

Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection

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The level of heterosubtypic immunity (Het-I) and the immune mechanisms stimulated by a heterosubtypic influenza virus infection were investigated in pigs. Pigs are natural hosts for influenza virus and, like humans, they host both subtypes H1N1 and H3N2. Marked Het-I was observed when pigs were infected with H1N1 and subsequently challenged with H3N2. After challenge with H3N2, pigs infected earlier with H1N1 did not develop fever and showed reduced virus excretion compared with non-immune control pigs. In addition, virus transmission to unchallenged group-mates could be shown by virus isolation in the non-immune control group but not in the group infected previously with H1N1. Pigs infected previously with homologous H3N2 virus were protected completely. After challenge with H3N2, pigs infected previously with H1N1 showed a considerable increase in serum IgG titre to the conserved extracellular domain of M2 but not to the conserved nucleoprotein. These results suggest that antibodies against external conserved epitopes can have an important role in broad-spectrum immunity. After primary infection with both H1N1 and H3N2, a long-lived increase was observed in the percentage of CD8⁺ T cells in the lungs and in the lymphoproliferation response in the blood. Upon challenge with H3N2, pigs infected previously with H1N1 again showed an increase in the percentage of CD8⁺ T cells in the lungs, whereas pigs infected previously with H3N2 did not, suggesting that CD8⁺ T cells also have a role in Het-I. To confer broad-spectrum immunity, future vaccines should induce antibodies and CD8⁺ T cells against conserved antigens.

Introduction

Influenza A virus expresses on its membrane two immunogenic, but variable 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 virus occurs, a phenomenon known as antigenic shift, and this may result in a pandemic. Current human influenza vaccines are updated annually to induce immune responses specific for the prevalent strains.

Antigenic drift is generally ascribed to immune pressure, i.e. the influence of antibodies induced by a previous infection, or

by vaccination. In pigs, antigenic drift of influenza viruses seems to be more limited than in humans (Brown *et al.*, 1997; Bikour *et al.*, 1995; Castrucci *et al.*, 1994). This is probably because pigs have a short life span, which does not cover more than one influenza epidemic, and vaccines are only infrequently applied. Nevertheless, antigenic drift of swine influenza A H3N2 viruses was demonstrated in the Netherlands and Belgium. This drift has led to a loss of cross-reactivity of recent field isolates with the human A/Port Chalmers/1/73 (H3N2) strain, which is the strain in the current swine influenza vaccine. Therefore, replacement of this strain by a more recent swine H3N2 isolate was recommended (de Jong *et al.*, 1999). However, the regular updating of influenza vaccines is costly and impractical. Antigenic shifts occur frequently in pigs (Castrucci *et al.*, 1993; Brown *et al.*, 1998; Zhou *et al.*, 1999), probably because pigs are highly susceptible to infection and have cell-surface receptors for

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viruses of both avian and human origin (Ito *et al.*, 1998). Moreover, it is conceivable that, if vaccines were to be applied more widely, acceleration of antigenic drift and antigenic shifts would soon give rise to escape variants.

Instead of the regular updating of vaccine strains, it might be possible to induce broadly reactive immune responses that could provide protection when the vaccine does not match circulating strains, perhaps even against a new subtype. Broadly reactive responses against influenza A virus have been studied extensively in mice (Kurimura & Hirano, 1973; Okuno *et al.*, 1994; Smirnov *et al.*, 1999; Webster & Askonas, 1980; Wraith & Askonas, 1985). Infection of mice with an influenza A virus of one subtype can induce partial protection against an infection with a virus of a different subtype (Epstein *et al.*, 1997; Flynn *et al.*, 1998; Liang *et al.*, 1994; Nguyen *et al.*, 1999; Schulman & Kilbourne, 1965; Werner, 1966). However, the immune mechanisms responsible for this broad protection, termed heterosubtypic immunity (Het-I), are still incompletely defined.

In this study, the level of Het-I to infection with an H3N2 virus after primary infection with an H1N1 virus and the immune mechanisms stimulated were investigated in pigs. The H3N2 virus was transmitted from humans to pigs during the 1968 Hong Kong pandemic and the H1N1 virus from birds to pigs in Northern Europe in 1979. In 1984, a genetic reassortment between the two subtypes occurred, resulting in an H3N2 virus carrying all the proteins of the avian H1N1 virus except for HA and NA (Castrucci *et al.*, 1993). Since then, the unchanged avian H1N1 virus and the reassortant human H3N2 virus have continued to co-circulate in the swine population in Europe. The fact that all proteins except for HA and NA are very similar between the two subtypes makes these European swine viruses ideal to study Het-I.

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). These viruses were isolated in primary cultures of porcine thyroid cells and then passaged three times in these cells and then twice in Madin–Darby canine kidney (MDCK, ECACC no. 84 121 903) cells. 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.

■ Pigs, immunization and challenge. Thirty Dutch Landrace pigs were obtained from the specific-pathogen-free herd of the Institute for Animal Science and Health. The pigs were divided into three groups of 10 and each group was housed in a separate room. At the age of 10 weeks, pigs were inoculated with live virus, in the nostrils, with an aerosol produced by nebulization of 2 ml culture supernatant, using an airbrush device (model no. 100LG; Badger). Pigs in the Het-I group were immunized with 10^8 TCID₅₀ of the field isolate A/Sw/Best/96 (H1N1). Pigs in the homologous immunity (Hom-I) group were immunized with A/Sw/Oedenrode/96 (H3N2). Pigs in the control group were inoculated with uninfected culture supernatant and were used as challenge controls.

At day 42 post-inoculation (p.i.), 6 weeks after primary infection, five of the pigs in each group were challenged with an aerosol of the field isolate A/Sw/Oedenrode/96 (H3N2). The other five pigs of each group were kept apart for 24 h and then reunited with their challenged group-mates to determine whether virus transmission occurred in the presence of Hom-I and Het-I and in the absence of immunity. The experiment was approved by the institute's ethical committee for experiments in animals.

