity of the expression products, Vero cells in 6 cm tissue culture plates were transfected with 15 μg of plasmid DNA by calcium phosphate coprecipitation (Graham & van der Eb, 1973). For immunofluorescence, cells were incubated at 37 °C for 48 h, fixed with 3% paraformaldehyde for 20 min, with 3% paraformaldehyde–0·1% Triton X-100 for 10 min and reacted for 30 min with glycoprotein-specific monoclonal antibodies (MAbs; B. Klupp & E. Weiland, unpublished results) followed by incubation with secondary FITC-conjugated goat anti-mouse IgG (Dianova). For radioimmunoprecipitation cells were incubated at 37 °C for 24 h and labelled overnight with 250 μCi Tran^{35}S-Label (ICN). Cell lysates were precipitated using MAbs as described (Lukács *et al.*, 1985). Precipitates were separated in SDS–10% polyacrylamide gels (Laemmli, 1970), and proteins were visualized by fluorography.

■ **Animal experiments.** Five-week-old seronegative crossbred piglets from different seronegative sows were obtained from local farms, housed in groups in isolation and fed commercial food and water *ad libitum*. They were injected intramuscularly (i.m.) or intradermally (i.d.) with a total of 1 ml of the plasmid preparation using a 21-gauge needle, or with 150 μl using a hypodermic battery-powered injector (IDAL). Blood samples were obtained from the vena jugularis externa, incubated overnight at 4 °C, centrifuged for 30 min at 3000 r.p.m., and supernatants were transferred into new tubes. Sera were inactivated for 30 min at 56 °C and stored at −20 °C.

For challenge experiments, animals were infected intranasally with 1 ml of PrV strains NIA-3 or 75V19 at the indicated titre. Clinical signs and rectal temperature were measured daily.

■ **Western blot analyses.** For Western blots either 5 μg of purified virions (Kopp & Mettenleiter, 1992) or 2 μg of gC purified on HiTrap heparin 1 ml columns (Pharmacia) were used per lane. Filters were incubated overnight with porcine serum at the indicated dilutions. Bound antibody was visualized after incubation with a peroxidase-conjugated secondary antibody by chemiluminescence (ECL; Amersham). As controls, a MAb directed against PrV gC (B. Klupp & E. Weiland, unpublished results) and a polyclonal rabbit anti-gD serum (Klupp *et al.*, 1992) were included.

■ **Neutralization assays.** Twofold dilutions of the test serum in medium were mixed with ca. 300 $TCID_{50}$ PrV in a 100 μl assay and incubated for 16 h at 37 °C. Thereafter, 100 μl of Vero cell suspension corresponding to $1–2 \times 10^4$ cells was added and the mixture was plated into 96-well microtitre plates. After 4 days at 37 °C cells were assayed for PrV-specific CPE. Titres are indicated as the $\log_2$ of the reciprocal of the highest dilution leading to complete neutralization, i.e. in which no CPE was observed.

To assay plaque reduction, inactivated sera were diluted 1:4 and mixed with approximately 500 p.f.u. of PrV in a 200 μl assay. Normal rabbit serum (5%) was added as a source of complement. After incubation for 1 h at 37 °C the volume was adjusted to 1 ml with MEM, and used for inoculation of MDBK cells in 6-well tissue culture plates. After 2–3 days incubation at 37 °C under semisolid medium, cells were fixed with formalin, stained with crystal violet, and plaques were counted. Plaque reduction was assessed compared to respective controls.

