

Characterization of the IgA and subclass IgG responses to neutralizing epitopes after infection of pregnant sows with the transmissible gastroenteritis virus or the antigenically related porcine respiratory coronavirus

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In this study, we have investigated the characteristics of secreted IgA and other classes of Ig induced after vaccination of sows with transmissible gastroenteritis virus (TGEV) or the antigenically related porcine respiratory coronavirus (PRCV). Both viruses induced the secretion of neutralizing antibodies of different classes in the sows' milk, but these protected suckling piglets against TGEV to different degrees. Quantitative differences in the induction of IgA by both viruses were found among the different viral antigenic sites and subsites of glycoprotein S. In TGEV-vaccinated sows, antigenic subsite A was the best inducer of IgA, followed by antigenic site D. After vaccination with PRCV, lower

levels of IgA were detected on colostrum and milk, antigenic site D and subsite Ab being the immunodominant sites. This quantitative difference in epitope recognition could explain the differences in newborn piglet protection found using Ig classes purified from the milk of sows immunized with both viruses. Apparently only IgA recognizing at least antigenic sites A and D confers good protection *in vivo*, whereas any Ig class recognizing only one antigenic site may neutralize the virus in cell culture. These results indicate that the formulation of a subunit vaccine against TGEV has to consider the inclusion of more than one antigenic site involved in virus neutralization.

Introduction

Transmissible gastroenteritis virus (TGEV) is a member of the *Coronaviridae* family. TGEV infects the gut epithelium of pigs, irrespective of age, and causes high mortality in neonates (Bohl, 1981; Siddell *et al.*, 1983). Pigs are agammaglobulinaemic at birth and depend upon ingestion of colostrum and milk antibodies to protect them against infection (Porter & Allen, 1972). Since 1984, TGEV seroconversion has been observed in the swine population of different European countries (Brown & Cartwright, 1986; Jestin *et al.*, 1987; Pensaert *et al.*, 1986; Yus *et al.*, 1989) and North America (Wesley *et al.*, 1990). This seroconversion is produced by the porcine respiratory coronavirus (PRCV), a non-enteropathogenic virus derived from TGEV (Laude *et al.*, 1993; Sánchez *et al.*, 1992). Both types of viruses have common antigenic determinants in at least the three major structural proteins S, N and M (Callebaut *et al.*, 1988; Rasschaert *et al.*, 1990; Sánchez *et al.*, 1990; Wesley *et al.*, 1990). The S protein of PRCV differs by a deletion of 224 to 227 amino acids in the N terminus compared to that of TGEV (Rasschaert *et al.*, 1990; Sánchez *et al.*, 1992; Wesley *et al.*, 1990). This deletion

may be related to the different tropism of the viruses (Sánchez *et al.*, 1992). This deletion means that antigenic sites B and C are eliminated in PRCV, whilst those designated as A and D, both involved in virus neutralization, remain as major antigenic sites, in common with TGEV (Delmas *et al.*, 1986; Jiménez *et al.*, 1986). Site A, subdivided in three antigenic subsites (Aa, Ab and Ac; Correa *et al.*, 1988) is the immunodominant site in pregnant sows that confers lactogenic protection, followed by antigenic site D (De Diego *et al.*, 1992).

The wide distribution of PRCV in the swine population has been accompanied by a marked reduction in the number of TGEV outbreaks and it has been proposed that PRCV behaves as a natural vaccine against TGEV (Enjuanes & van der Zeijst, 1994). Generally it is accepted that PRCV-induced lactogenic antibodies protect pigs against TGEV to a lesser degree than TGEV-induced antibodies. The immune response induced by PRCV and TGEV, conditioned by the route of antigen presentation and stimulation of the bronchus-associated lymphoid tissue (BALT) or the gut associated lymphoid tissue (GALT) respectively, remains unexplored. The protection of suckling piglets against an enteric TGEV infection is based on the uptake of specific lactogenic

antibodies, mainly of the IgA class, in the milk of TGEV-immune mothers (Bohl, 1981). It has been reported that PRCV reinfections during pregnancy or early lactation cause the induction of lactogenic anti-TGEV IgA in naturally infected sows (Callebaut *et al.*, 1990; Van Deun *et al.*, 1990). The efficiency with which TGEV- or PRCV-induced lactogenic IgA protects suckling piglets against an enteric TGEV infection could be conditioned by different characteristics of the IgA induced in the gut or respiratory compartments of the mucosal immune system.

In the present work, we have investigated the induction of the different Ig classes by the virus S glycoprotein epitopes involved in neutralization, in TGEV- and PRCV-vaccinated sows with different piglet protection rates. We have tried to define the characteristics of the *in vivo* protective IgA response that should be taken into account when designing a hypothetical subunit vaccine.

