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# Vaccination of pigs with a DNA construct expressing an influenza virus M2–nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus

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**In mice, vaccines inducing antibodies to the extracellular domain of the M2 protein (M2e) can confer protection to influenza A virus infection. Unlike the surface glycoproteins, haemagglutinin and neuraminidase, this domain of M2 is highly conserved and is therefore a potential broad-spectrum immunogen. In this study, the protection conferred by vaccines inducing antibodies to M2e was evaluated in a challenge model for swine influenza in pigs. A protein resulting from the fusion between M2e and the hepatitis B virus core protein (M2eHBc), with or without adjuvant, was evaluated. In addition, a DNA construct expressing a fusion protein between M2e and influenza virus nucleoprotein (M2eNP) was evaluated to see if the broad-spectrum protection conferred by antibodies could be further enhanced by T helper cells and cytotoxic T cells. All vaccines induced an antibody response against M2e, and the M2eNP DNA vaccine additionally induced an influenza virus-specific lymphoproliferation response. However, after challenge with a swine influenza virus (H1N1), no protection was observed in the vaccinated groups compared with the non-vaccinated control group. On the contrary, vaccinated pigs showed more severe clinical signs than the control pigs. The M2eNP DNA-vaccinated pigs showed the most severe clinical signs and three out of six pigs died on days 1 and 2 post-challenge. These results indicate that antibodies to M2e, especially in combination with cell-mediated immune responses, exacerbate disease. Thus, clinical signs after infection should be observed closely in further studies using M2e as an immunogen and caution should be exercised in using M2e in humans.**

## Introduction

Influenza A virus expresses two highly immunogenic, but variable, transmembrane proteins: haemagglutinin (HA) and neuraminidase (NA). In humans, new epidemic strains arise every 1–2 years as a result of selected point mutations in these two surface glycoproteins, a phenomenon known as antigenic drift. Sometimes, an exchange of the HA and/or NA gene segment with an animal influenza virus occurs, a phenomenon known as antigenic shift, and this may result in a flu pandemic.

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Current human influenza vaccines are updated yearly to induce immune responses specific for the prevalent strains. These vaccines are efficacious when the HA of the vaccine strain matches that of the circulating virus, but not when it differs as a result of antigenic drift or shift.

Another transmembrane protein is the M2 protein. This protein is relatively invariant from strain to strain, and a number of studies have shown that it is a potential broad-spectrum immunogen. Vaccination with the M2 protein in the absence of other influenza virus proteins reduced virus infection (Frace *et al.*, 1999; Slepushkin *et al.*, 1995), and therapeutic treatment with a mAb specific for the extracellular domain of M2 (M2e) was shown to reduce pulmonary virus titres by 100- to 1000-fold in mice (Mozdzanowska *et al.*, 1999; Treanor *et al.*, 1990). In a previous study in pigs, we observed that the antibody response to M2e, which was low after a primary influenza virus H3N2 infection, was clearly boosted by a subsequent infection with H1N1, indicating that

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antibodies to M2e may have a role in heterosubtypic immunity (Heinen *et al.*, 2001). In contrast to the HA protein, the M2 protein is not accessible to antibody and therefore antibodies to M2e are not virus-neutralizing. However, they can bind to the M2e on infected host cells and reduce virus replication by interfering with virus budding (Zebedee & Lamb, 1989) and by mediating the killing of infected lung epithelial cells by complement or by cells of the innate immune system (antibody-dependent cytotoxicity) (Mozdzanowska *et al.*, 1999). In addition, antibodies that bind before budding of the virions could hinder them from infecting cells or could enhance the uptake by phagocytic cells via the Fc receptor.

Because the M2 protein itself seems to be a weak immunogen and only antibodies to the extracellular domain of the protein can be expected to protect, Neirynck *et al.* (1999) combined this highly conserved domain with the hepatitis B virus core protein (Neirynck *et al.*, 1999). The resulting fusion protein (M2eHBc) facilitated the presentation of M2e and induced partial immunity in mice. In the present study, we investigated the protection conferred by this fusion protein, with or without adjuvant, in a challenge model for swine influenza in pigs. In addition, we evaluated whether the broad-spectrum protection conferred by antibodies could be further enhanced by inducing T helper and cytotoxic T lymphocytes (CTLs). T helper cells can mediate recovery from influenza virus infection by the promotion of an antiviral antibody response (Mozdzanowska *et al.*, 1997; Topham & Doherty, 1998) and CTL response (Riberdy *et al.*, 2000). CTLs have been proposed to be the major mediator of heterosubtypic immunity and many studies have indeed shown that CTLs contribute to protection in mice (Epstein *et al.*, 2000; Mozdzanowska *et al.*, 2000; Topham & Doherty, 1998; Ulmer *et al.*, 1998). Moreover, a previous study we performed indicated that CTLs also have a role in heterosubtypic immunity in pigs (Heinen *et al.*, 2001). After infection, a large proportion of these CTLs recognize conserved epitopes of the nucleoprotein (NP). In mice, more than 15% of CTLs in the lungs after primary and more than 65% after secondary heterosubtypic influenza infection were shown to be specific for the immunodominant $NP_{366–374}$ peptide (Doherty & Christensen, 2000, Flynn *et al.*, 1998, 1999). Therefore, we made a DNA construct expressing a fusion protein between M2e and influenza virus NP (M2eNP). It was expected that the expression of M2eNP by DNA vaccination would induce an antibody response to the conserved M2e, as well as a T helper and CTL response to the conserved NP, and would confer a better protection than the M2eHBc fusion protein.