■ **Lymphoproliferation assay.** Porcine peripheral blood mononuclear cells (PBMC) were isolated by ficoll gradient centrifugation (density 1·077; Serotech) at 800 ***g*** for 30 min. The pellet was resuspended in RPMI 1640 medium (GibcoBRL) supplemented with 10% FCS, sedimented by centrifugation, and resuspended again. Cells were stored at −196 °C. For *in vitro* stimulation, 2×10^5 PBMC per well were incubated in the presence of UV-inactivated wild-type or gC-negative PrV at 1×10^6 particles per well. As a control, supernatants of homogenized noninfected cells were used. Because of the cytolytic activity of PrV, virus was inactivated for 8 min by UV irradiation at 254 nm (HNS 30W; Osram) and loss of infectivity was controlled by titration. After 4 days cells were labelled for 18 h with 1 μCi of [^{3}H]thymidine (ICN) per well, harvested with a semiautomatic cell harvester (Skatron Instruments) and incorporated radioactivity was measured by scintillation counting after addition of Rotifluorescint scintillator (Roth). The lymphocyte index of proliferation was calculated as counts per minute after stimulation with antigen divided by counts per minute after stimulation with noninfected cell homogenate.

Results

Construction of expression plasmids

The complete gC open reading frame plus additional ca. 1·4 kbp of downstream sequences were excised from a genomic 4·2 kbp *Pst*I fragment by cleavage with *Nco*I (Robbins *et al*., 1986; Schreurs *et al*., 1988) and subcloned into plasmid vector pUC 20 (Boehringer Mannheim). After cleavage with *Hin*dIII and *Xba*I, the gC fragment was cloned into the multiple cloning site of pRc/CMV, resulting in expression plasmid gC-CMV. The bicistronic expression unit encompassing the gD and gI genes, which are transcribed into a single mRNA (Kost *et al*., 1989), was excised from genomic *Bam*HI fragment 7 by *Bam*HI and *Dra*I cleavage after conversion of the *Bst*XI restriction site in front of the gD gene into a *Bam*HI site using synthetic linkers (Rauh & Mettenleiter, 1991). The fragment was subcloned into plasmid pVL1393 (PharMingen). It was then excised with *Hin*dIII and *Not*I and inserted into appropriately cleaved expression vector pRc/CMV, yielding plasmid gDgI-CMV.

To verify identity of the expression products, Vero cells were transfected with gC-CMV and gDgI-CMV and analysed by immunofluorescence and radioimmunoprecipitation. Approximately 1% of cells reacted with MAbs against gC and gD, respectively, whereas pRc/CMV-transfected cells, as expected, did not exhibit specific fluorescence. Radioimmunoprecipitation using MAbs demonstrated that expression products comigrated with authentic gC and gD from PrV-infected cell lysates (data not shown). Since gI-specific immunological reagents were not available, expression of gI from the bicistronic construct could not be verified.

Immunization of pigs

In trial experiments, both gC-CMV and gDgI-CMV have been shown to induce a corresponding antibody response when injected into mice by various routes (data not shown). To test the potential of a DNA vaccine in the natural host of PrV, two groups of four piglets each were immunized i.m. four times with 50 μg of gC-CMV or gDgI-CMV, respectively. As a control, one animal was immunized four times with parental pRc/CMV vector without an inserted gene. Reactivities in the Western blot of sera of gC-CMV-vaccinated animals taken 2 weeks after the fourth immunization are shown in Fig. 1, and reactivities of gDgI-CMV-vaccinated animals are depicted in Fig. 2. The development of neutralizing antibodies is presented in Fig. 3. We conclude that after the fourth immunization all animals showed the presence of specific antibodies, and that titres of gDgI-CMV-vaccinated pigs were approximately 1 $\log_2$ higher than those of the gC-CMV-vaccinated animals.