Methods

Cells, viruses and virus titration. The attenuated strain PUR46 and the virulent strain MAD88 of TGEV, and the BEL85-83 strain of PRCV (Laviada *et al.*, 1988; Sánchez *et al.*, 1990) were grown in swine testicular (ST) cells. Infectious virus for *in vivo* immunization was grown on confluent cell monolayers infected with TGEV MAD88 or PRCV BEL85-83 at an m.o.i. of 0.5 in medium supplemented with 2% fetal bovine serum. After 20 h (20% c.p.e.), medium containing extracellular virus was harvested. Cellular debris was removed from the fluid by low-speed centrifugation. Supernatants were supplemented with 20% fetal bovine serum and stored at -70°C . After titration by the TCID₅₀ method (Reed & Muench, 1938) these inocula were used for vaccination and challenge. The virus was also titrated to determine p.f.u./ml as described by Jiménez *et al.* (1986) and used in seroneutralization tests. Briefly, 10-fold serial dilutions of virus were made in medium and applied to confluent ST cells in 24-well tissue culture plates. After 1 h of virus adsorption, the inoculum was replaced with medium containing 2% fetal bovine serum and 0.7% agarose and the cells were incubated at 37°C for 2 to 3 days. They were then fixed with 10% formaldehyde, stained with 0.1% crystal violet and the plaques counted.

Partially purified virus preparations were obtained by using confluent monolayers infected with TGEV PUR46 or MAD88 at an m.o.i. of 5. When cells reached 80 to 90% c.p.e., extracellular virus was harvested as described above. Virions were then semi-purified by centrifugation through a continuous sucrose gradient (60 to 20%) and the viral band was sedimented. The pellet was resuspended in buffer (0.001 M-Tris-HCl, 0.1 M-NaCl, 1 mM-disodium EDTA, pH 7.5) and its titre was determined antigenically in microtitre plates with a hyperimmune serum. These virus preparations were used for competitive (c)-ELISA.

Vaccination and challenge procedures. Six pregnant Large White sows, each weighing about 100 kg, were orally inoculated twice (at 84 and 104 days of pregnancy) with 2.5×10^9 TCID₅₀ of virulent TGEV MAD88. Two sows with the same characteristics were oronasally inoculated twice with an aerosol containing 2.5×10^9 TCID₅₀ of PRCV BEL85-83 on the same days of pregnancy as the TGEV-inoculated sows. One pregnant sow was left unimmunized as a negative control.

Piglets born from these sows (7 days old) were isolated, orally exposed to 6.4×10^8 TCID₅₀ of TGEV MAD88 and then breast fed 1 h later.

One pregnant Large White sow was inoculated with virulent TGEV MAD88 as described for the first group of sows. The piglets born from this sow were isolated on day 2 after farrowing and fed cows' milk treated with gentamicin (at a final concentration of 40 µg/ml) that had been allowed to stand at 4°C overnight before use. When the piglets were 7 days old they were challenged with virulent TGEV MAD88 as described above.

Antibodies. All sows were bled before each immunization and on the day of farrowing. Colostrum and milk samples from each sow were collected on the day of farrowing, on the day after farrowing (day 1) and on the day of challenge (day 7). For the sow whose piglets were isolated on day 2 after farrowing, only colostrum samples were collected. Colostrum and milk samples were treated as described (Abou-Youssef & Ristic, 1972). Briefly, casein and free fat were removed by centrifugation at $10000 \times g$ for 3 h. The whey was further clarified by centrifugation at $10000 \times g$ for 3 h. Lipoproteins were precipitated by adding 0.02 ml of 10% dextran sulphate 500 and 0.1 ml of 1 M-CaCl₂ per ml of whey. The precipitate was removed by centrifugation at $1500 \times g$ for 10 min.

The previously characterized TGEV-specific monoclonal antibodies (MAbs; Correa *et al.*, 1988, 1990; Jiménez *et al.*, 1986) were kindly provided by Dr L. Enjuanes (Centro Nacional de Biotecnología, CSIC, Madrid, Spain). The porcine Ig class-specific MAbs, anti-IgA, anti-IgG₁ and anti-IgG₂, were kindly provided by Dr C. R. Stokes (Bristol University, Bristol, U.K.).

The different Ig classes, IgA, IgG₁ and IgG₂, were purified by affinity chromatography from milk whey obtained from three TGEV-inoculated sows, numbers 4, 6 and 7, which protected their piglets after challenge, and from the two PRCV-inoculated sows, numbers 8 and 9, which conferred partial protection to their piglets. The class-specific MAbs were purified from ascites fluid by affinity chromatography in a Sepharose 4B-Protein A column (Pharmacia) and coupled to Sepharose CL-4B (Pharmacia), as described by Sánchez *et al.* (1991). Briefly, 5 mg MAb/ml gel were mixed with buffer (0.1 M-NaHCO₃ and 0.5 M-NaCl, pH 8.3) for 4 h at room temperature, excess ligand was washed away with PBS and the remaining active groups blocked with 0.2 M-glycine for 2 h. The gel was washed with PBS followed by 0.05 M-diethylamine, and again with PBS. The gel was then poured into a column and stored at 4°C . Milk whey (2 to 4 ml) diluted 1:1 in PBS was applied to the columns and washed with PBS until no protein was eluted. The bound Igs were eluted with 0.05 M-diethylamine. Fractions of 1 ml were collected, immediately neutralized with 100 µl of 2 M-Tris-HCl pH 3 and dialysed against PBS. The purity of the isolated Igs was tested by SDS-PAGE and ELISA and quantified by determination of A₂₈₀ readings.

ELISA. Serial twofold dilutions of purified milk Ig were used to coat four rows of microtitre plate wells, starting with 2 µg Ig/well. Wells were blocked with blocking buffer (0.05% Tween 20 and 30% fetal bovine serum in PBS, pH 7.2) for 1 h at 37°C . Biotin-conjugated class-specific MAbs, anti-IgA, anti-IgG₁ and anti-IgG₂, at the predetermined optimum dilutions, were added to one row and incubated for 1 h at 37°C . The wells were then incubated with a streptavidin-peroxidase conjugate (Amersham) and developed with 3-dimethylaminobenzoic acid 3-methyl-2-benzothiazolinone hydrazone substrate (Sigma). The reaction was stopped by addition of H₂SO₄. Finally, the A₆₂₀ values were read spectrophotometrically.