■ Clinical observations and sampling of pigs. Rectal temperatures of all pigs were measured with a thermometer (C 402 Terumo Digital Clinical, Vetin-Aacofarma) and oropharyngeal fluid was collected daily for 8 days following primary infection (days 0–8 p.i.) and challenge inoculation (days 42–50 p.i.). Blood was collected from the five challenged pigs at days 0, 3, 7, 10, 18, 25, 31, 38, 46, 50, 53 and 59 p.i. and from the five contact pigs at days 38, 46, 50, 53 and 59 p.i. Serum was collected to determine HI antibody titres in the HI assay and titres of IgG antibodies specific for the extracellular domain of M2 (M2e) and for the nucleoprotein (NP) in ELISAs. Heparinized blood was collected for the isolation of peripheral blood mononuclear cells (PBMC) to be used in a T-cell proliferation assay. Bronchoalveolar lavage fluid (BALF) and nasal swabs (NS) (Medical Wire & Equipment Co.) of the challenged pigs were collected at days 0, 2, 4, 8, 11, 15, 22, 29, 39, 44, 46, 50, 53 and 57 p.i. To collect NS and BALF, animals were anaesthetized by injection (ketamine, midazolam, medetomidine). To avoid excessive interference with virus transmission, no BALF was collected from the contact pigs. NS was collected to determine antibody titres in the anti-NP IgA ELISA and BALF to monitor the changes in phenotypes of BALF cells by flow cytometry.

■ Virus isolation. Tenfold 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 $5\ \mu\text{g}/\text{ml}$ trypsin). Dilutions were inoculated on MDCK cells in microtitre plates, which were incubated at 37°C and examined for 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 in 25 ml tissue culture flasks. Virus titres were calculated by the Spearman–Kärber method.

■ 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) or A/Sw/Oedenrode/96 (H3N2).

■ ELISA for IgG specific for M2e. The sequence encoding the complete M2 protein was determined for A/Sw/Best/96 (H1N1) and A/Sw/Oedenrode/96 (H3N2). The first 25 N-terminal amino acid residues that form the extracellular domain of the M2 protein (M2e) were the same for both subtypes. A synthetic peptide with the amino acid sequence MSLLEVEVTPTRNGWECRY was made and a conjugate of this peptide with keyhole lipid haemocyanin was used to coat 96-well ELISA plates (Costar EIA/RIA). Plates were then blocked with 1% BSA in PBS, incubated with 2-fold 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 absorbance of 1.0.

■ ELISAs for IgG and IgA specific for NP. ELISAs to measure influenza virus NP-specific IgG and IgA antibodies in pigs were described recently (Heinen *et al.*, 2000). 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 was described previously (van Leengoed & Kamp, 1989). Approximately 30 ml PBS was added to the BALF to give a total volume of 50 ml. 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), MHC II (clone MSA3, IgG2a), CD14 (clone MY4, Ig2b) (Coulter) and CD163 (clone CVI 517.2, IgG2b). The MAbs used to differentiate lymphoid cells were directed against CD2 (clone MSA4, IgG2a), CD3 (clone ppt3, IgG1), CD4 (clone 74-12-4, IgG2b), CD5 (clone b53b7, IgG1), CD6 (clone a38b2, IgG1), CD8 (clone 295/33, IgG2a) and $\gamma\delta$ T cell receptor (TCR- $\gamma\delta$) (clone ppt16, IgG2b). These MAbs were used previously to analyse changes in the phenotype of leukocytes in the BALF of pigs infected with porcine reproductive and respiratory syndrome virus (Samsom *et al.*, 2000). After incubation, the cells were washed three times with FACS buffer and then incubated for 30 min on ice with the appropriate FITC- or PE-conjugated goat anti-mouse IgG isotype-specific antibodies, diluted in FACS buffer. Subsequently, the cells were washed three times, re-suspended 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, PBMC were isolated from heparinized blood samples by centrifugation onto Lymphoprep (Nycomed Pharma A), and were washed twice with PBS. The isolated PBMC were seeded in 96-well flat-bottom plates (M29, Greiner) at a density of 5×10^5 cells per well in 100 μ l medium (RPMI 1640 containing 10% porcine serum, 2 mM L-glutamine, 50 μ M β -mercaptoethanol, 200 U/ml penicillin, 200 μ g/ml streptomycin and 100 U/ml mycostatin). To the PBMC, 100 μ l medium containing 10^5 TCID₅₀ influenza A/Sw/Best/96 (H1N1) or A/Sw/Oedenrode/96 (H3N2), a control sample prepared from non-infected cells (mock control) or 5 μ g/ml concanavalin A (vitality control) were added in quadruplicate. After 4 days of incubation at 37 °C in a 5% CO₂ atmosphere, the cultures were pulsed with 0.4 μ Ci [³H]thymidine (Amersham). After 4 h of incubation, cells were harvested and the incorporated radioactivity was measured in a Betaplate scintillation counter (Wallac). Proliferation was expressed as the number of counts (mean of quadruplicate) of influenza virus-stimulated PBMC minus the number of counts of the mock control-stimulated PBMC (Δ c.p.m.).

Results

Clinical signs and virus excretion

Primary inoculation of pigs by aerosol with 10^8 TCID₅₀ A/Sw/Oedenrode/96 (H3N2) or A/Sw/Best/96 (H1N1) virus into the nostrils caused acute disease. Eight of ten H3N2-inoculated pigs and all H1N1-inoculated pigs developed fever (≥ 40 °C) for at least 1 day between day 1 and day 6 p.i. Infection with the H1N1 virus was more severe than with the H3N2 virus, as more pigs had fever for a longer time. Mean