Nine weeks after the first immunization, i.e. at a time when all animals had seroconverted (see Figs 1–3), intranasal lethal challenge with 5×10^6 p.f.u. of the highly pathogenic PrV strain NIA-3 was performed. The control animal, all animals

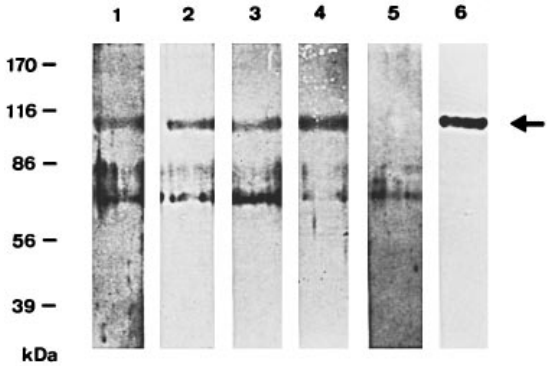

Fig. 1. Western blot of sera from gC-CMV-immunized pigs. Four piglets were immunized with 50 μg of gC-CMV four times at biweekly intervals (lanes 1–4). As a control, one animal was injected with parental vector pRc/CMV (lane 5). Sera obtained 2 weeks after the fourth immunization were analysed by Western blot on purified gC. Reactivity of an anti-gC MAb is shown in lane 6. Arrow denotes position of gC.

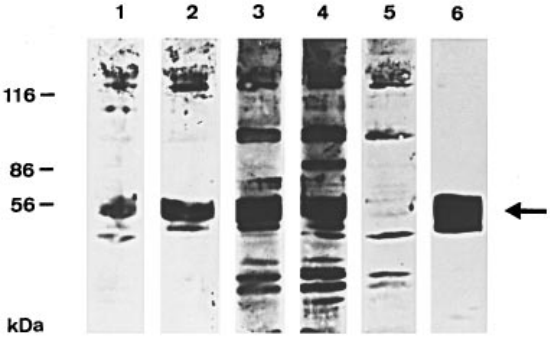

Fig. 2. Western blot of sera from gDgI-CMV-immunized pigs. Four piglets were immunized with 50 μg of gDgI-CMV four times at biweekly intervals (lanes 1–4). One pRc/CMV-vaccinated animal served as a control (lane 5). Sera were obtained 2 weeks after the fourth immunization and were analysed by Western blot on purified PrV virions. Reactivity of an anti-gD polyclonal rabbit serum is shown in lane 6. Arrow denotes position of gD.

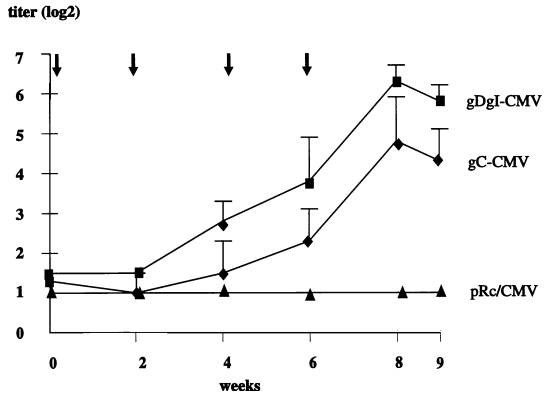

Fig. 3. Development of neutralizing antibodies in gC-CMV- and gDgI-CMV-vaccinated pigs. Neutralizing antibody titres in sera of gC-CMV- and gDgI-CMV-vaccinated pigs were determined. Vertical arrows denote times of vaccination. As a control, one animal was injected with parental vector pRc/CMV. Titres are plotted exponentially in $\log_2$ steps.

Table 1. Vaccination and challenge experiments in pigs

	Pig	Vaccinated with§	Survival‖
Expt I*	1–4	50 μg gC-CMV i.m.[a]	2/4
	5–8	50 μg gDgI-CMV i.m.[a]	0/4
	9	50 μg pRc/CMV i.m.[a]	0/1
Expt II†	1–2	1 μg gC-CMV i.d.[a]	0/2
Group I	3–4	10 μg gC-CMV i.d.[a]	0/2
	5–6	50 μg gC-CMV i.d.[a]	0/2
	7–8	1 μg gC-CMV i.m.[a]	0/2
Group II	9–10	10 μg gC-CMV i.m.[a]	1/2
	11–12	50 μg gC-CMV i.m.[a]	1/2
	13–14	1 μg gC-CMV i.d.[b]	1/2
Group III	15–16	10 μg gC-CMV i.d.[b]	1/2
	17–18	50 μg gC-CMV i.d.[b]	0/2
	19–20	50 μg pRc/CMV[a]	0/2
	21	2 ml inactivated i.m.[a]	1/1
Expt III‡	1–6	50 μg gC-CMV i.d.[b]	6/6
	7–8	50 μg gC-CMV i.m.[a]	2/2
	9	50 μg pRc/CMV i.m.[a]	0/1
	10–13	untreated	2/4