Competition ELISA. Competition assays were performed essentially as described previously (De Diego *et al.*, 1992) for competition radioimmunoassays (RIA). Semipurified TGEV PUR46, at the optimum concentration (about 0.44 µg per well), was used to coat microtitre plate wells. The wells were incubated for 1 h at 37°C with 200 µl of blocking solution (5% BSA in PBS). In the two-step assay, an adequate amount of competitor-purified Igs, diluted in 0.1% BSA-PBS buffer to a final volume of 200 µl per well, was added and incubated

overnight at 4 °C. The plates were washed with PBS containing 0.05% Tween 20 and pretitrated TGEV-specific MAbs (hybridoma culture supernatants) were added and incubated for 1 h at 37 °C. For one-step competition assays, the competitor antibody and the MAb were incubated simultaneously in the well for 2 h at 37 °C. The plates were washed again and a biotin-labelled anti-mouse Ig (Amersham) at optimum concentration in dilution buffer containing 5% TGEV-negative pig serum was added. After 1 h incubation at 37 °C the plates were developed as described above for ELISA. Results were expressed as the percentage of competition according to the following formula: competition % = 100 - binding % where binding % = (A_{620} value obtained in the presence of competitor antibody A_{620} obtained in the absence of competitor antibody) × 100. Each assay was done in triplicate.

Virus neutralization in vitro. A plaque neutralization assay was performed as described by Jiménez *et al.* (1986). Twofold dilutions of the purified Ig classes (starting with 75 µg) were incubated with 10³ p.f.u. of TGEV PUR46 in medium at 37 °C for 1 h. The virus-antibody mixture was titrated and the number of p.f.u. was determined as described above. As a control, virus samples were incubated with medium and processed by the same protocol. The neutralization percentage was determined according to the following formula: neutralization % = 100 - (p.f.u. in the presence of antibody / p.f.u. in the absence of antibody) × 100.

For the determination of the kinetics of neutralization, 50 µg of purified Ig classes diluted in medium were incubated with 10³ p.f.u. of TGEV PUR46 and titrated on ST cells at various times. The number of p.f.u. and the neutralization percentages were determined as described above.

For the reversibility of the neutralization assays, 100 µg of the purified Ig classes was incubated with 10³ p.f.u. of TGEV PUR46 for 1 h at 37 °C. The virus-antibody mixture was diluted 1:100 and immediately titrated on ST cells. After a 2 to 3 day incubation period, the number of p.f.u. was determined. Each assay was performed in triplicate.

In vivo protection assays. Piglet inoculation for protection assays was carried out basically as described by Wesley *et al.* (1988). Briefly, 3- to 5-day-old piglets, obtained from TGEV-seronegative sows, were isolated and fed gentamicin-treated cows' milk during the experiment. The daily ration of treated milk was divided into two 25 ml feedings and one 60 ml feeding during the first 2 days and gradually increased to 60 ml per feeding.

The challenge dose, 10³ p.f.u. of TGEV MAD88 in 1 ml of medium, was incubated with an excess of antibody (250 µg of purified Ig classes) for 1 h at 37 °C and given orally to piglets. The virus-antibody inocula were non-infectious when titrated on ST cells. Control animals received the challenge virus diluted to the same volume with medium. After challenge, piglets were left unfed for a period of 30 min after which regular feedings were resumed. The pigs were observed for the onset of clinical signs during the week following challenge. The percentage protection was then determined, a protected pig being one that did not develop clinical signs. A minimum of four piglets were used in each assay.

Results

Epitope specificity of secreted Ig classes and subclasses induced after antigen presentation in GALT by TGEV

IgA and IgG subclasses IgG₁ and IgG₂ were purified from colostrum and milk obtained on day 1 after farrowing of two TGEV-vaccinated sows (numbers 4 and 6) that protected 100% of their piglets from death

after the challenge and from one sow (number 7) that protected 80% of its offspring. In order to determine which of the TGEV epitopes involved in virus neutralization (epitopes belonging to antigenic sites A, B and D of the S protein) were the best inducers of IgA and to ascertain the selective epitope induction of other Ig classes during the immune response, cELISAs were performed. Different concentrations of the purified Ig classes were tested to determine whether they would block the binding of MAbs specific for sites B and D and subsites Aa, Ab and Ac (Fig. 1). In sow 4, the specific secretory antibody response of different Ig classes against the studied epitopes was slightly higher than in the other two sows, as deduced from the competition percentages shown by all purified Ig classes and subclasses. In all three sows, both antigenic sites A and D were the best inducers of IgA, site A being a better IgA inducer than site D. Both sites were also good inducers of IgG in colostrum and milk, especially in sows number 6 and 7, which presented a higher IgG, rather than IgA, response. Interestingly, for sow 4, which was able to protect some of its piglets from infection, the immune response to the TGEV epitopes tested was mainly of the IgA class, with blocking percentages, for 2.5 µg, higher than 70% for all three A subsite- and for site D-specific MAbs.