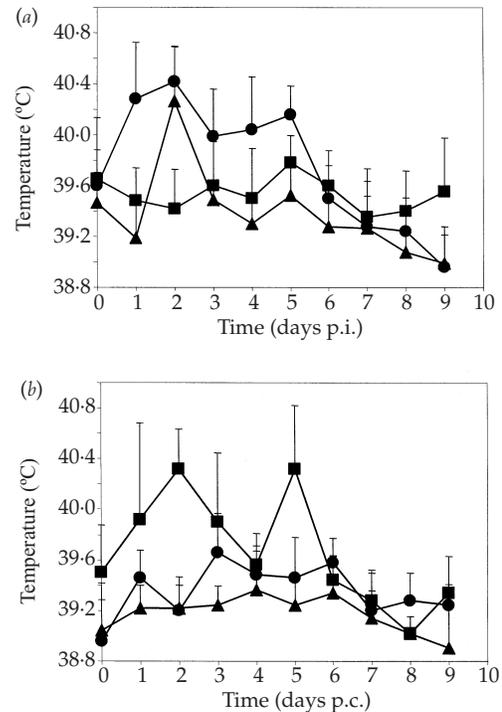


Fig. 1. (a) Mean temperatures of control pigs (■) and pigs infected with influenza virus A/Sw/Best/96 (H1N1) (●) or A/Sw/Oedenrode/96 (H3N2) (▲) ($n = 10$). Later, of the 10 pigs in each group, five were challenged and five were used as contact pigs. Contact pigs were separated before their group-mates were challenged and were then reunited after 24 h. (b) Mean temperatures of pigs after challenge with influenza A/Sw/Oedenrode/96 (H3N2) virus, 6 weeks after primary infection with A/Sw/Best/96 (H1N1) (●) or A/Sw/Oedenrode/96 (H3N2) (▲) and of non-immune control pigs (■). Results are presented as means \pm SD ($n = 5$). Only temperatures of challenged pigs are presented. No fever was detected in the contact pigs in any of the three groups. p.c., Post-challenge.

temperatures of all pigs are presented (Fig. 1a). In both groups, pigs excreted virus for 5 or 6 days after infection (Table 1).

After challenge inoculation with H3N2 at day 42 p.i., fever was measured in each of the five challenged pigs in the control group but not in the groups that were previously immunized with H1N1 (Het-I group) or H3N2 (Hom-I group) (Fig. 1b). In addition, virus excretion and transmission to the contact animals was reduced in the Het-I group compared with the control group (Table 1), indicating that infection with H1N1 confers heterosubtypic protection against infection with H3N2. Pigs in the Hom-I group did not excrete any virus after challenge.

No fever was detected in the contact pigs in the Hom-I or in the Het-I group or in the contact pigs of the control group (data not shown).

HI responses

No cross-reactivity of HI antibodies from H1N1-infected pigs was observed with the H3N2 virus, or vice versa, after primary infection (Fig. 2a, b).

Table 1. Virus titres in oropharyngeal swabs of pigs after primary infection with A/Sw/Best/96 (H1N1) or A/Sw/Oedenrode/96 (H3N2) and after subsequent challenge of pigs with A/Sw/Oedenrode/96 (H3N2)

Titres are given as $\log_{10}(\text{TCID}_{50}/\text{ml})$. < 1.6, Virus detected in 1 ml sample; -, no virus detected. Day 1 post-challenge is day 43 p.i.

Animal	Time (days p.i.)								Time (days post-challenge)							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
A/Sw/Best/96 (H1N1)																
1	4.4	-	3.6	< 1.6	2.1	< 1.6	-	-	2.6	< 1.6	-	-	-	-	-	-
2	2.5	< 1.6	< 1.6	2.5	2.8	< 1.6	-	-	-	< 1.6	-	-	-	-	-	-
3	4.6	< 1.6	< 1.6	< 1.6	3.2	-	-	-	< 1.6	< 1.6	-	-	-	-	-	-
4	2.6	-	< 1.6	< 1.6	< 1.6	-	-	-	< 1.6	-	-	-	-	-	-	-
5	2.3	< 1.6	2.3	< 1.6	< 1.6	-	-	-	< 1.6	< 1.6	-	-	-	-	-	-
A/Sw/Oedenrode/96 (H3N2)																
1	2.5	-	2.6	-	2.6	-	-	-	-	-	-	-	-	-	-	-
2	3.4	-	< 1.6	2.3	< 1.6	< 1.6	-	-	-	-	-	-	-	-	-	-
3	2.7	< 1.6	< 1.6	< 1.6	< 1.6	-	-	-	-	-	-	-	-	-	-	-
4	3.3	2.2	2.3	< 1.6	< 1.6	< 1.6	-	-	-	-	-	-	-	-	-	-
5	2.1	< 1.6	< 1.6	2.6	< 1.6	-	-	-	-	-	-	-	-	-	-	-
Non-immunized controls																
Challenged pigs																
1									< 1.6	< 1.6	< 1.6	< 1.6	< 1.6	-	-	-
2									< 1.6	< 1.6	< 1.6	-	-	-	-	-
3									< 1.6	< 1.6	-	< 1.6	-	-	-	-
4									2.7	-	3.7	-	-	-	-	-
5									2.5	-	-	-	-	-	-	-
Contact pigs*																
6									-	-	< 1.6	-	< 1.6	2.0	-	-
7									-	-	< 1.6	< 1.6	-	< 1.6	-	-
8									-	-	< 1.6	-	-	-	-	-
9									-	-	2.6	-	-	-	-	-
10									-	-	2.6	-	-	< 1.6	-	-

* Virus was only detected in contact pigs in the non-immunized control group and not in contact pigs in the other groups. The five contact pigs were separated before their group-mates were challenged and were then reunited after 24 h.

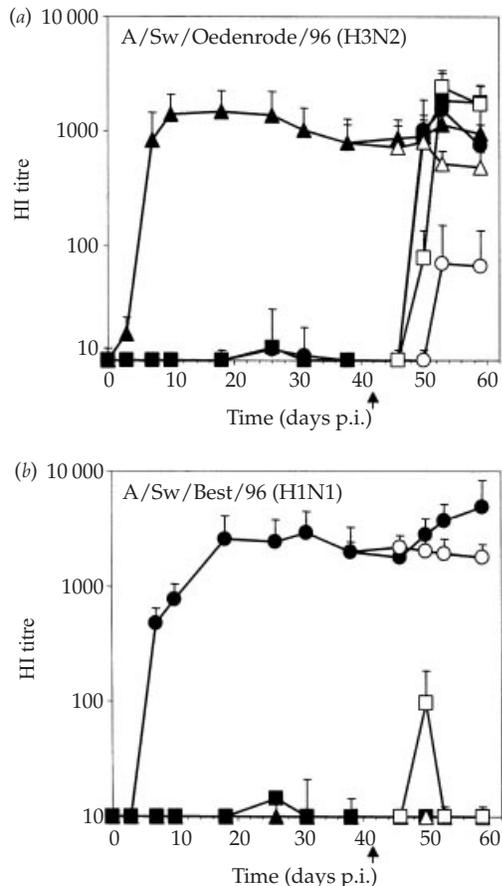


Fig. 2. Mean HI titres against the virus strains A/Sw/Oedenrode/96 (H3N2) (a) and A/Sw/Best/96 (H1N1) (b) in serum of pigs. At day 0 p.i., pigs were inoculated with 10^8 TCID₅₀ A/Sw/Best/96 (H1N1) (●) or A/Sw/Oedenrode/96 (H3N2) (▲) or with non-infected cell supernatant (■). At day 42 p.i., pigs were challenged with A/Sw/Oedenrode/96 (H3N2). Open symbols represent contact pigs in the three groups. Contact pigs were separated before their group-mates were challenged and were then reunited after 24 h. Results are presented as means \pm SD ($n = 5$).