* Animals were vaccinated intramuscularly by syringe four times at biweekly intervals with the indicated plasmids. Nine weeks after the first immunization animals were intranasally challenged with 5×10^6 p.f.u. of PrV strain NIA-3.

† Animals were vaccinated three times at biweekly intervals with the indicated amounts of plasmid either i.d. by syringe (group I), i.m. by syringe (group II) or i.d. by injector (group III). One hundred days after the first immunization, animals were intranasally challenged with 10^5 p.f.u. of PrV strain NIA-3.

‡ Animals were vaccinated three times at biweekly intervals with the indicated amounts of plasmid either i.m. by syringe or i.d. by injector. Two months after the first immunization animals were intranasally challenged with 10^5 p.f.u. of PrV strain 75V19.

§ Vaccination by syringe (*a*) or injector (*b*) is indicated.

‖ Protection as assessed by number of survivors compared to total number of animals is recorded.

vaccinated with gDgI-CMV, and two of the four gC-CMV-injected pigs succumbed to the infection at days 5 and 6 post-infection (p.i.). In contrast, two of the gC-CMV-vaccinated pigs survived the challenge infection without exhibiting any central nervous system disorders (Table 1, experiment I).

Immunization with different amounts of DNA by various routes

To test the influence of different amounts of DNA and different routes of application, 18 piglets in three separate groups were vaccinated three times at 2-week intervals either i.d. using a syringe (group I), i.m. by syringe (group II) or i.d. with an injector (group III). Within each group, two animals each were injected with 1, 10 and 50 μg of gC-CMV at each vaccination. As controls, two piglets were immunized with parental vector pRc/CMV, and one animal was immunized with an inactivated commercially available vaccine at the same intervals. Four weeks after immunization all gC-CMV-vaccinated animals developed gC-specific antibodies as analysed by Western blotting, except for one piglet immunized i.d. by syringe with 1 μg of gC-CMV (data not shown). Plaque reduction assays showed a dose response relative to the amount of DNA used for immunization irrespective of the route of application (Fig. 4). However, i.d. vaccination by injector (Fig. 4, columns 13–18) appeared to induce higher titres of neutralizing antibodies compared to i.d. (Fig. 4, columns 1–6) or i.m. application by syringe (Fig. 4, columns 7–12). Preimmune serum from two randomly selected pigs did not exhibit any neutralizing activity (Fig. 4, columns A, B). None of the other preimmune sera led to any reduction in virus titres, nor did serum from the two pRc/CMV-vaccinated animals.

One hundred days after the first immunization animals were infected with 1×10^5 p.f.u. of PrV strain NIA-3 (Table 1, experiment II). Both control animals injected with pRc/CMV died (days 3 and 5 p.i.). Also, all animals vaccinated i.d. by syringe died between days 4 and 6. In contrast, one each of the animals vaccinated with 1 and 10 μg of gC-CMV i.d. by injector, as well as two animals immunized i.m. with 10 and 50 μg of gC-CMV survived. As observed before, none of the surviving animals developed any central nervous symptoms. The animal which had been immunized with the commercial inactivated vaccine survived but developed severe central nervous and respiratory disorders, high fever and growth retardation. These results confirm that vaccination with gC-CMV confers partial protection of piglets against a lethal challenge with the highly pathogenic NIA-3 strain of PrV.