No significant differences in competition percentages were found between the two subclasses of IgG, which were equally induced by the different epitopes tested; site A being a slightly higher inducer of IgG₁, as shown by competition percentages obtained at low antibody concentrations (5 and 2.5 µg).

Epitope specificity of secreted Ig classes and subclasses induced after antigen presentation in the respiratory tract-associated lymphoid tissue by PRCV

IgA and IgG subclasses IgG₁ and IgG₂ were purified from colostrum and milk obtained on day 1 after farrowing from two PRCV-vaccinated sows (numbers 8 and 9). The animals conferred protective lactogenic immunity against TGEV of 62.5% and 28.5% piglet protection after challenge, respectively. In order to determine whether there were any similarities with GALT-induced responses in the Ig class induction by the different epitopes mentioned above, cELISAs were performed (Fig. 2). The induction in GALT of specific antibodies against the epitopes studied was much higher than the induction after stimulation of respiratory lymphoid tissue. This was evident from the much lower competition percentages shown by equal amounts of purified Ig classes, obtained from PRCV-vaccinated sows, as compared to TGEV-vaccinated sows. As was found for TGEV-vaccinated sows, both antigenic sites A and D were the best inducers of IgA, site D and subsite

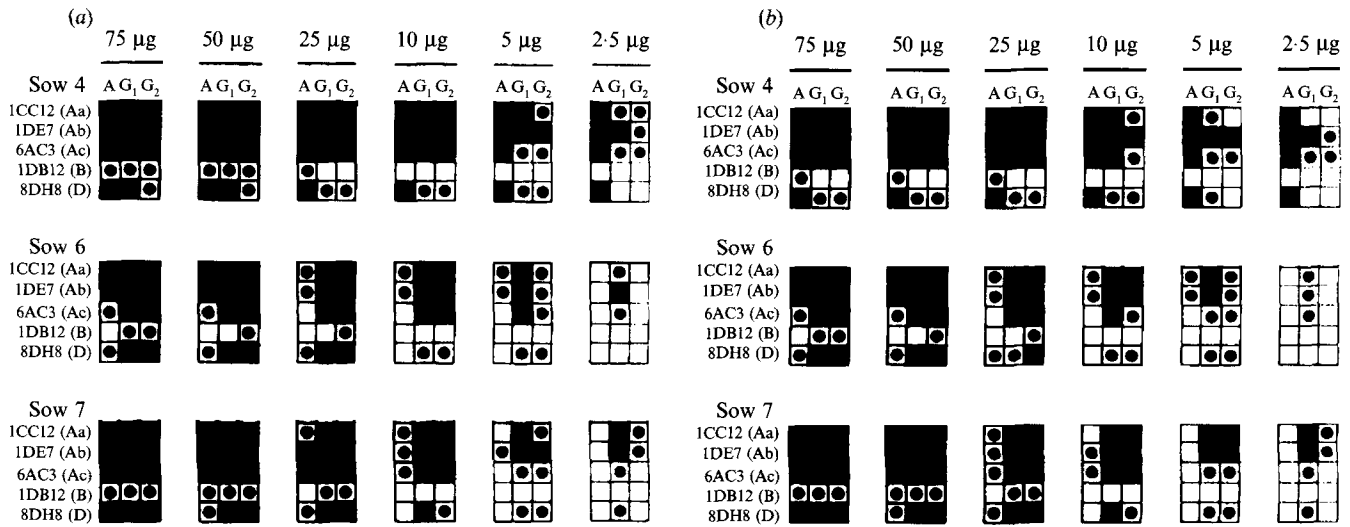


Fig. 1. Two-step competition binding assays to TGEV of different dilutions of IgA, IgG₁ and IgG₂ purified from colostrum (a) and milk (b) of TGEV-vaccinated sows (numbers 4, 6 and 7), with MABs directed to S glycoprotein antigenic sites A, B and D. MABs 1CC12, 1DE7 and 6AC3 define antigenic subsites Aa, Ab and Ac, respectively. Symbols: □, ◻ and ■ represent competition percentages of 0 to 35%, 36 to 70% and 71 to 100%, respectively.

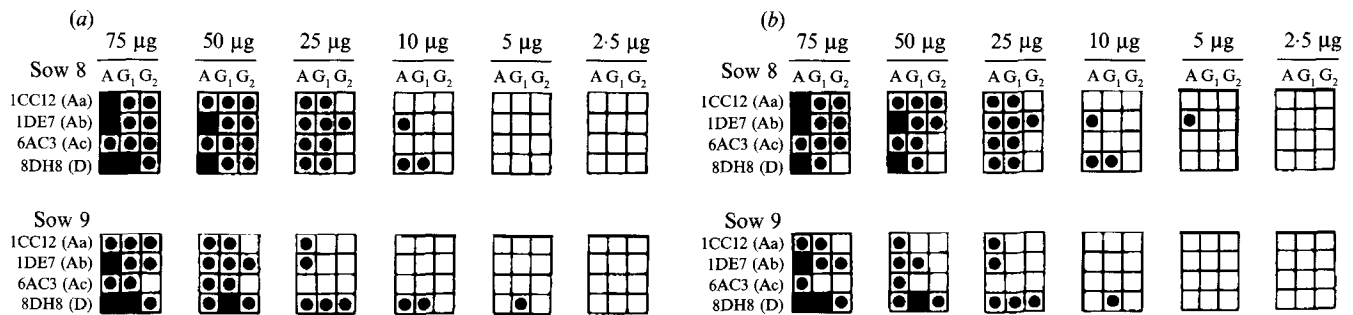


Fig. 2. Two-step competition binding assays to TGEV of different dilutions of IgA, IgG₁ and IgG₂ purified from colostrum (a) and milk (b) of PRCV-vaccinated sows (numbers 8 and 9), with MABs directed to S glycoprotein antigenic sites A and D. Symbols represent the same competition percentages as in Fig. 1.