After challenge, the challenged pigs in the Het-I group developed an H3N2-specific HI response that was very similar, in both kinetics and magnitude, to the response developed by the non-immune control pigs (Fig. 2a). However, contact pigs in the Het-I group showed no detectable HI response (2/5) or only a very low HI response (3/5).

A slight increase was also observed in the HI titre in the challenged pigs in the Het-I group to H1N1 (Fig. 2b), indicating that some cross-reactive HI antibodies were produced after heterosubtypic infection.

M2e-specific serum IgG response and NP-specific serum IgG and nasal IgA response

After primary infection with H1N1 or H3N2, a low IgG response to M2e was observed. This response was 3-fold higher after H1N1 infection than after H3N2 infection, which was also observed for the anti-NP IgG response. After

challenge, the anti-M2e titre increased 25-fold in the challenged pigs in the Het-I group, whereas it did not increase in the Hom-I group (Fig. 3a). This strong IgG booster response in the Het-I group against M2e was not observed against the conserved NP. No increase in the M2e IgG titre was observed in the contact pigs in the Het-I group.

The kinetics of the NP-specific IgG and IgA antibody responses after primary infection with H1N1 were very similar to those seen after infection with H3N2. However, H1N1-infected pigs showed a higher serum IgG response (Fig. 3b), whereas H3N2-infected pigs showed a higher nasal IgA response (Fig. 3c).

After challenge infection with H3N2, a slight secondary NP-specific IgG response in the challenged pigs was observed in the Het-I group but not in the Hom-I group (Fig. 3b). No secondary IgG response was detected in the contact pigs of the Het-I group.

After challenge infection, a secondary IgA response was observed in the Het-I group, which was stronger than that in the Hom-I group (Fig. 3c). No secondary IgA response was observed in the contact pigs of the Hom-I group or in the Het-I group.

Flow cytometric analysis of BALF cells

With the MAbs used, two phenotypes of myeloid cells were differentiated: SWC3⁺ CD163⁻ CD14⁺ MHCII⁻ cells (neutrophil phenotype) and SWC3⁺ CD163⁺ CD14⁺ MHCII⁺ cells (monocyte and macrophage phenotype) (Fig. 4a).

By comparing the kinetics and percentages of lymphoid cells stained with all combinations of MAbs, five phenotypes of lymphoid cells were distinguished. These five phenotypes could all be seen separately when a combination of MAbs against CD5 and CD8 was used (Fig. 4b). The lymphoid phenotypes were: CD2⁺ CD3⁻ CD4⁻ CD5⁻ CD6⁻ CD8^{low} TCR- $\gamma\delta$ ⁻ (NK phenotype), CD2⁺ CD3⁺ CD4⁻ CD5⁺ CD6⁺ CD8^{high} TCR- $\gamma\delta$ ⁻ (CTL phenotype), CD2⁺ CD3⁺ CD4⁺ CD5^{high} CD6⁺ CD8^{-/low} TCR- $\gamma\delta$ ⁻ (Th phenotype), CD2⁺ CD3⁺ CD4⁻ CD5^{low} CD6⁻ CD8^{-/low} TCR- $\gamma\delta$ ⁺ (TCR- $\gamma\delta$ ⁺ T-cell phenotype) and a heterogeneous population of CD5⁻ CD8⁻ cells (-/- T cells).

As reported previously (Samsom *et al.*, 2000), a population of large, highly autofluorescent cells (LHAC) and a population of small, low-autofluorescent cells (SLAC) were distinguished and these were analysed separately (Fig. 4a). It was shown that the LHAC consisted solely of myeloid (SWC3⁺) cells. However, a large proportion of the SLAC at days 2 and 4 after infection was also SWC3⁺. These were SWC3⁺ CD163⁻ neutrophils, which massively infiltrated the lung (Fig. 4a), as described previously (Haesebrouck & Pensaert, 1986; Van Reeth *et al.*, 1999). Corrections were therefore made such that the neutrophils were subtracted from the lymphoid population and added to the myeloid population and the percentages of the different cell phenotypes within the two populations could be calculated. Because the cell numbers collected from the