Longevity of antibody response

To analyse the duration of the antibody response, three piglets were immunized three times with 50 μg of gC-CMV at biweekly intervals. Four weeks after the first immunization all animals had seroconverted. Blood was collected every second week, and the presence of gC-specific antibodies was assayed by Western blot and plaque reduction assay. Specific antibodies were detectable for up to 9 months after immunization with the experiment still in progress (data not shown).

Assessment of cellular immune response

Two animals were vaccinated three times with 50 μg of gC-CMV at biweekly intervals. Fifteen weeks after the first immunization PBMC were isolated and stimulated *in vitro* with UV-inactivated wild-type or gC-negative PrV. Since gC-negative PrV exhibits an approximately 50-fold-lower specific infectivity compared to wild-type PrV (Schmidt *et al.*, 1997), input virus was standardized according to particle number. Control wells were stimulated with UV-treated homogenates of noninfected cells. After 4 days of incubation [^{3}H]thymidine was added and its incorporation was analysed. As a positive control, PBMC from two reconvalescent pigs which had been vaccinated with attenuated PrV and subsequently survived a challenge infection were also stimulated with the same

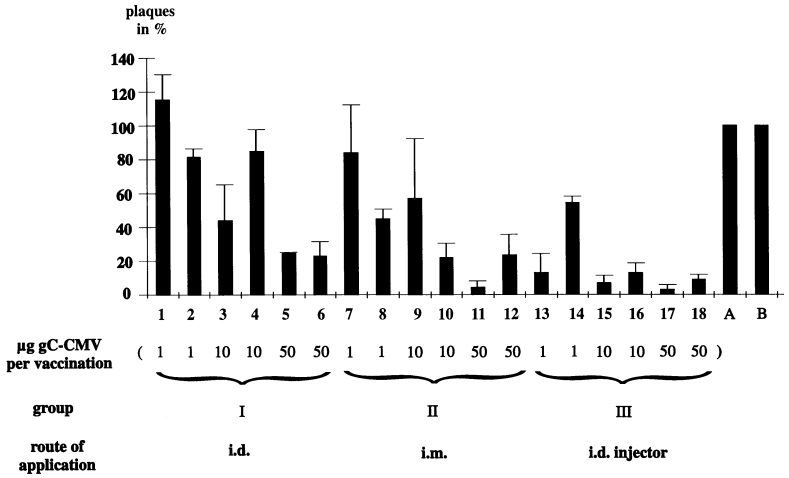

Fig. 4. Neutralizing antibodies in sera of pigs vaccinated by different routes. Sera of animals which had been vaccinated i.d. by syringe (group I, columns 1–6), i.m. by syringe (group II, columns 7–12) or i.d. by injector (group III, columns 13–18) were collected 2 weeks after the third immunization with the indicated amount of DNA. Immunizations were carried out at biweekly intervals. Columns A and B demonstrate lack of neutralizing activity in preimmune sera from two randomly selected pigs.

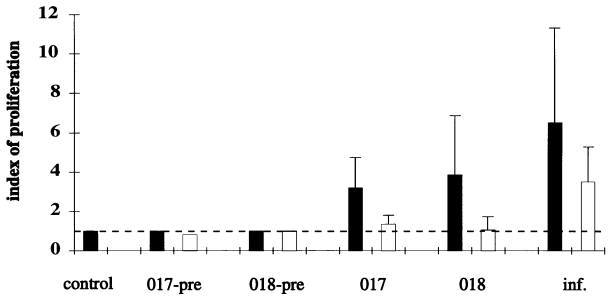

Fig. 5. Lymphoproliferation assay. PBMC from two pigs (017 and 018) which had been vaccinated three times with 50 µg of gC-CMV were isolated and stimulated *in vitro* with UV-inactivated wild-type (black columns) or gC-negative PrV (white columns). As a positive control PBMC from two pigs which had been vaccinated with an attenuated live PrV vaccine and subsequently challenged with wild-type PrV were also tested (inf.), and average stimulation is indicated. 'Control' represents values of nonspecific proliferation after stimulation with noninfected cell lysate. Results using preimmune PBMC from the two vaccinated animals are shown in columns '017-pre' and '018-pre'. Average values and standard deviations were calculated from five independent assays each.