Ab (defined by MAb 8DH8 and 1DE7, respectively) being the best IgA inducers, with blocking percentages of over 70% using 75 µg. Both antigenic sites also induced IgG subclasses, but to a lesser extent than class IgA. Site D was the best inducer of secreted IgG. As was observed for TGEV-induced antibodies, there was a slight preference towards the induction of IgG₁.

A schematic representation of the specificity of secreted Ig classes against S protein in milk after stimulation of the different compartments of the mucosal immune system in pregnant sows is shown in Fig. 3. When virus stimulation took place in GALT, it resulted in a similar IgA antibody immune response against all antigenic A subsites and site D. Virus replication in the lung induced a preferential IgA response against subsite Ab, among A subsites, and site D. Both antigenic sites A and D were also good inducers of IgG present in milk from TGEV-vaccinated sows. The antigenicity of site B

(only present in TGEV) was lower than that of the other sites and subsites, since the competition percentages shown by all purified Ig classes were never higher than 50%.

In vitro virus neutralization and in vivo protection assays with secreted purified Ig classes

The *in vitro* and *in vivo* consequences of antibody recognition of antigenic sites and subsites in protection were tested by analysing IgA and IgG with different epitope specificities obtained from the milk of sows after stimulation with TGEV or PRCV. We used IgA purified from milk collected on day 1 after farrowing from sows 4 and 7 (TGEV-vaccinated) and sow 8 (PRCV-vaccinated), and IgG and IgA purified from the milk of sow 7 collected on days 1 and 7 after farrowing, respectively.

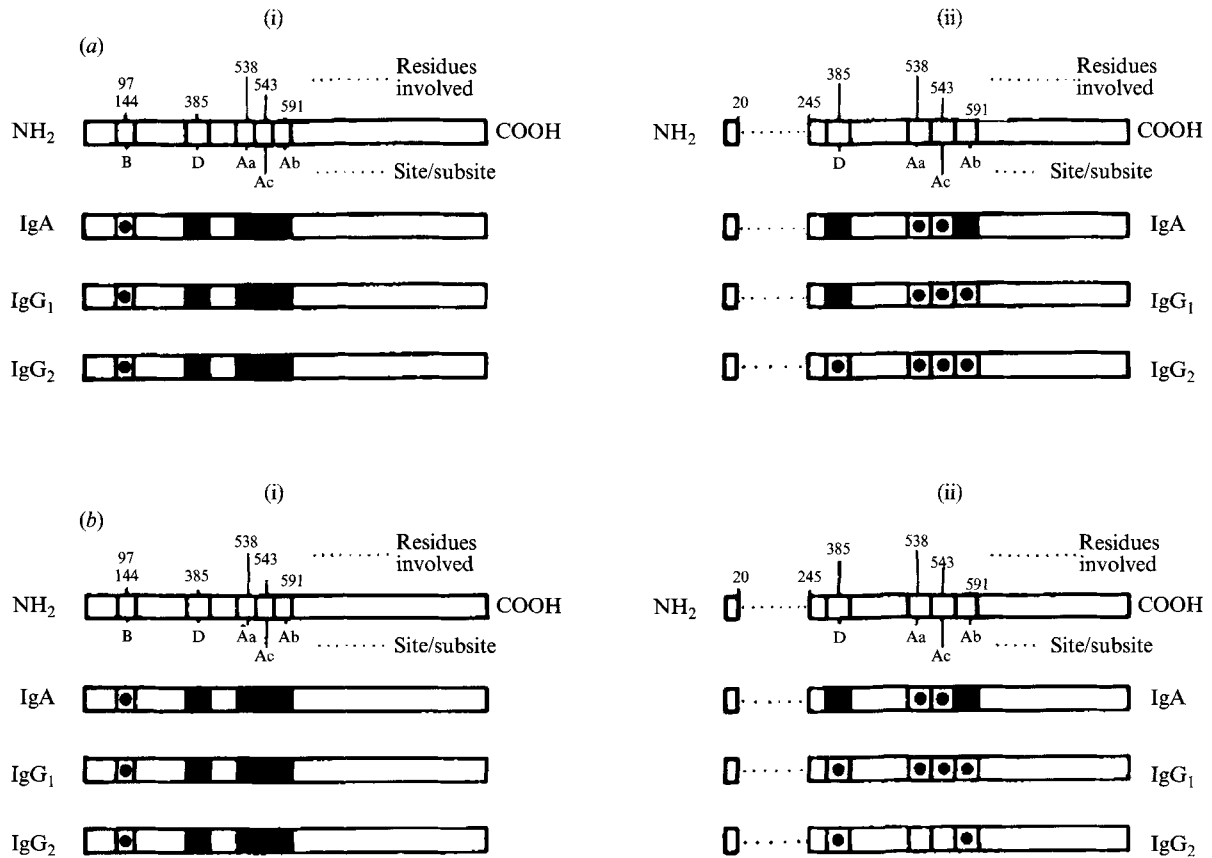


Fig. 3. Schematic representation of antigenic site and subsite specificity of Ig classes and subclasses on the sequences of TGEV (i) and PRCV (ii) S protein after vaccination of sows with both viruses. The data in this figure correspond to the mean of the competition values obtained with 75 µg of IgA, IgG₁ and IgG₂ purified from colostrum (a) and milk (b) and the MAbs defining the different antigenic sites and subsites. Symbols indicate the same competition percentages as in Fig. 1.