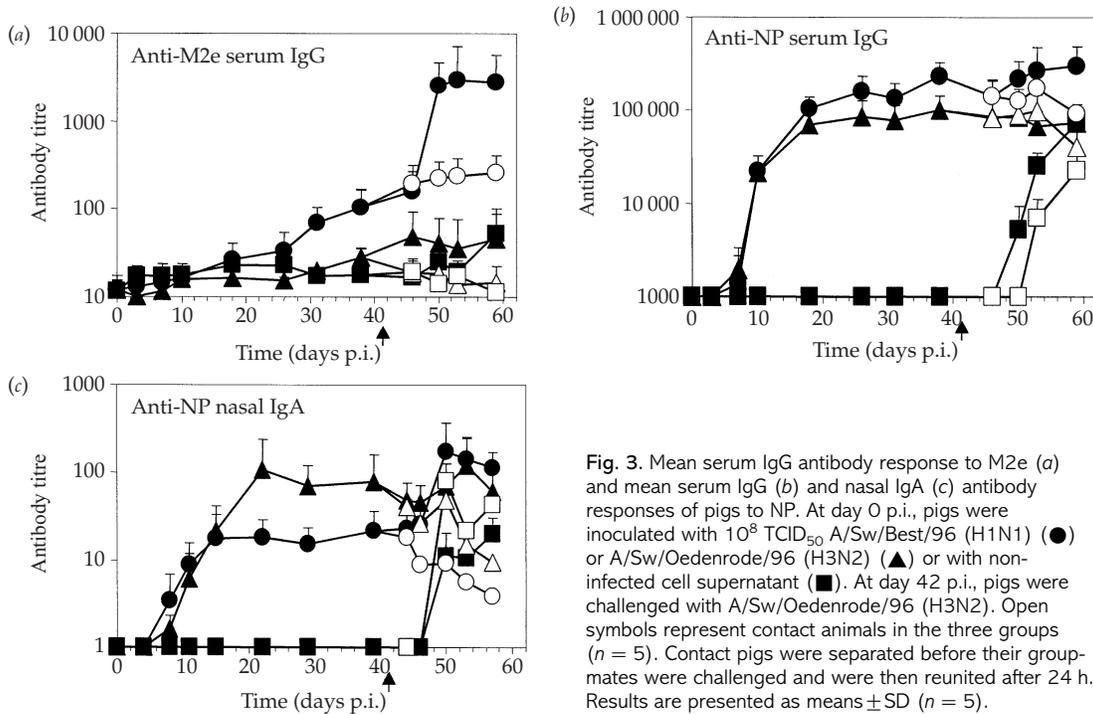


Fig. 3. Mean serum IgG antibody response to M2e (a) and mean serum IgG (b) and nasal IgA (c) antibody responses of pigs to NP. At day 0 p.i., pigs were inoculated with 10^8 TCID₅₀ A/Sw/Best/96 (H1N1) (●) or A/Sw/Oedenrode/96 (H3N2) (▲) or with non-infected cell supernatant (■). At day 42 p.i., pigs were challenged with A/Sw/Oedenrode/96 (H3N2). Open symbols represent contact animals in the three groups ($n = 5$). Contact pigs were separated before their group-mates were challenged and were then reunited after 24 h. Results are presented as means \pm SD ($n = 5$).

BALF of pigs varied too much between samples, we did not use the cell counts to calculate the absolute numbers of cells of each phenotype.

All changes in the phenotype of the BALF myeloid and lymphoid leukocyte populations after primary and secondary infection of the pigs are shown (Fig. 5a, b). After primary infection with H1N1 or H3N2, an acute inflammatory response characterized by a massive increase of neutrophils from 6 to 40% (day 2 p.i.) of all leukocytes in the lungs was observed. After challenge, the infiltration was the same in the non-immune control group, reduced in the Het-I group and absent in the Hom-I group (Fig. 5a).

From day 8 p.i., the percentage of lymphocytes in the lungs of both H1N1- and H3N2-infected pigs was higher than in the control pigs and remained elevated until day 42 p.i. Further analysis of the different phenotypes within the lymphocyte population shows the following sequence of events after H3N2 infection: (i) an increase in the percentage of NK cells starting at day 2 p.i., (ii) a peak in percentage of Th cells between days 4 and 11 p.i., (iii) a massive infiltration of CTLs on day 8 p.i., leading to a decrease in the percentage of NK cells at this time-point, (iv) the temporary disappearance of the CTLs from the lungs around day 11 p.i. and (v) a long-lived increase in the percentage of CTLs in the lungs and the decrease in NK cells to the pre-infection level. The same sequence of events was also observed after H1N1 infection, but developed faster than after H3N2 infection (Fig. 5b).

Upon challenge infection on day 42 p.i., a second increase in the percentage of CTLs was observed in the Het-I group but not in the Hom-I group. The secondary infiltration of CTLs

after H3N2 challenge infection (day 42 p.i.) in the Het-I group was not observed to be any faster than after primary infection in the control group, and even later than after primary H1N1 infection (day 0 p.i.) (Fig. 5b).

Influenza virus-specific T-cell response

After infection with both H1N1 and H3N2, an influenza virus-specific T-cell response of PBMC was measured in the lymphoproliferation assay from day 7 p.i. onwards (Fig. 6a). The T-cell proliferation was partially subtype-specific. PBMC of pigs infected with H1N1 virus showed a higher response when stimulated with A/Sw/Best/96 (H1N1) than with A/Sw/Oedenrode/96 (H3N2), while the opposite was true for PBMC of pigs infected with H3N2 (Fig. 6a, b).

Upon secondary challenge infection on day 42 p.i., an increase in the T-cell proliferation response was observed in the Het-I group but not in the Hom-I group. In both the challenged pigs (Fig. 6a) and the contact pigs (Fig. 6b) in the Het-I group, a secondary lymphoproliferation response was observed.

The vitality of PBMC was the same throughout the experimental period in all groups.

Discussion

Heterosubtypic immunity (Het-I), the immunity induced by infection with an influenza virus of one subtype against a subsequent infection with a virus of another subtype, has been observed in mice (Epstein *et al.*, 1997; Flynn *et al.*, 1998; Liang