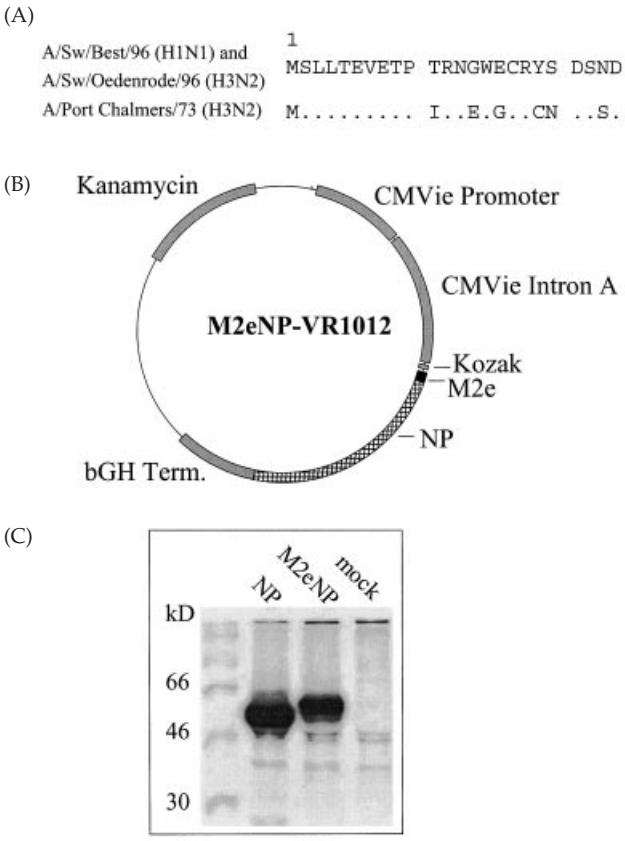


Fig. 1. (A) Alignment of the amino acid sequences of the extracellular domain of the M2 protein (M2e) from European swine and human influenza viruses. (B) Schematic representation of the expression vector M2eNP–VR1012. The expression vector was constructed from pVR1012, a mammalian expression vector containing the HCMV immediate-early transcriptional enhancer, promoter and intron A regulatory elements and the poly(A) signal and transcription termination sequences of the bovine growth hormone in a pUC18 backbone. The gene fragments encoding M2e and the NP of influenza virus A/Sw/Oedenrode/96 were fused and cloned into the multiple cloning site of the vector. (C) Analysis of the M2eNP fusion protein. Influenza virus-infected minipig kidney cells and M2eNP–VR1012-transfected or non-transfected SK-6 cells were incubated with $^{35}S$-labelled cysteine and methionine. Proteins from the cell lysates were immunoprecipitated with an influenza virus NP-specific mAb and analysed by SDS–PAGE. The M2eNP fusion protein is larger than the wild-type NP (59 and 56 kDa, respectively).

## Methods

■ **Propagation of influenza virus.** The influenza virus strains A/Sw/Best/96 (H1N1) and A/Sw/Oedenrode/96 (H3N2) were isolated from pneumonic lung tissue of pigs from outbreaks of influenza during a recent field survey (Loeffen *et al.*, 1999). Except for the HA and NA, all proteins of these two viruses are very similar because in 1984 a genetic reassortment between the two subtypes occurred in pigs, in which the H3N2 virus acquired all other proteins from the H1N1 virus (Castrucci *et al.*, 1993). The viruses were isolated in primary cultures of porcine thyroid cells, then passaged three times in these cells and then passaged twice in Madin–Darby canine kidney (MDCK) cells (ECACC No. 84 121 903). Virus stocks were produced and stored at −70 °C until used as inoculum or as antigen in haemagglutination inhibition (HI) assays, ELISAs and T cell proliferation assays.

■ **M2e–hepatitis B virus core fusion protein (M2eHBc).** Purified M2eHBc (Neirynck *et al.*, 1999) was kindly provided by Sabine Neirynck (Flanders Interuniversity Institute for Biotechnology). The amino acid sequence of the M2e is derived from a human influenza virus strain and is present in almost all presently known human influenza A virus field strains. This sequence differs from the M2e of European swine influenza viruses in six out of the 23 amino acids that form the M2e (Fig. 1a).

■ **Construction, preparation and analysis of the M2eNP DNA construct.** The two regions of gene segment 7 encoding the complete M2 protein were amplified by RT–PCR from RNA of influenza virus strains A/Sw/Oedenrode/96 (H3N2) and A/Sw/Best/96 (H1N1). Primers used were Uni 12 (5′ AGCAAAAGCAGG 3′) and V7 166R (5′ TAGCCACTCCATGAGAGCC 3′), and V7 675 (5′ ATGAGAACAGT-TGGGACTC 3′) and V7 1007R (5′ TTACTCCAGCTCTATGTTGAC 3′). The region of gene segment 5 encoding the complete NP of influenza virus A/Sw/Oedenrode/96 (H3N2) was amplified using the primers Uni12 and V5 1546R (5′ GAAACAAGGGTATTTTTCT 3′). The NP was cloned into *Escherichia coli* using a pGEM-T cloning kit (Promega). The nucleotide sequences of M2 and NP were determined using the PRISM Ready Dye T Deoxy Terminator cycle sequencing kit and the ABI PRISM 310 Genetic Analyser (Perkin-Elmer). These sequences were sent to GenBank and the accession numbers are AF385294 and AF385295 for the nucleotide and amino acid sequence of the M2 protein of A/Sw/Best/96 (H1N1), respectively, and AF385296 and AF385297 for the M2 protein and AF385293 for the NP of A/Sw/Oedenrode/96 (H3N2).