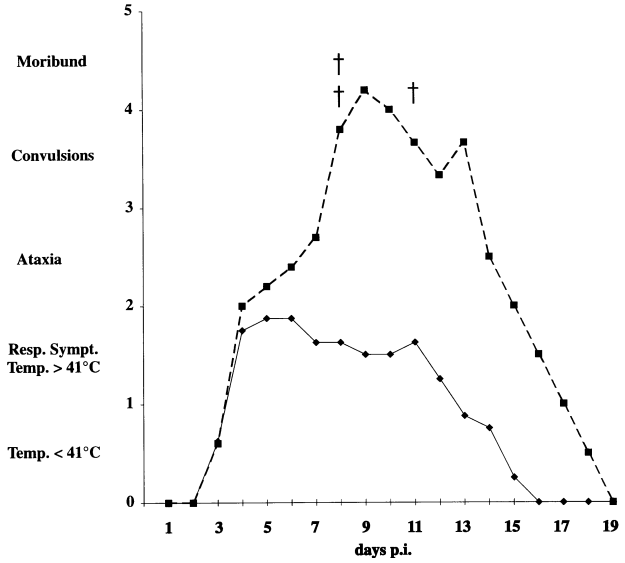

Fig. 6. Clinical score in vaccinated and nonvaccinated pigs after challenge infection with PrV-75V19. Animals vaccinated either i.d. (six animals) or i.m. (two animals) three times at biweekly intervals with 50 µg of gC-CMV (◆), and four age-matched untreated control animals and one animal injected with parental pRc/CMV (■) were challenged intranasally with 10^5 p.f.u. of PrV strain 75V19. Every animal in each group was observed for symptoms of Aujeszky's disease twice a day and assigned a clinical score according to the stages indicated on the *y*-axis, correlating to five levels of severity. Mean values are depicted. Three of the control animals died between days 6 and 8 p.i. as indicated.

antigens. Compared to the preimmune sera (Fig. 5, 017-pre and 018-pre), PBMC from the two gC-CMV-immunized animals (Fig. 5, 017 and 018) were stimulated three- to sixfold by wild-type PrV, but not by gC-negative PrV. In contrast, PBMC from two reconvalescent animals were stimulated eight- to tenfold by wild-type PrV and also responded with a three- to fivefold stimulation to gC-negative PrV (Fig. 5, inf.). Uninfected cell homogenate did not result in increased incorporation of [^{3}H]thymidine (Fig. 5, control). These results show that immunization with gC-CMV induced cellular immune reponses.

Immunization with gC-CMV and challenge with PrV-75V19

For routine vaccine testing PrV strain 75V19 (Andries *et al.*, 1978), which is slightly less virulent than NIA-3, is widely used. Therefore, we analysed the efficacy of gC-CMV immunization in protection against PrV-75V19 challenge. To this end, six animals were vaccinated three times at biweekly intervals i.d. by injector, and two animals were injected i.m. by syringe with 50 µg of gC-CMV. One animal was i.d. immunized with parental pRc/CMV. Two months after the first immunization animals were intranasally challenged with 10^5 p.f.u. of PrV-75V19 (Table 1, experiment III). As additional controls, four untreated age-matched animals were included in the experiment. Three of the five control animals succumbed to the disease at days 6 and 8 p.i. The surviving two control animals exhibited severe central nervous symptoms, but finally recovered. In contrast, all vaccinated animals survived the infection, and none of them developed central nervous

symptoms. A cumulative index of clinical manifestation is shown in Fig. 6. Severity of clinical symptoms was assessed as (1) elevated temperature of above 40 and below 41 °C; (2) high fever above 41 °C correlated with respiratory distress; (3) ataxia; (4) convulsions; and (5) moribund state/death. These results show that triple immunization with 50 μg of gC-CMV results in full protection of pigs against lethal infection with PrV-75V19 and, for the first time, demonstrate the usefulness of DNA immunization in protecting an economically important large animal species against a lethal virus disease.