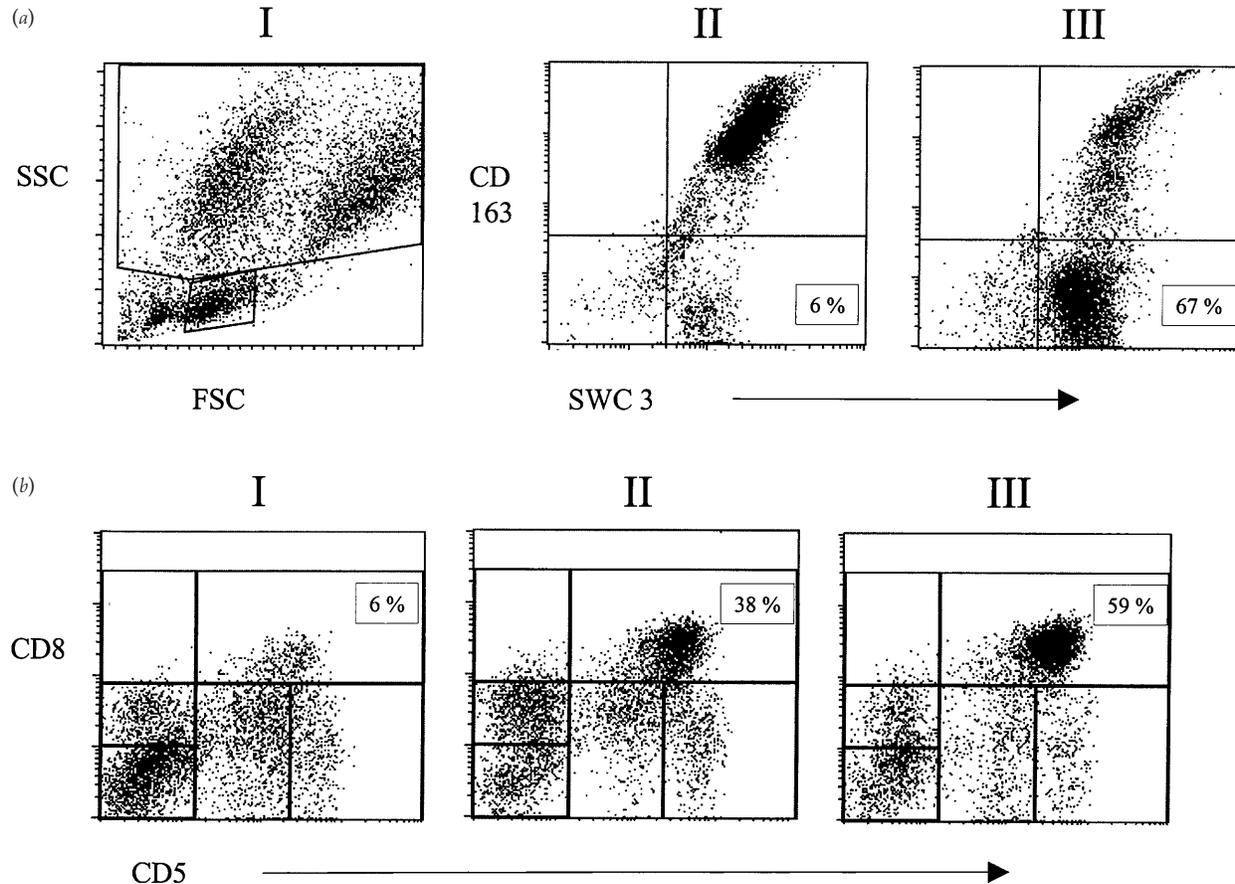


Fig. 4. (a) Flow cytometry analysis of leukocytes collected from the lungs of pigs. Plots show the two gates separating the lymphocytes from other leukocytes (I) and the percentage of SWC3⁺ CD163⁻ neutrophils in the lungs of non-immune pigs, 2 days after inoculation with non-infected cell supernatant (II) or influenza virus-infected cell supernatant (III). (b) Flow cytometry analysis of lymphocytes collected from the lungs of pigs after inoculation with non-infected cell supernatant (I), primary inoculation with 10^8 TCID₅₀ A/Sw/Best/96 (H1N1) (II) and subsequent challenge with A/Sw/Oedenrode/96 (H3N2) (III). Cells were double-stained with MAbs directed against CD5 and CD8. These plots of representative samples clearly show the infiltration of CD5⁺ CD8^{high} CTLs.

et al., 1994; Nguyen *et al.*, 1999). In humans, it was shown to be weak (Steinhoff *et al.*, 1993), although occasional examples have been reported (Sonoguchi *et al.*, 1985). Here, we report the existence of clear Het-I in pigs, using swine viruses of two subtypes that are currently co-circulating in the pig population in Europe. Infection with a swine influenza virus of subtype H1N1 was shown to protect pigs partially against infection with a virus of subtype H3N2. After challenge with H3N2, pigs infected previously with H1N1 did not get fever and showed reduced virus excretion compared with non-immune control pigs. As a consequence, less virus was transmitted to the unchallenged group-mates and virus excretion by these pigs could not be detected by virus isolation. Although Het-I does not provide the complete protection against infection seen with Hom-I, it could be sufficient to halt the spread of an influenza virus infection in a pig population in the field.

The predominant role of HA- and NA-specific virus-neutralizing IgA and IgG antibodies in protection against secondary infection with a homologous influenza A virus is

well established. However, the immune mechanisms responsible for Het-I are incompletely defined. In the present study, the responses of different immune mechanisms to primary and secondary infection were monitored to gain insight into the immunity underlying the suboptimal protection against infection with a heterologous or, in our case, heterosubtypic virus. This insight might suggest approaches to enhance the induction by vaccination of responses leading to broad protection.

HI antibodies after infection with H1N1 did not cross-react with the H3N2 virus in the HI assay and, therefore, pre-challenge HI antibodies do not seem to be responsible for the observed Het-I. However, after challenge with H3N2, an increase in HI titre was also observed against H1N1, which suggests that cross-reactive HI antibodies are produced. HA-specific virus-neutralizing antibodies that cross-react between subtypes have been reported (Okuno *et al.*, 1994; Smirnov *et al.*, 1999). In addition, helper T cells specific for influenza virus internal proteins can enhance the HI antibody response (Russell