The first 24 N-terminal amino acid residues (MSLLTEVETPTRNGW-ECRYSDSND) that form M2e were identical for the swine influenza H1N1 and H3N2 virus strains and differed in six residues from the universal human sequence (Fig. 1A). A 102 residue oligonucleotide was synthesized, which comprised the complete sequence encoding swine M2e downstream of a Kozak motif for initiation of translation in vertebrates (Kozak, 1987). A 106 residue antisense strand was also synthesized and the two oligonucleotides were annealed together by heating for 2 min at 95 °C and subsequent cooling, thus resulting in a 102 bp fragment with a sticky end. For directional cloning, a *Bcl*I restriction site was added at the 5′ end of the fragment. The sticky 3′ end could be directly ligated on to the DNA fragment encoding the NP after restriction of this fragment with *Sml*I. The fragment resulting from this fusion was cloned into the *Xba*I and *Bcl*I cloning sites of the eukaryotic expression vector pVR1012 (Vical), downstream of the human cytomegalovirus (HCMV) promoter (Fig. 1B). The pVR1012 plasmid lacking the M2eNP DNA insert served as a control. Plasmids were propagated in the HB101 strain of *E. coli* and purified using Qiagen columns.

Transient expression of the M2eNP fusion protein was confirmed in confluent monolayers of SK-6 cells (Kasza *et al.*, 1972), using mAbs against the NP (ATCC No. HB 65, H16-L10-4R5) and M2e (14C2-S1-4), kindly provided by Walter Gerhard (The Wistar Institute). The correct size of the fusion protein was confirmed by radio-immunoprecipitation (RIP), using the mAb against the NP and subsequent SDS–PAGE (Fig. 1C).

■ **Pigs, immunization and challenge.** Twenty-four Dutch Landrace pigs were obtained from the SPF herd of the Institute for Animal Science and Health. They were assigned to four groups of six pigs and each group was housed in a separate room. Starting at the age of 10 weeks, all pigs were inoculated three times at 3 week intervals with 2 ml inoculum. Pigs in the M2eHBc group were immunized intramuscularly with 50 μg protein in saline. Pigs in the M2eHBc plus adjuvant group were immunized intramuscularly with 50 μg protein in adjuvant (CoVaccine). Pigs in the M2eNP DNA group were injected intradermally, behind the ear, with 200 μg of M2eNP-encoding plasmid. Pigs in the control group were injected with 200 μg of empty plasmid.

At post-inoculation day (PID) 70, 4 weeks after the third vaccination, all pigs were challenged with the field isolate A/Sw/Best/96 (H1N1). Pigs were challenged with an aerosol produced by nebulization of 2 ml culture supernatant containing $10^8$ $TCID_{50}$ virus, using an air brush device (Badger, No. 100LG, Franklin, IL, USA). The institute's ethics committee for experiments in animals approved the experiment.

■ **Clinical observations and sampling.** Clinical signs and body temperature of all pigs were monitored and oropharyngeal fluid was collected daily for 8 days following challenge inoculation (PID 70–78). The following clinical signs were scored: laboured breathing, abdominal breathing, anorexia, apathy and coughing. Blood was collected at PID 0, 3, 7, 10, 17, 24, 31, 38, 45, 52, 66, 73, 77, 80 and 87. Serum was collected to determine the titres of HI antibodies in the HI assay and of IgG antibodies specific for M2e and NP in ELISAs. Heparinized blood was collected for the isolation of peripheral blood mononuclear cells (PBMCs) to be used in a T cell proliferation assay. Bronchioalveolar lavage fluid (BALF) and nasal swabs (NS) were collected at PID 66, 72, 74, 78 and 88. To collect NS and BALF, animals were anaesthetized by injection (ketamine, midazolam, medetomidine). NS was collected to determine antibody titres in the anti-NP IgA ELISA, and BALF was used to monitor the changes in phenotypes of BALF cells by flow cytometry.

■ **Virus isolation.** Ten-fold serial dilutions, starting at a dilution of 1:10, of oropharyngeal fluid were prepared in cell culture infection medium (McCoy's medium without serum, supplemented with 5 μg/ml trypsin). Dilutions were inoculated on MDCK cells in microtitre plates, which were incubated at 37 °C and examined for a cytopathic effect after 4 days. Of the samples that were negative in the microtitre assay, 1 ml was tested for the presence of virus by inoculating a monolayer of MDCK cells in 25 ml tissue culture flasks. Virus titres were calculated by the Spearman–Kärber method.

■ **Haemagglutination inhibition (HI) assay.** The HI assay was performed essentially as described previously (Kendal *et al.*, 1982), using 0·5% chicken erythrocytes for haemagglutination and four haemagglutinating units of A/Sw/Best/96 (H1N1).

■ **ELISA for IgG specific for M2e and for IgG and IgA specific for NP.** The ELISA to detect IgG specific for M2e was recently described (Heinen *et al.*, 2001). A synthetic peptide with the amino acid sequence MSLLTEVETPTRNGWECRY of the M2e of the swine influenza viruses was made and a conjugate of this peptide with keyhole limpet haemocyanin was used to coat 96-well ELISA plates (Costar EIA/RIA, cat. no. 3590, Costar, Cambridge, UK). Plates were then blocked with 1% BSA in PBS, incubated with twofold serial dilutions of serum samples starting at a dilution of 1:20, incubated with a mAb against swine IgG1 (23.49.1) conjugated to HRP and then incubated at room temperature with chromogen/substrate solution. The absorbance at 450 nm was read with an ELISA reader (Spectra Reader, SLT Labinstruments) and antibody titres were expressed as the reciprocal of the sample dilution still giving an optical density (OD) value of 1·0.

The ELISAs to measure influenza virus NP-specific IgG and IgA antibodies in pigs were as described previously (Heinen *et al.*, 2001). Absorbance and antibody titres were determined as described for the anti-M2e IgG ELISA.