Discussion

Immunization with naked DNA is being regarded as holding great promise for improvement of existing vaccines. In a wide range of model systems of infectious disease, mainly in mice, the efficacy of DNA immunization in protecting animals to a varying degree from clinical manifestation of disease has been observed. However, practical application of this new technique requires studies in relevant natural infectious agent–host systems, but only rather limited information is available in this respect.

We show here that immunization with DNA constructs encoding envelope glycoproteins of the alphaherpesvirus PrV induces a humoral immune response in pigs, the natural host of the virus. We also demonstrate that animals vaccinated with a gC expression plasmid were protected from lethal challenge with two virulent PrV strains.

Natural virus–host systems in which DNA vaccination has been shown to induce some level of protection include avian influenza virus (Fynan *et al.*, 1993; Robinson *et al.*, 1993) and Newcastle disease virus (NDV; Sakaguchi, *et al.*, 1996) infection of chicken, murine cytomegalovirus (MCMV; Gonzales *et al.*, 1996) and lymphocytic choriomeningitis virus (LCMV; Pedroza *et al.*, 1995; Yokohama *et al.*, 1995) infection of mice, and BHV-1 infection of cattle (Cox *et al.*, 1993). Whereas in the latter system immunization with constructs expressing envelope glycoprotein D was shown to induce an antibody response and to partially protect bovines from respiratory distress, chickens were partially protected from lethal challenge by influenza and Newcastle disease viruses by immunization with genes encoding the envelope glycoproteins haemagglutinin and fusion protein, respectively. In contrast, only low levels of protection were observed after immunization of mice with plasmids expressing the major immediate early protein pp89 of MCMV, and protection against persistent LCMV infection was considered incomplete (Pedroza *et al.*, 1995; Yokohama *et al.*, 1995). Compared to these studies, our experiments yielded a comparable or even better level of protection.

It is interesting to note that in our system partial protection could be achieved with as little as 1–10 μg of plasmid DNA per dose, and that increasing the dose to 50 μg per injection resulted in seroconversion of 100% of the animals. In contrast, in the BHV-1 study 500 μg of plasmid was used per dose (Cox *et al.*, 1993), chickens were injected with 100 μg of NDV fusion protein-expressing plasmid (Sakaguchi *et al.*, 1996) or 100–200 μg of influenza haemagglutinin construct (Fynan *et al.*, 1993; Robinson *et al.*, 1993), and mice received 50 μg of pp89 plasmid (Gonzales *et al.*, 1996) or 100–200 μg of LCMV nucleoprotein expression vector (Pedroza *et al.*, 1995; Yokohama *et al.*, 1995). Thus, the amount of DNA administered in our study compares favourably to those used in other natural virus–host systems, especially when taking into account the size of the target animal, and holds promise for a practical application of DNA vaccination in protecting livestock from infectious diseases.

Studies in large animal species are hampered by the high costs of acquiring and keeping the animals. Therefore, the number of animals used in our experiments, though relatively low when compared to the possibilities if small animal models were used, approached the upper limit of our capacity. Despite the difficulty for statistical evaluation, our combined data clearly show the potency of DNA vaccination in preventing symptoms of Aujeszky's disease after challenge infection. When evaluating the data it has to be taken into account that the challenge PrV strain NIA-3 is one of the most highly virulent strains described, and that in our experience only animals vaccinated with live attenuated viruses are able to resist a challenge infection under our conditions without major clinical signs. It is interesting to note, in this context, that one animal included in our studies which had been vaccinated three times with a commercially available inactivated vaccine (see Table 1) exhibited severe central nervous symptoms after challenge infection, though it eventually recovered. This indicates that DNA vaccination provides a level of protection which equals or even surpasses that of an inactivated vaccine. Since one of the major objections against a more widespread use of live attenuated vaccines relates to questions of biological safety, this can possibly be overcome by using DNA vaccines.