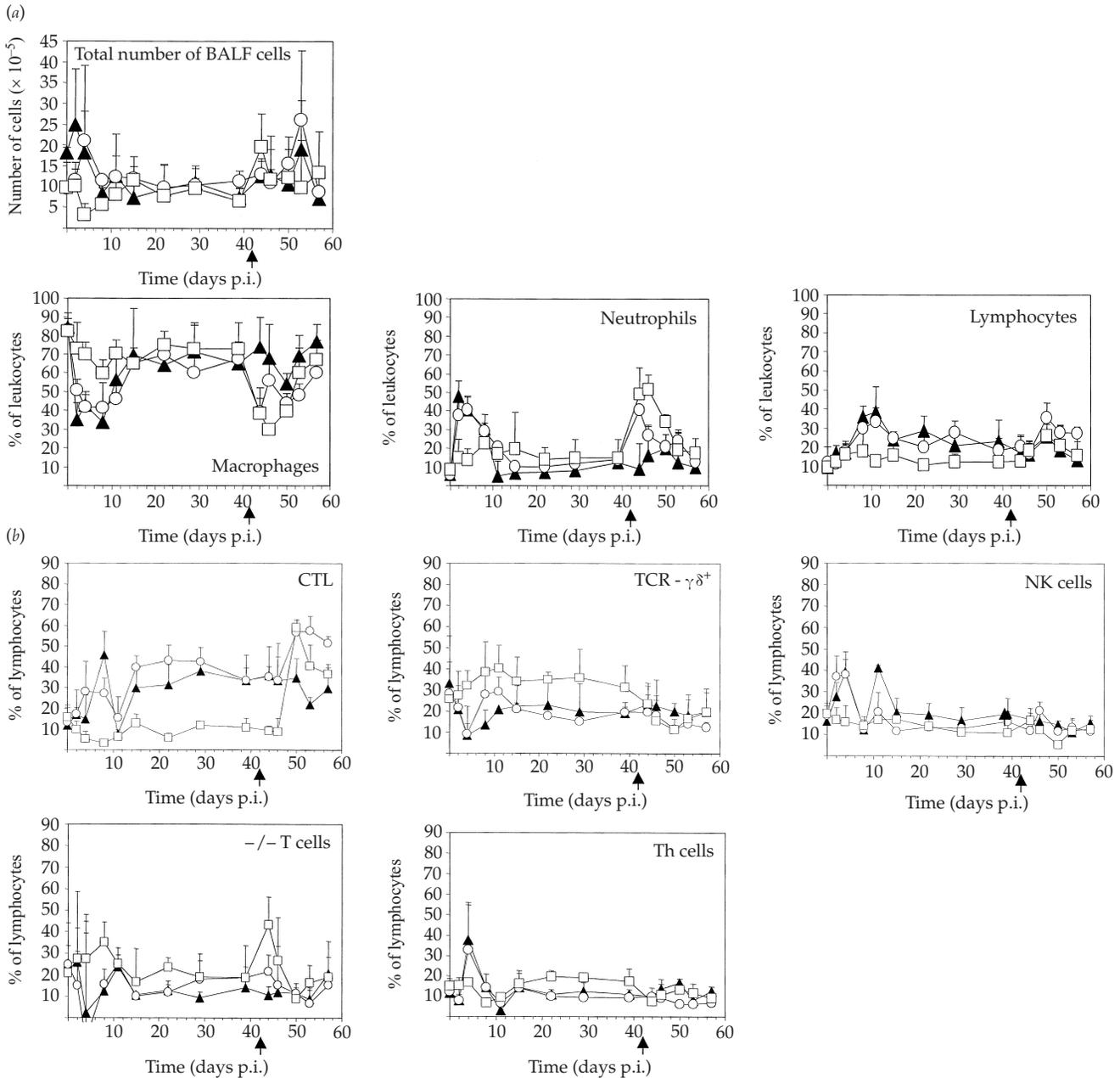


Fig. 5. (a) Changes in the leukocyte population in the lungs of pigs ($n = 5$). At day 0 p.i., pigs were inoculated with 10^8 TCID₅₀ A/Sw/Best/96 (H1N1) (○) or A/Sw/Oedenrode/96 (H3N2) (▲) or with non-infected cell supernatant (□). At day 42 p.i., pigs were challenged with A/Sw/Oedenrode/96 (H3N2). (b) Changes in the lymphocyte population in the lungs of pigs ($n = 5$). At day 0 p.i., pigs were inoculated with 10^8 TCID₅₀ A/Sw/Best/96 (H1N1) (○) or A/Sw/Oedenrode/96 (H3N2) (▲) or with non-infected cell supernatant (□). At day 42 p.i., pigs were challenged with A/Sw/Oedenrode/96 (H3N2). Results are presented as means \pm SD ($n = 5$).

& Liew, 1979). In this manner, subtype-specific antibodies will be produced faster and can contribute to Het-I, in addition to cross-reactive antibodies. In the present study, the kinetics and magnitude of the H3-specific HI antibody response after challenge of pigs in the Het-I group was the same as in the non-immune control group, which seems to disagree with this concept. However, pre-existing immunity can enhance the

response to challenge infection, but can also inhibit the response by reducing antigen exposure. Therefore, it is difficult to compare the response to challenge infection with the response to injection with a fixed quantity of antigen, as studied by Russell & Liew (1979).

Interestingly, the antibody response to the conserved extracellular domain of the M2 protein (M2e) was clearly

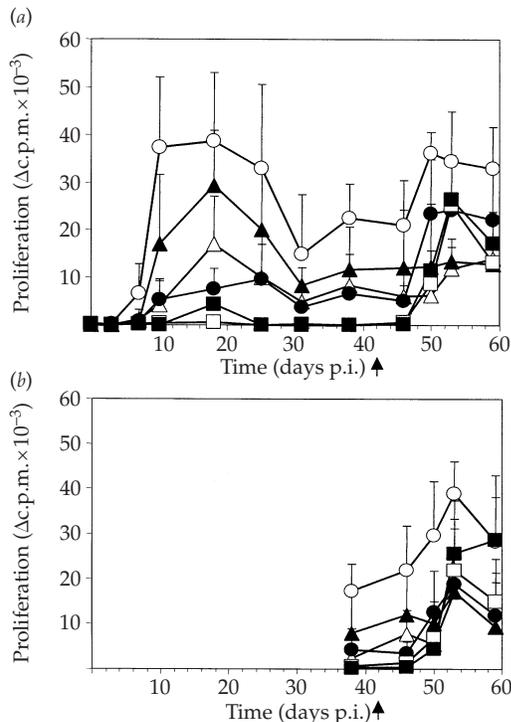


Fig. 6. (a) Mean influenza virus-specific lymphocyte proliferation responses of PBMC of pigs when stimulated with A/Sw/Oedenrode/96 (H3N2) (closed symbols) or A/Sw/Best/96 (H1N1) (open symbols). At day 0 p.i., pigs were inoculated with 10^8 TCID₅₀ A/Sw/Best/96 (H1N1) (●, ○) or A/Sw/Oedenrode/96 (H3N2) (▲, △) or with non-infected cell supernatant (■, □). At day 42 p.i., pigs were challenged with A/Sw/Oedenrode/96 (H3N2). (b) Mean influenza virus-specific lymphocyte proliferation responses of PBMC of contact pigs in the three groups. Contact pigs were separated before their group-mates were challenged and were then reunited after 24 h. Results are presented as means \pm SD ($n = 5$).

