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Cooperation between transmissible gastroenteritis coronavirus (TGEV) structural proteins in the in vitro induction of virus-specific antibodies

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Abstract

Following infection of haplotype defined NIH-miniswine with virulent transmissible gastroenteritis coronavirus (TGEV), isolated mesenteric lymph node $CD4^+$ T-cells mounted a specific proliferative response against infectious or inactivated purified virus in secondary in vitro stimulation. A specific, dose-dependent response to the three major recombinant viral proteins: spike (S), membrane (M), and nucleoprotein (N), purified by affinity chromatography, was characterized. Induction of in vitro antibody synthesis was analyzed. The purified recombinant viral proteins induced the in vitro synthesis of neutralizing TGEV-specific antibodies when porcine TGEV-immune cells were stimulated with each of the combinations made with two of the major structural proteins: S + N, S + M, and to a minor extent with M + N, but not by the individual proteins. S-protein was dissociated from purified virus using NP-40 detergent and then micellar S-protein oligomers (S-rosettes) were formed by removing the detergent. These occurred preferentially by the association of more than 10 S-protein trimmers. These S-rosettes in collaboration with either N or M-proteins elicited TGEV-specific antibodies with titers up to 84 and 60%, respectively, of those induced by the whole virus. N-protein could be partially substituted by a 15-mer peptide that represents a T helper epitope previously identified in N-protein (Antón et al. (1995)). These results indicate that the induction of high levels of TGEV-specific antibodies requires stimulation by at least two viral proteins, and that optimum responses are induced by a combination of S-rosettes and the nucleoprotein. Copyright © 1996 Elsevier Science B.V.

Keywords: Coronavirus; TGEV; Antibody synthesis

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1. Introduction

Porcine transmissible gastroenteritis is a highly contagious enteric disease of swine caused by transmissible gastroenteritis virus (TGEV), which belongs to the Coronaviridae family and has a positive-stranded polyadenylated RNA genome of 28.5 kb (Eleouet et al., 1995; Mendez et al., 1996). The disease usually causes only a transient diarrhea in adults but is a major cause of death in piglets under 2 weeks of age (Saif and Bohl, 1986). The viral genome encodes three major structural proteins: S, M, and N-proteins, composed of 1447, 262, and 382 amino acids (aa), respectively (Enjuanes and Van der Zeijst, 1995). A fourth structural protein has more recently been described, the small membrane (sM) protein (Godet et al., 1992). Protein S is the major inducer of TGEV neutralizing antibodies (Jiménez et al., 1986; Laude et al., 1986).

Lactogenic immunity, induced in sows after natural infection or oral immunization, appears to be the most important mechanism protecting newborn piglets against TGEV infection (Bohl and Saif, 1975). Protection can be provided by antibodies, without a requirement for cytotoxic responses. Newborn animals can be protected by artificial lactogenic immunity using IgG and sIgA immunoglobulin fractions from immune colostrum or serum (De Diego et al., 1992; Stone et al., 1977; Wesley et al., 1988). Therefore, it is of interest to identify immunogenic structures formed by viral components which elicit strong antibody immune responses.

TGEV is a T-cell dependent antigen (Bullido et al., 1989). For effective activation of the humoral immune response, determinants recognized by both B and T helper (Th) cells are required. The antigenic structure of the S-protein has been defined for the B-cell compartment. In this protein, four antigenic sites (A, B, C, and D) have been identified (Correa et al., 1988; Delmas et al., 1990; Gebauer et al., 1991). In swine, TGEV induces antibodies which bind to all these antigenic sites (Correa et al., 1988).

Although the T-cell responses to the whole virus using TGEV and porcine respiratory coronavirus (PRCV) have been defined (Brim et al., 1994), little information is available on the identification of well defined T-cell epitopes of TGEV (Antón et al., 1995; Enjuanes, 1995). S, N, and M-proteins from coronavirus are relevant targets for cellular immune recognition (Enjuanes, 1995). N-protein is frequently involved in T-cell responses to coronaviruses (Bergmann et al., 1993; Stohlman et al., 1992; Stohlman et al., 1995). The nucleoprotein of infectious bronchitis virus (IBV) is recognized by IBV-specific murine T-cell hybridomas (Boots et al., 1991) and T-lymphocytes from IBV immune chicken (Boots et al., 1992). An immunodominant epitope located in the carboxyterminal end of the N-protein of mouse hepatitis virus (MHV) contributes to protection by $CD4^+$ T-cells (Heemskerk et al., 1994; Wege et al., 1993). N-protein of MHV also contains an epitope recognized by cytotoxic T-lymphocytes (Bergmann et al., 1993). S (Flory et al., 1993; Körner et al., 1991) and M (Mobley et al., 1992; Xue et al., 1995) proteins also have been involved in cellular immune responses to MHV infection in mice.