DNA immunization is thought to induce both humoral and cellular immunity, providing access of endogenously synthesized antigens to the MHC class I- and class II-restricted pathways (Ciernik *et al.*, 1996; Justewicz *et al.*, 1995; Raz *et al.*, 1994). Proliferation assays of PBMC from gC-CMV-immunized animals showed a specific stimulation by UV-irradiated wild-type PrV, but not by gC-negative PrV. This indicates that the response was indeed gC-specific, as has to be expected after immunization with the gC expression construct. We realize that our tests did not uncover which population(s) of PBMC were actually stimulated to proliferate. Nevertheless, the demonstration of specific proliferation shows that cellular immune responses were indeed elicited by our DNA construct.

Glycoproteins C and D have been shown to represent major targets for the immune response of pigs against a PrV infection (reviewed by Mettenleiter, 1996). Especially, gD has repeatedly been tested for efficacy in subunit or vector-expressed form and shown to provide significant protection

against the clinical manifestation of Aujeszky's disease (Eloit *et al.*, 1988; Marchioli *et al.*, 1987). MAbs against gD are most potent in neutralizing virus infectivity in the presence and absence of complement (Coe & Mengeling, 1990; Marchioli *et al.*, 1988; Wathen & Wathen, 1984). Whereas anti-gC MAbs have also been shown to exhibit virus-neutralizing activity (Coe & Mengeling, 1990; Hampl *et al.*, 1984), and a major portion of the neutralizing activity in sera from reconvalescent pigs appears to be directed against gC (Ben-Porat *et al.*, 1986), gC also represents a major antigen for porcine cytotoxic T-cells (Zuckermann *et al.*, 1990). This might explain the different outcome of the challenge experiments of animals vaccinated with gC or gDgI constructs. Both groups mounted a neutralizing antibody response with higher titres in the gDgI-immunized animals. However, only the gC-CMV-immunized pigs showed a significant level of protection. Lack of protection after immunization of pigs with gD constructs despite the induction of neutralizing antibodies has also been observed by others (Monteil *et al.*, 1996). It has been demonstrated that cytotoxic T-cells are important in limiting herpesvirus infection in mice (reviewed by Martin *et al.*, 1988; Reddehase, 1989). Thus, respective T-cell responses against gC in pigs might have been responsible for the level of protection observed after immunization with the gC construct.

In our experiments seroconversion was observed irrespective of the route of application of expression plasmid gC-CMV. However, i.d. application by injector appeared to be most efficient. The latter finding, i.e. that immunization of pigs can be performed with aqueous solutions of DNA in PBS using a battery-powered intradermal application device, is of particular relevance. This form of application can be used for vaccinating large numbers of animals with little effort, which is important for any practical use of DNA vaccination in livestock.

A particular advantage of DNA immunization appears to be the possibility to mix cocktails of different expression plasmids to simultaneously vaccinate against a number of infectious diseases. So far, we have only tried immunization with single plasmids encoding either gC or gDgI. However, we are currently constructing expression plasmids for all of the known PrV glycoproteins. It is conceivable that by mixing these different plasmids we will be able to enhance the animal's immune response. In addition, work is in progress to clone genes for protective antigens of other porcine viruses into expression plasmids and try to use them in combinations with the PrV plasmids.

In summary, we have demonstrated the protective efficacy of DNA vaccination of large animals against lethal viral challenge. The low amounts of DNA necessary and the ease of application hold great promise for introducing DNA vaccination into disease prevention in livestock.

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