boosted by the heterosubtypic infection, as the serum anti-M2e antibody titre increased 25-fold between day 4 and day 8 post-challenge. In comparison, the antibody titre to the also conserved NP only increased 2-fold. Therapeutic treatment with an M2e-specific MAb was shown to reduce pulmonary virus titres 100- to 1000-fold in mice (Mozdzanowska *et al.*, 1999; Treanor *et al.*, 1990). It is therefore conceivable that pre- and post-challenge anti-M2e antibodies play a significant role in the early reduction of virus replication. The M2-specific antibody response in convalescent humans has been reported to be low and not consistently detectable (Black *et al.*, 1993). The present study shows that, in pigs, the anti-M2e antibody response to a primary influenza virus infection is indeed low compared with the response after heterosubtypic infection. The current porcine vaccine does not induce a detectable anti-M2e antibody response (unpublished result). Enhancement of the anti-M2e response by vaccination may provide increased Het-I, as was suggested previously for humans (Mozdzanowska *et al.*, 1999; Treanor *et al.*, 1990). Interestingly, it was shown that, in the proper circumstances, vaccination with the extracellular domain of the M2 protein in the absence

of other influenza virus proteins can reduce virus infection in mice (Neirynek *et al.*, 1999).

The percentage of CD8⁺ CTLs in the lungs of pigs increased from approximately 10% before primary infection to more than 30% after primary infection and more than 50% after secondary infection. The secondary challenge only increased the percentage of CTLs in the lungs of heterosubtypically immune pigs, indicating that CTLs play a role in Het-I. The CD8⁺ T cell arm of the cellular response has been proposed to be the major mediator of Het-I, and many studies have indeed proved that CTLs contribute to protection in mice (Epstein *et al.*, 2000; Mozdzanowska *et al.*, 2000; Topham & Doherty, 1998; Ulmer *et al.*, 1998). A large proportion of these cells recognizes conserved epitopes of NP. More than 15% of CTLs in the lungs after primary infection and more than 65% after heterosubtypic challenge were shown to be specific for the immunodominant NP₃₆₆₋₃₇₄ peptide (Doherty & Christensen, 2000; Flynn *et al.*, 1998, 1999). Moreover, challenge was performed 7 months after priming of mice, removing any concern that CTL memory is of short duration. However, it was also shown that the secondary CTL response develops in the mesenteric lymph nodes and that it takes at least 4–5 days before the effectors are available in the infected respiratory tract of mice. In the present study, the infiltration of CTLs into the lungs after secondary infection with the H3N2 virus was not observed any earlier than after primary H3N2 infection and even later than after primary H1N1 infection. Such a delay could be too long for CTLs to make a major contribution to clearance of the 'hit-and-run' influenza virus. In line with this idea, it was shown that the CTL response was capable of handling all but a very low challenge dose of influenza virus in the absence of antibody (Riberdy *et al.*, 1999). In the present study, the contact pigs in the Het-I group showed a similar lymphoproliferation response as the challenged pigs, but hardly any serum HI antibody response, anti-M2e antibody response or nasal anti-NP antibody response. Therefore, it seems that the contact pigs were indeed able to cope with the low dose of transmitted virus via a T-cell response rather than a B-cell response.

An infiltration of NK cells into the lungs was observed between day 0 and day 15 after primary infection. These cells probably kill influenza virus-infected epithelial cells in the early stage of primary infection in a non-specific manner but, at a later stage of the primary infection, as well as in the early stage of secondary infection, they are possibly targeted to infected host cells by antibodies. Anti-M2e MAbs and anti-NA MAbs that do not have virus-neutralizing activity were suggested to target effectors of the innate immune system to infected cells in mice (Mozdzanowska *et al.*, 1999). An influx of CD4⁺ CD8^{-low} Th cells was observed between day 0 and day 11 p.i. CD4⁺ T cell-mediated recovery from influenza virus infection was reported to be mediated by the promotion of an antiviral antibody response (Mozdzanowska *et al.*, 1997; Topham & Doherty, 1998) and a CTL response (Riberdy *et al.*, 2000). In

addition, CD4⁺ T cells have been suggested to kill infected cells in an MHCII-restricted manner (De Bruin *et al.*, 2000; Yasukawa *et al.*, 1988). The role of TCR- $\gamma\delta^+$ T cells in protection is unclear. In mice, they have been reported to increase greatly in frequency during the recovery phase from influenza pneumonia (Doherty *et al.*, 1989), but this was not observed in pigs in the present study.

The lymphoproliferation response increased after challenge in the Het-I group, but not in the Hom-I group. The pigs in the Hom-I group are probably so well protected that the challenge virus is cleared from the lungs without inducing a secondary lymphoproliferation response. The increase in lymphoproliferation seems to correspond to the increase in the percentage of CTLs in the lungs, which was also observed only in the Het-I group. However, in a previous experiment, in which pigs were primary-infected and then challenged with the homologous virus 9 weeks later, a secondary lymphoproliferation response, but no increase in the percentage of CTLs in the lungs, was observed (unpublished results). In Hom-I, lymphoproliferation probably mainly supports an antibody response whereas, in Het-I, it also supports a CTL response. The lymphoproliferation response was subtype-specific, as it was higher when PBMC from infected pigs were stimulated with the homologous virus than with the heterosubtypic virus. This could be due to the fact that the proliferation depends partially on T cells and on antigen presentation by B cells that recognize epitopes of the glycoproteins.

In conclusion, the present study shows Het-I in pigs. Furthermore, it provides additional evidence for the notion that cross-reactive antibodies and CTLs both play a role in Het-I. Het-I provides a much lower level of protection than Hom-I because antibodies, antigen-presenting B cells, Th cells and part of the CTLs are subtype-specific and because the less-protective antibody response to conserved epitopes is low. In particular, the antibody response to the conserved M2e after primary infection is suboptimal. This makes M2e an attractive candidate for vaccine-induced broad-spectrum immunity. Additional conserved epitopes to which protective immunity can be induced may be discovered in the future. Although Het-I does not provide the complete protection against infection seen with Hom-I, it caused a pronounced reduction in virus replication and fever. Prevention of all virus replication after challenge may not be a realistic goal in cross-protection, but a significant reduction in virus replication and clinical disease may well be. For humans, such a level of protection could be vital in the event that the vaccine strain does not match the circulating strain or in a future pandemic. For pigs, the level of protection could be sufficient to prevent virus spread if the vaccine strain does not match the circulating strain.

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