■ **Flow cytometric analysis of BALF cells.** The technique that was used to obtain BALF has been previously described (van Leengoed & Kamp, 1989). Approximately 30 ml of PBS was added to the BALF to give a total volume of 50 ml. The BALF cells were collected by centrifugation at 300 ***g*** for 10 min at 4 °C and washed once with 50 ml PBS. Cells were then suspended in 1 ml PBS containing 2% heat-inactivated bovine serum and 0·01% sodium azide (FACS buffer) and the total number of recovered cells was determined. Cells were spun down in 96-well U-bottomed microtitre plates by centrifugation at 230 ***g*** for 3 min. The supernatant was discarded and the cells were incubated for 30 min on ice with various combinations of mAbs to leukocyte differentiation antigens. The mAbs used to differentiate myeloid cells

were directed against the following cell markers: SWC3 (clone 74-22-15, IgG1) and CD163 (clone CVI 517.2, IgG2b). The mAbs used to differentiate lymphoid cells were directed against: CD4 (clone 74-12-4, IgG2b), CD5 (clone b53b7, IgG1), CD6 (clone a38b2, IgG1) and CD8 (clone 295/33, IgG2a). These mAbs have been previously used to analyse changes in the phenotype of leukocytes in the BALF of pigs infected with influenza virus (Heinen *et al.*, 2001). After incubation, the cells were washed three times with FACS buffer and then incubated for 30 min on ice with the appropriate fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated goat anti-mouse IgG isotype-specific antibodies, diluted in FACS buffer. Subsequently, the cells were washed three times, resuspended in FACS buffer and transferred to tubes. Fluorescence was measured using a FACScan flow cytometer (Becton Dickinson).

■ **T cell proliferation assay.** The T cell proliferation assay to measure influenza virus-specific T cell responses of pigs was performed essentially as described for pseudorabies virus (Kimman *et al.*, 1995). Briefly, PBMCs were isolated from heparinized blood samples by centrifugation on to Lymphoprep (Nycomed Pharma A) and were washed twice with PBS. The isolated PBMCs were seeded in 96-well flat-bottomed plates (M29, Greiner) at a density of $5 \times 10^5$ cells per well in 100 µl medium (RPMI 1640 containing 10% porcine serum, 2 mM L-glutamine, 50 µM $\beta$-mercaptoethanol, 200 U/ml penicillin, 200 µg/ml streptomycin and 100 U/ml mycostatin). To the PBMCs, 100 µl medium containing $10^5$ $TCID_{50}$ influenza virus A/Sw/Best/96 (H1N1), 2 µg/ml purified M2eHBc fusion protein, a control sample prepared from non-infected cells (mock control) or 5 µg/ml Con A (vitality control) were added in quadruplicate. After incubation for 4 days at 37 °C in a 5% $CO_2$ atmosphere, the cultures were pulsed with 0·4 µCi [$^3$H]thymidine (Amersham). After incubation for 4 h, cells were harvested and the incorporated radioactivity was measured in a Betaplate scintillation counter (Wallac, EG&G Instruments). Proliferation was expressed as the number of counts (mean of quadruplicate) of antigen-stimulated PBMCs minus the number of counts of the mock-control-stimulated PBMCs (delta counts).

■ **Statistics.** Comparisons of mean clinical score, temperatures, virus excretion and changes in the phenotype of cells in the BALF between immunized groups and control groups and comparison of T cell proliferation when PBMCs were stimulated with ConA before and after challenge were evaluated by two-sided Student's *t*-tests. Probability ($P$) values $< 0{\cdot}05$ were considered significant.

## Results

### Clinical signs and virus excretion

Unexpectedly, clinical signs after challenge were more severe in all immunized groups compared with the control group. In the M2eNP DNA group, clinical signs were extremely severe and one pig died on post-challenge day (PCD) 1 and two more died on PCD 2, during the lung lavage. The clinical signs of laboured breathing, abdominal breathing, anorexia, apathy and coughing were all given a score of 0 if absent and 1 if present. The mean accumulated clinical scores of all groups are presented in Fig. 2(A). The highest clinical score was found in the M2eNP DNA group, in which three pigs died.

In all groups, pigs developed fever ($\geqslant$ 40 °C) for at least 1 day between PCD 0 and 6. The mean temperatures in the different groups did not correlate with the clinical signs. On the contrary, pigs in the control group showed the highest mean temperatures, whereas in the M2eNP DNA group, mean temperatures were the lowest (Fig. 2B).