The specificities of the purified Ig classes tested are shown in Fig. 4. A comparison between one-step and two-step competition assays with an excess (75 µg) of purified Ig was carried out, in order to ascertain not only the quantity but also the quality of the specific secreted antibodies. IgA, specific for site A, from milk collected on day 1 from sows number 4 and 7 blocked the binding of the MAbs with competition percentages higher than 77% in both one- and two-step assays. Similar results were observed with this antigenic site using IgG purified from the milk sample obtained from sow 7. However, IgA from day 1 showed a slight increase in the blocking of MAbs 1DB12 and 8DH8 (specific for sites B and D respectively) when the assays were performed in two steps, rather than in one step, with competition percentages rising from 22% to 47% for site B and 66% to 78% for site D.

IgA purified from milk collected on day 7 from sow number 7 showed a much more efficient blocking of MAb binding when it was allowed to bind to virus before MAbs were present in the reaction. This result indicates a somewhat lower dissociation rate of the specific

secreted antibodies present in this sample than those obtained on day 1. This was especially remarkable with antibodies specific for subsites Aa and Ab, with blocking percentages rising from 58% to 76% and 40% to 78% respectively, and for site D, with an increase in competition percentage of 24% to 61%. Blocking percentages of subsite Ac-specific MAb were 53% and 69% in one-step and two-step assays, respectively.

Blocking percentages of MAb 8DH8 obtained with site D-specific PRCV-induced IgA did not significantly vary in one- or two-step assays, giving results of 73% and 71%, respectively. However, differences were observed in the competition obtained with antigenic subsite Ab MAb, competition percentages varying from 37% to 71% when the technique was performed in one or two steps respectively, and subsite Ac MAb with competition percentages varying from 23% to 56%.

To investigate further the differences between the purified Ig classes induced after vaccination with TGEV or PRCV and their consequences, virus neutralization and *in vivo* protection assays were performed. For the *in vitro* assay, plaque reduction analysis was carried out

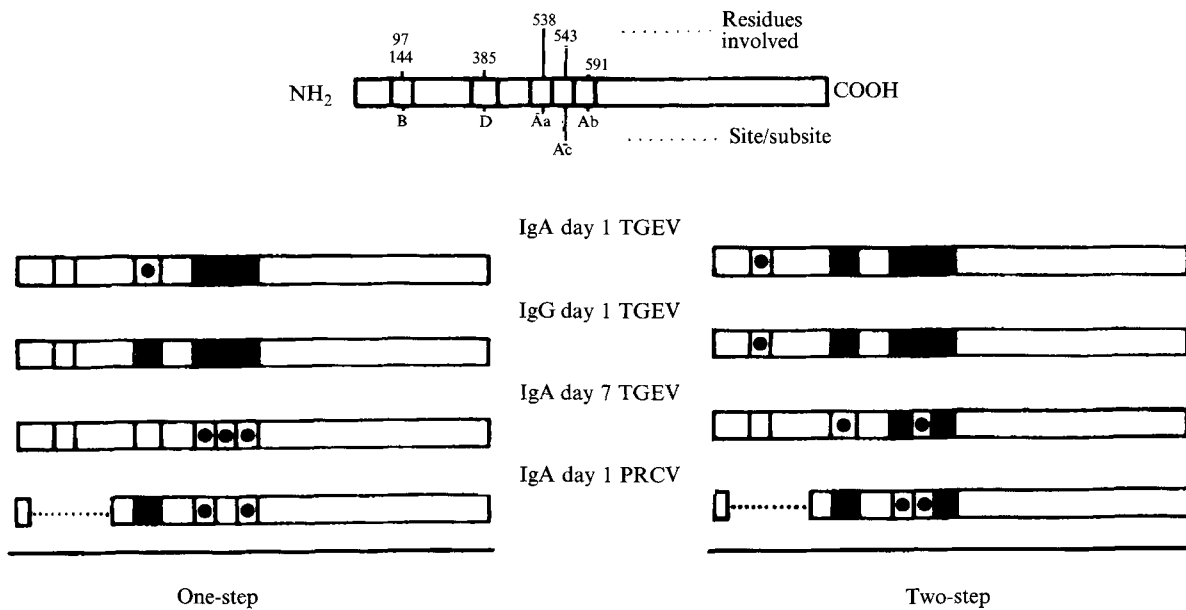


Fig. 4. Schematic representation of competition percentages obtained against the different antigenic sites and subsites, localized on the S protein sequence, with purified milk Ig classes and subclasses obtained from TGEV- or PRCV-vaccinated sows after one- or two-step competition assays with MABs. The samples employed (75 µg) for these assays were IgA purified from milk collected on day 1 after farrowing from TGEV-vaccinated sows 4 and 7 (mean value) and PRCV-vaccinated sow 8, and purified IgG and IgA obtained from the milk of sow 7 collected on days 1 and 7 after farrowing, respectively. Symbols represent the same competition percentages as in Fig. 1.