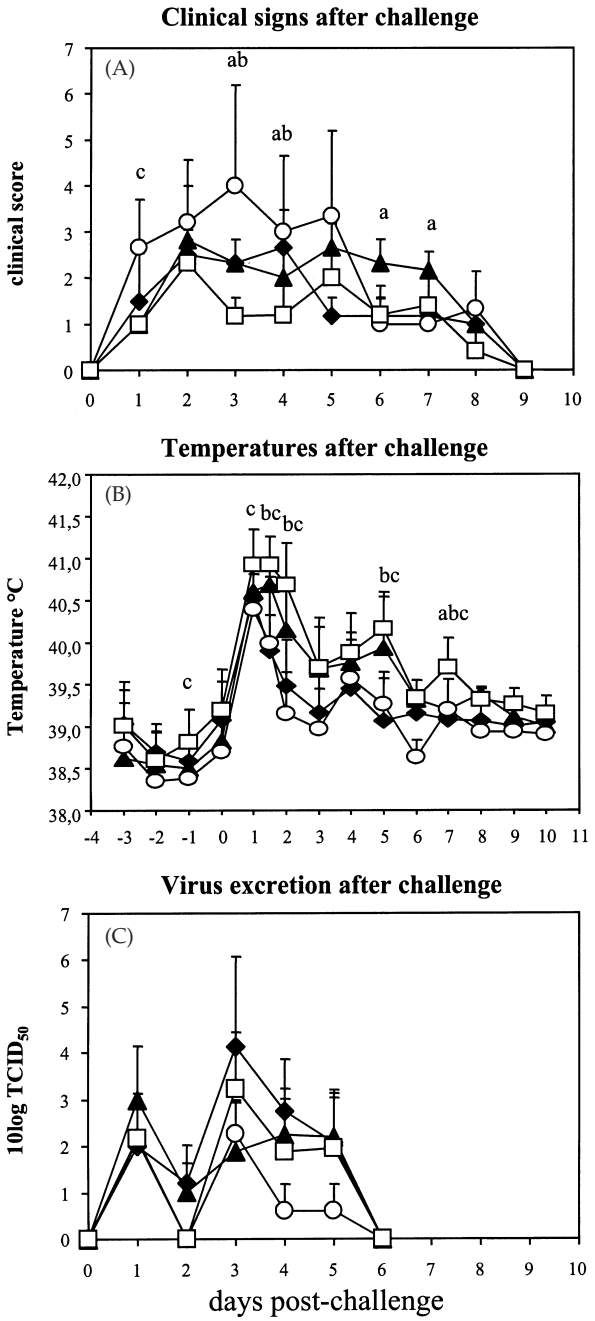


Fig. 2. Mean clinical score (A), temperatures (B) and virus excretion (C) of pigs immunized with M2eHBc fusion protein (▲), M2eHBc fusion protein + adjuvant (◆), M2eNP DNA (○) and of control pigs (□), after challenge infection. Pigs were immunized three times at 3 week intervals and challenged 4 weeks after the third immunization with influenza virus A/Sw/Best/96 (H1N1). In the M2eNP DNA-immunized group, three pigs died on PCD 1 and 2. Results are presented as the mean ± SD ($n = 6$). The means in each immunized group were compared with the mean in the control group. Significant differences ($P < 0{\cdot}05$) of the mean in the M2eHBc fusion protein (a), M2eHBc fusion protein + adjuvant (b) and M2eNP DNA (c) immunized groups are indicated.

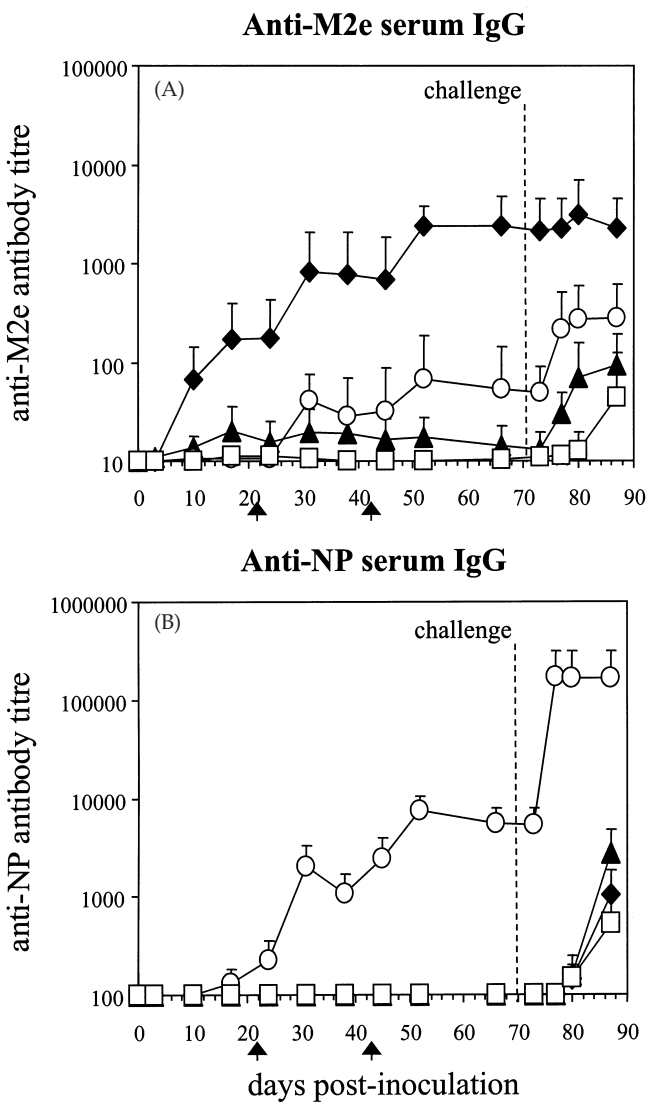


Fig. 3. Mean serum IgG antibody response to M2e and the influenza virus NP of pigs immunized with M2eHBc fusion protein (▲), M2eHBc fusion protein + adjuvant (◆), M2eNP DNA (○) or control DNA (□). Pigs were immunized (arrows) three times at 3 week intervals and challenged 4 weeks after the third immunization with influenza virus A/Sw/Best/96 (H1N1). In the M2eNP DNA-immunized group, three pigs died on PCD 1 and 2. Results are presented as the mean ± SD ($n = 6$).

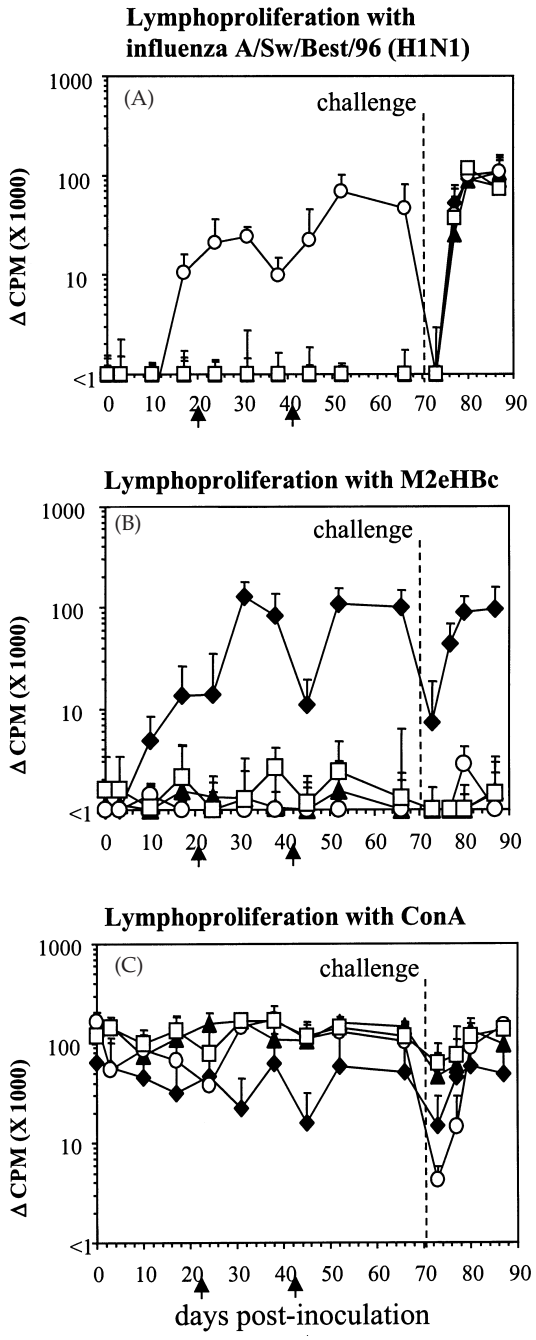


Fig. 4. Mean lymphocyte proliferation responses of PBMCs of pigs when stimulated with influenza virus A/Sw/Best/96 (H1N1), M2eHBc protein or ConA. Pigs were immunized with M2eHBc fusion protein (▲), M2eHBc fusion protein + adjuvant (◆), M2eNP DNA (○) or control DNA (□). Pigs were immunized (arrows) three times at 3 week intervals and challenged 4 weeks after the third immunization with influenza virus A/Sw/Best/96 (H1N1). In the M2eNP DNA-immunized group, three pigs died on PCD 1 and 2. Results are presented as the mean ± SD ($n = 6$).

No significant reduction in virus excretion was observed in the immunized groups compared with the control group (Fig. 2C). In all groups, pigs excreted virus from PCD 1–5.

### M2e-specific serum IgG, NP-specific serum IgG and nasal IgA and HI antibody responses

In all immunized groups, an antibody response against M2e was detected by ELISA after vaccination. The response was highest in the M2eHBc protein plus adjuvant group, intermediate in the M2eNP DNA group and low in the M2eHBc without adjuvant group (Fig. 3A).

After challenge, an increase in the antibody titre to M2e was observed, which was fastest in the M2eNP DNA group, probably as a result of the promotion of the antibody response by T helper cells in this group.