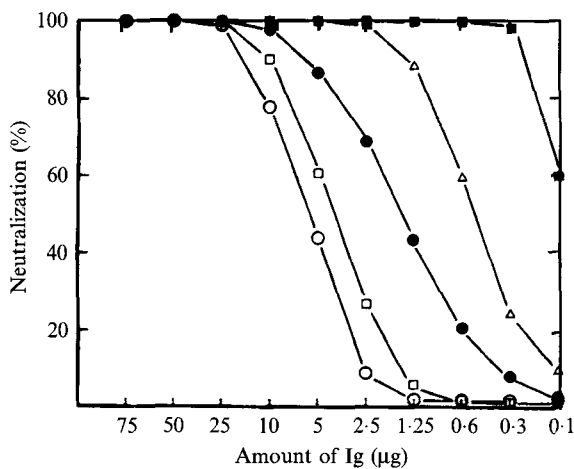


Fig. 5. *In vitro* protective properties of purified immunoglobulin classes. Neutralization curves obtained by a plaque reduction assay (10^2 p.f.u.) with IgA purified from milk collected on day 1 after farrowing from TGEV-vaccinated sow 4 (■) and sow 7 (□) and PRCV-vaccinated sow 8 (●), purified IgG obtained from the milk of sow 7 collected on day 1 (△) and IgA obtained from the milk of sow 7 collected on day 7 after farrowing (○).

with identical concentrations of Ig (Fig. 5). Secreted IgA purified from milk obtained on day 1 from sow 4 showed the highest neutralization titre. IgA purified from milk obtained on days 1 and 7 from TGEV-vaccinated sow 7 was capable of protecting cells *in vitro* to a similar extent, although with a lower neutralization titre, than that from

sow 4. IgG from milk collected on day 1 from sow 7 showed a higher titre than IgA obtained from the same milk. Finally, PRCV-induced IgA showed a higher *in vitro* neutralization titre than TGEV-immune sow 7 IgA purified from the milk obtained at day 1 after farrowing (Fig. 5).

Additional differences among the Ig samples analysed were found when the kinetics and reversibility of neutralization were studied (data not shown). TGEV-induced IgA from day 7 milk showed slower kinetics of neutralization than the other Igs studied, only reaching 100% neutralization after a 30 min incubation period. IgA and IgG purified from milk collected from this group of sows on day 1 neutralized 100% of viral infectivity after 1 min of the reaction. PRCV-induced IgA achieved complete viral neutralization after 2 min of reaction. These results may indicate a lower association rate of TGEV-induced IgA on day 7 and PRCV-induced IgA from day 1 milk, as was also suggested by the competition results against some antigenic sites.

Experiments on neutralization reversibility, performed by diluting the virus-antibody complexes to allow their dissociation, showed that neutralization of TGEV was reversed more than 20% only when the neutralizing antibody was IgA obtained from day 7 milk from sow 7, whereas neutralization by all other antibodies proved irreversible under the experimental conditions used (data not shown).

Table 1. Protection of piglets against TGEV using various Ig samples*

Origin	Ig class	Number of piglets	Percentage of protected piglets
TGEV-immunized sows			
Milk day 1 (sow 4)	IgA	4	100
Milk day 1 (sow 7)	IgA	6	100
Milk day 7 (sow 7)	IgA	6	0
Milk day 1 (sow 7)	IgG	4	50
PRCV-immunized sows			
Milk day 1 (sow 8)	IgA	6	33

* Ig samples were the same as those used in Fig. 5.

In vivo experiments on piglet protection were carried out with purified Igs (Table 1). While all purified Ig samples tested were able to protect cells *in vitro*, as shown above, only IgA purified from milk obtained on day 1 after TGEV-vaccination (sows 4 and 7) was capable of protecting 100% of the piglets. All piglets treated with IgA obtained from sow 7 milk collected on day 7, which had a similar neutralization titre *in vitro* as day 1 IgA, but different epitope specificity (partial recognition of site A and lower recognition of site D), developed typical clinical signs of transmissible gastroenteritis. PRCV-induced IgA, which also showed differences in antigenic site recognition with respect to TGEV-vaccinated sows' IgA (partial recognition of antigenic site A), was capable of protecting about 30% of piglets from TGEV infection. TGEV-induced IgG, very efficient both in competition and *in vitro* neutralization assays, protected 50% of the piglets assayed.

Finally, a group of piglets that received only colostrum from a TGEV-immunized sow was challenged with virulent virus 1 week after farrowing. This sow presented a high titre of TGEV-specific colostral antibodies, showing good recognition of antigenic sites A and D, similar to sow 4 (data not shown). All piglets showed mild transmissible gastroenteritis but were able to recover and, therefore, survived the exposure to virulent TGEV. This result indicates that continuous milk ingestion was not necessary for protection against TGEV in these piglets, which were challenged 7 days after specific antibody ingestion.

Discussion

Infection of sows during pregnancy with PRCV or TGEV causes an induction or rise in titre of lactogenic IgA, which neutralizes TGEV *in vitro* to a similar extent (De Diego *et al.*, 1992; Van Deun *et al.*, 1990). However, the main point that remains uncertain is the cause of the different efficiency with which lactogenic IgA induced in the gut and the respiratory tract protect suckling piglets

against enteric TGEV infection. Therefore, in this paper we have investigated the differences between secretory immune responses against TGEV neutralizing epitopes after TGEV and PRCV vaccination of pregnant sows.