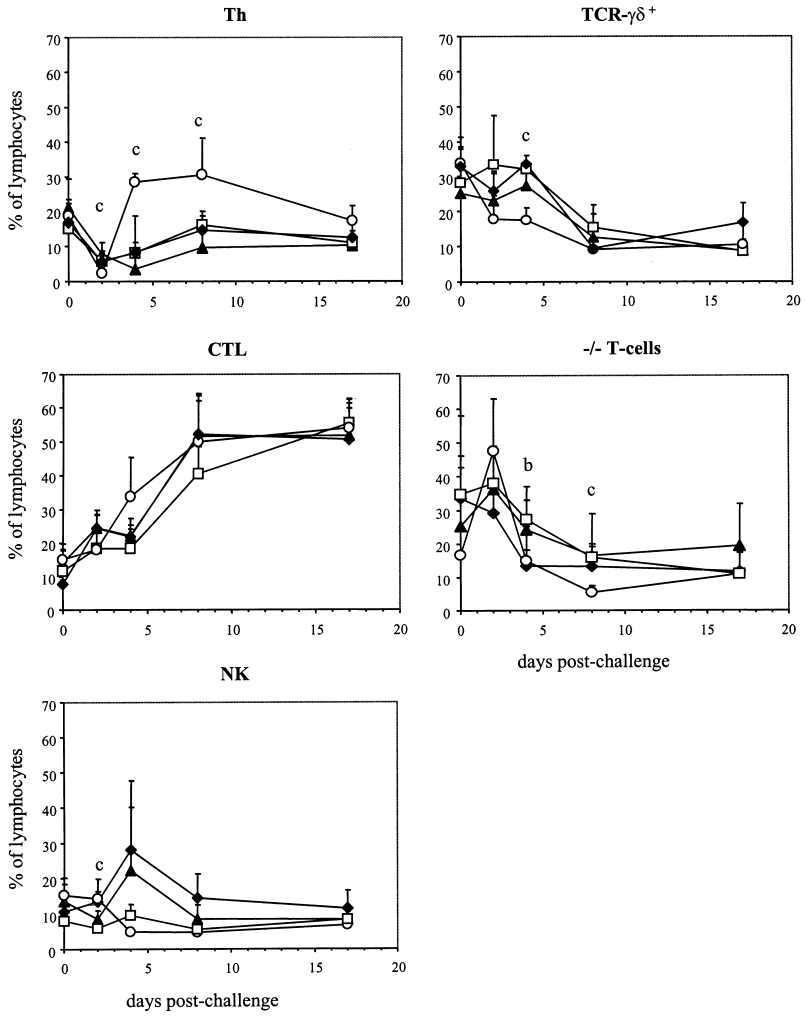


Fig. 5. Changes in the lymphocyte population in the lungs after challenge of pigs immunized with M2eHBc fusion protein (▲), M2eHBc fusion protein + adjuvant (◆), M2eNP DNA (○) or control DNA (□). Pigs were immunized three times at 3 week intervals and challenged 4 weeks after the third immunization with influenza virus A/Sw/Best/96 (H1N1). In the M2eNP DNA-immunized group, three pigs died on PCD 1 and 2. Results are presented as the mean ± SD ($n = 6$). The means in each immunized group were compared with the mean in the control group. Significant differences ($P < 0{\cdot}05$) of the mean in the M2eHBc fusion protein (a), M2eHBc fusion protein + adjuvant (b) and M2eNP DNA (c) immunized groups are indicated.

Also, an antibody response against NP was detected in the M2eNP DNA group. The titre against the NP did not correlate with the titre against M2e. In pigs with high titres against the NP, low titres against M2e were found and vice versa, showing an individual preference for epitopes. Nevertheless, all pigs were able to respond to the peptide because all pigs showed a detectable antibody response to M2e in the M2eHBc + adjuvant group, and in a previous study, all pigs sequentially infected with influenza H1N1 and H3N2 virus showed a detectable response (Heinen *et al.*, 2001).

After challenge, the titre against NP in the M2eNP DNA group quickly increased to a maximum between day 4 and 7 after challenge, which was faster than in the other groups (Fig. 3B). No nasal IgA response against the NP was detected before challenge. After challenge, all groups responded with an anti-NP IgA response, which, like the serum IgG response, was fastest in the M2eNP DNA group (results not shown).

No significant differences between groups were observed in the HI response after challenge (results not shown).

## T cell proliferation

Before challenge, a proliferation response of PBMCs was observed only in the M2eNP DNA group when cells were stimulated with influenza virus A/Sw/Best/96 (Fig. 4A). When cells were stimulated with M2eHBc protein a response was observed only in the M2eHBc plus adjuvant group (Fig. 4B). The absence of a proliferation response of PBMCs from M2eNP DNA-vaccinated pigs when stimulated with M2eHBc, and of M2eHBc-vaccinated pigs when stimulated with in-

fluenza virus, indicates that T cells are only stimulated by the carrier protein and not by the M2e peptide.

After challenge, the antigen-specific proliferation response decreased strongly, especially in the M2eNP DNA group (Fig. 4A). All groups developed a similar proliferation response to stimulation with influenza A/Sw/Best/96 after challenge with this virus.

The lymphoproliferation response of PBMCs when stimulated with ConA dropped significantly after challenge in all immunized groups ($P < 0{\cdot}001$ for M2eHBc and M2eHBc+ adjuvant, $P < 0{\cdot}01$ for M2eNP group), but not in the control group ($P > 0{\cdot}05$) (Fig. 4C).

### Flow cytometric analysis of BALF cells

With the mAbs used, we distinguished two phenotypes of myeloid cells: $SWC3^+CD163^-$ cells (neutrophil phenotype) and $SWC3^+CD163^+$ cells (monocyte and macrophage phenotype), and five phenotypes of lymphoid cells: $CD4^-$**CD5**$^-CD6^-$**CD8**$^{low}$TCR-$\gamma\delta^-$ (**NK** phenotype), $CD4^-$**CD5**$^+CD6^+$**CD8**$^{high}$ (**CTL** phenotype), $CD4^+$**CD5**$^{high}CD6^+$**CD8**$^{-/low}$ (**Th** phenotype), $CD4^-$**CD5**$^{low}CD6^-$**CD8**$^{-/low}$ (**TCR-**$\gamma\delta^+$ phenotype) and a heterogeneous population of **CD5**$^-$**CD8**$^-$ cells (**−/− T cells**). Because the cell numbers collected from the BALF of pigs varied too much between samples, we could not calculate the absolute numbers of cells of each phenotype.

The most relevant observation was a higher percentage of T helper cells after challenge in the M2eNP DNA group than in the other groups (Fig. 5). This percentage was significantly ($P < 0{\cdot}05$) higher than in the control group on PCD 4 and 8. In addition, the percentage of CTLs was higher on PCD 4 in the M2eNP DNA-vaccinated group than in the other groups. Although this difference was not significant, it was observed even though the percentage of T helper cells was also higher in this group.

In the M2eHBc protein group and the M2eHBc protein plus adjuvant group, the percentage of NK cells on PCD 4 was higher than in the control group, but this difference was not significant. No significant differences in the absolute number of cells collected from the BALF or in the phenotype distribution of the myeloid leukocyte populations after challenge were observed between the groups (results not shown).

## Discussion

The experimental vaccines evaluated in the present study induced the desired immune responses. All vaccines induced an antibody response to the extracellular domain of the M2 protein of influenza A virus and the M2eNP DNA vaccine additionally induced an NP-specific lymphoproliferation response. However, no protection was observed when the vaccinated pigs were challenged with a swine influenza virus. Clinical signs and virus excretion were not reduced in the vaccinated groups compared with the non-vaccinated control group. On the contrary, the vaccinated pigs showed more severe clinical signs after challenge than the control pigs. In particular, the M2eNP DNA-vaccinated pigs showed severe signs and three out of six pigs died on days 1 and 2 post-challenge. Previous studies in mice have suggested that enhancement of the antibody response to the M2 protein by vaccination may provide broad-spectrum immunity to humans (Mozdzanowska *et al.*, 1999; Neirynck *et al.*, 1999; Treanor *et al.*, 1990), and we have suggested the same for pigs (Heinen et al., 2001). However, the results of the present study in pigs indicate that antibodies to M2e can also exacerbate disease. Therefore, clinical signs after infection should be observed closely in further studies using M2 as an immunogen and caution should be exercised in using M2 in humans.

Vaccination of pigs with M2eHBc fusion protein did not confer protection against influenza virus challenge infection, which does not agree with results of a previous study in which mice were partially protected after immunization with the same material (Neirynck *et al.*, 1999). The difference in results could be caused by several reasons. One reason could be that in the previous study a human challenge virus was used with the same amino acid sequence of the M2e used for immunization, while in the present study a swine influenza challenge virus was used in which the sequence of the M2e differed (Fig. 1A). Nevertheless, antibodies induced by the immunization were shown to bind to the peptide with the amino acid sequence of the swine influenza challenge virus in the ELISA. If the sequence difference caused the absence of protection after vaccination, then this would mean that the spectrum of protection conferred by immunization with M2e does not include viruses with the swine influenza virus M2e sequence. A second reason could be that, in the present study, pigs instead of mice were used, and that pigs were challenged by aerosol instead of intranasal instillation and the dose of challenge differed. Since the pig is a natural host of influenza virus, the swine influenza model seems a better model than the mouse model to study vaccination strategies not only for pigs, but also for humans. The course of infection with influenza A virus in pigs is similar to that in humans. In fact, the same strains can infect both pigs and humans, and pigs have been implicated as a mixing reservoir for the generation of new pandemic strains (Scholtissek, 1995). In contrast, influenza virus challenge in rodents typically leads to lethal pulmonary infection and protection is scored by survival rather than progression of infection.

From the results obtained in this study, it is uncertain which immune mechanism underlies the observed exacerbation of disease. However, pigs in which only antibodies against M2e were induced showed a severity of clinical signs after challenge infection that was intermediate between the severity seen in the non-immunized control group and the M2eNP DNA-immunized group in which both antibodies to M2e and influenza virus-specific T cells were induced. These findings

suggest that the antibodies to M2e are involved, while in the M2eNP DNA group, T helper cells played a role in the exacerbation, probably by stimulating inflammatory responses. In previous studies with NP DNA vaccination in mice (Epstein *et al*., 2000; Ulmer *et al*., 1998; Zebedee & Lamb, 1989), but also in pigs (Macklin *et al*., 1998), no exacerbation of disease was observed after challenge, indicating that induction of T cell immunity alone does not result in exacerbation of disease. In contrast with antibodies against the HA protein, the antibodies against M2e are not virus-neutralizing (VN), i.e. do not prevent the attachment of influenza virions to the surface of lung epithelial cells (Zebedee & Lamb, 1989). Therefore, many cells will become infected and will express the M2 protein on the membrane surface. Binding of the antibodies to the M2 will thus promote the death of infected cells via several cell-targeting (CT) mechanisms: (i) the antibodies can promote contact with phagocytes either by reduction in surface charge, by adherence directly via the Fc or via complement factor C3 bound to the Fc; (ii) the antibodies can promote contact with and killing by NK cells (antibody-dependent cellular cytotoxicity); or (iii) the antibodies can activate the full complement system, producing direct membrane damage. Initially, we expected these immune mechanisms to operate in protection against influenza infection. However, in the present study, they may have become overwhelmed because the virus challenge dose was too high to cope with in the absence of VN antibodies and the immune mechanisms caused damage rather than providing protection. Because the M2-specific antibodies do not prevent initial infection, it is likely that they are most effective in the early stage of challenge infection when only a few cells are infected, and they may need to be present in the respiratory tract in sufficient amounts prior to challenge. Induction of antibodies that act via CT mechanisms as well as CTLs may be more effective by mucosal than by parenteral vaccination.

Possibly, enhancement of disease by heterosubtypic immune mechanisms could also occur in natural infections with influenza virus. Heterotypic non-neutralizing antibodies have been suggested to mediate enhancement of dengue infections, causing dengue haemorrhagic fever and dengue shock syndrome (Chen *et al*., 2001; Morens, 1994; Yang *et al*., 2001). Did immunity to the previous pandemic strain perhaps underlie the extraordinarily high mortality in healthy people between the age of 20 and 30 during the 1918 'Spanish flu' pandemic?

Vaccination with the DNA construct expressing a fusion protein of M2e joined to the N terminus of the NP induced a detectable antibody response to M2e. This shows that the NP does present the M2e peptide to B cells and indicates that the peptide is exposed on the surface of the fusion protein. Indeed, a mAb to the peptide (14C2; Zebedee & Lamb, 1989) was shown to bind to the fusion protein in an ELISA (result not shown). To our knowledge, this is the first study to report the presentation of a peptide to the immune system by fusion to influenza virus NP. In addition, the DNA vaccination with the M2eNP construct induced a lymphoproliferation response similar to that seen after infection. Elevated percentages of T helper cells as well as CTLs in the lungs of pigs 4 days post-challenge compared with non-immunized pigs indicate that the DNA vaccination indeed induces cellular immune responses in addition to an antibody response.

In conclusion, the present study does not exclude the possibility that M2e can be used as an immunogen to broaden the spectrum of protection conferred by current influenza vaccines. However, it does indicate that antibodies to M2e can exacerbate clinical signs of disease and that caution should therefore be exercised. Ways of inducing broad-spectrum immunity other than by vaccination with M2e and/or NP only might be safer. For example, successive infection with an attenuated H1N1 strain and an H3N2 strain will induce both M2e and probably other subtype cross-reactive IgA and IgG antibodies, as well as CTLs, in the lungs. Although such drastic vaccination strategies are not applicable to pig farming, they might be applied as a strategy of immunization in case of a future human pandemic.

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