A major objective of this study was to determine which of the known antigenic sites on TGEV or PRCV glycoprotein S, the main inducer of neutralizing antibodies (Delmas *et al.*, 1986; Garwes *et al.*, 1978; Jiménez *et al.*, 1986), were good inducers of IgA in colostrum and milk from immune sows. In this work we have shown differences in the induction of secreted Ig classes by different antigenic sites as a consequence of vaccination of pregnant sows with TGEV or PRCV, with sites A and D being the best inducers of IgA. We cannot disregard the fact that other, less well studied, viral epitopes may be relevant to the induction of secretory antibodies after infection by both viruses. However, those analysed in this work are the best characterized at present (Correa *et al.*, 1988, 1990; Gebauer *et al.*, 1991; Jiménez *et al.*, 1986) and seem to be the immunodominant sites (De Diego *et al.*, 1992; Jiménez *et al.*, 1986).

An adequate interpretation of the relative antibody response against the different viral epitopes by cELISA requires that the MAbs used, which bind the different antigenic sites, have similar isotype and relative affinities. All the MAbs used were type γ_1 , with the exception of MAb 1CC12, which was γ_{2a} . The relative affinities of the MAbs were shown by a RIA study of the binding of identical amounts of the purified Ig to TGEV. In the same assay, both the plateau (maximum binding) and the titres of all the MAbs were very similar (De Diego *et al.*, 1992).

Interestingly, the differences in epitope recognition of antibodies found after TGEV or PRCV vaccination seem to be due to the different tropism of the viruses, but not to unequal levels of virus replication. It has been described that PRCV replicates in the lung to the same extent as TGEV does in the gut (Cox *et al.*, 1990; using the same PRCV strain as us). Recently a controversy has arisen, concerning whether BALF is really an integral structure of the lung and the common mucosal immune system, since it has been found that BALF is not a constitutive structure in many mammals and seems to play a minor role in the lung immune system (Pabst, 1992). A further study of the mucosal immune system of the pig remains to be carried out, but these findings might account for the lower secretory response against PRCV shown by pregnant sows when compared to the response obtained against TGEV.

In order to investigate the factors, other than epitope recognition, influencing the passive protection of piglets further, we studied the characteristics of isolated milk Ig classes in terms of affinity and neutralization capacities *in vitro* and *in vivo*. Purified IgA from milk collected on day

1 after farrowing from TGEV-vaccinated sows, which recognized both sites A and D with high affinity, was able to protect 100% of piglets assayed, in experiments carried out under saturating conditions. In contrast, purified IgA from milk collected on day 7, which recognizes mainly site A and shows low association and dissociation rates, was not able to protect the piglets under the same saturating conditions. Affinity differences observed among IgA of day 1 and 7 could also explain both the retarded kinetics of neutralization and its reversibility by dilution of virus-antibody complexes. Purified IgA from the PRCV-immune sow mainly bound to site D, with high affinity, whereas it needed a two-step competition assay to significantly block the binding of at least one antigenic A subsite-specific MAb. Competitions performed with neutralizing MAbs against antigenic site D showed similar percentages of binding inhibition, with competitor antibodies, as those observed with the non-neutralizing MAb presented in this work, both in TGEV and PRCV-vaccinated sows (data not shown). Interestingly, the *in vivo* protection rate for this purified IgA only reached about 30%.

These findings suggest that both antigenic sites A and D may play an important role in the lactogenic protective immune response to TGEV, the induction of IgA in milk by both antigenic sites being necessary for *in vivo* protection. For *in vitro* protection the binding of antibodies to just one site would seem to be required. This requirement for antibodies to bind to more than one antigenic site involved in virus neutralization for lactogenic protection of newborn piglets agrees with the results obtained by Wesley *et al.* (1988), who observed a lack of protection *in vivo* with neutralizing MAbs specific for S and M glycoproteins.

We were also interested in the selective epitope induction of other Ig classes, mainly IgG, which is present in colostrum and milk during early lactation at high concentrations (colostrum and early milk contain more IgG than IgA) and therefore cannot be disregarded as a major protective Ig (Stone *et al.*, 1977). Our results suggest that IgG may play an important role in lactogenic immunity. The lower *in vivo* protection rate shown by IgG antibodies when compared to IgA might be due to the fact that this isotype is less resistant to enzymatic digestion. Furthermore, the gastrointestinal tract of a 3- to 5-day-old piglet may not be representative of the gastrointestinal tract of the 1- to 2-day-old piglet in terms of levels of degradative enzymes and absorption of Igs. The high levels of IgG observed in colostrum probably play an even more important role than suggested by the results from challenge of 3- to 5-day-old piglets.

To conclude, we have been able to confirm that IgA is, as it has been described (Bohl, 1981; McGhee, 1992;

Moxley *et al.*, 1989; Saif & Bohl, 1979), the most important class of Ig in passive protection of neonatal pigs. However, our results suggest that IgA must recognize at least antigenic sites A (including all subsites) and D with high affinity to be protective. In our experience, this kind of IgA is induced only when virus epitopes significantly stimulate GALT as consequence of TGEV infection. All these factors should be taken into account when designing a strategy of immunization against TGEV with a subunit vaccine.

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