Collaboration between B and T-cell antigenic determinants may lead to the induction of optimum immune responses to coronaviruses. In fact, S and N-proteins independently elicited protection to MHV, as shown by using recombinant adenovirus expressing these proteins, but co-immunization with both S and N-proteins provided the best results (Wesseling et al., 1993).

In this paper we report the induction of T-cell responses by TGEV and its major structural proteins. In addition, it is shown that co-stimulation with at least two structural viral proteins is necessary for the in vitro induction of TGEV-specific neutralizing antibodies by TGEV-immune porcine lymphocytes. Optimum immunogenic responses were elicited by in vitro co-stimulation with S-rosettes and N-protein.

2. Materials and methods

2.1. Animals

Ten to 13-day-old inbred miniature swine expressing three independent homozygous SLA hap-

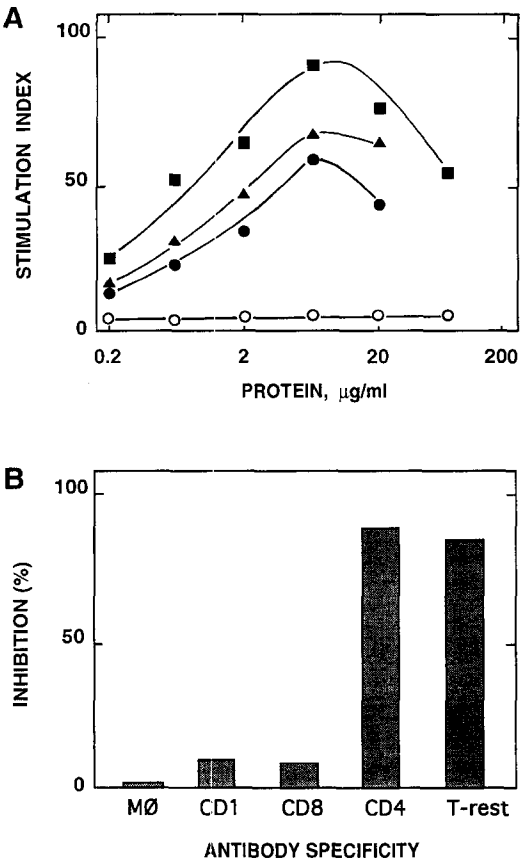

Fig. 1. Antigen dose-dependency of the blastogenic response to TGEV. (A) The reactivities of nylon wool-fractionated MLN cells from TGEV-infected miniswine collected 5 days after the last booster dose are shown. Cells from TGEV immune miniswine expressing *aa* (▲), *cc* (●) or *dd* (■) haplotypes were cultured with dilutions of purified TGEV (closed symbols) or MHV (○). Data shown represent mean stimulation indices of assays with MLN cells from 5 *dd*, 4 *aa* and 2 *cc* haplotype miniswine. Background values of control cultures were similar for cells with the three haplotypes, ranged between 350 and 500 counts per minute and the standard errors of triplicate determinations were less than 20% of the mean. (B) Inhibition of T-cell proliferation of MLN cells from TGEV-infected *dd* miniswine with MAbs specific for porcine leukocyte surface markers. TGEV-primed MLN cells (4×10^5 cells/well) were cultured for 4 days with 7 μg/ml of purified TGEV. Supernatants from hybridoma cultures of each monoclonal antibody were added at the beginning of culture to a final concentration of 10%. Inhibition of antigen stimulated proliferation by MAbs was calculated as % inhibition = [1 − (SI in the presence of virus plus MAb/SI in the presence of virus)] × 100. The results are the mean of three experiments with triplicate cultures.

lotypes (Sachs et al., 1976), $SLA^{a/a}$ (*aa*), $SLA^{c/c}$ (*cc*) and $SLA^{d/d}$ (*dd*), reared at Granja Cantoblanco de Animales de Laboratorio (Comunidad de Madrid, CSIC), were used for viral infection. One-month-old *dd* haplotype animals were used for immunization with purified virus.

2.2. Cell lines and viruses

The epithelial swine testicle cell line (ST) (McClurkin and Norman, 1966) was used to grow attenuated (PUR46-MAD) and virulent (MAD88) strains of TGEV (Sánchez et al., 1990). PUR46-MAD was purified through two sucrose gradients as previously described (Correa et al., 1988) and used for in vivo immunizations and for in vitro stimulations. The virus used in the in vitro stimulations was inactivated by UV irradiation unless otherwise indicated. Virus titration was performed as previously described (Jiménez et al., 1986). MHV A59 strain (kindly provided by K. Holmes) was grown in 3T3 cells and purified as described (Sánchez et al., 1990).

2.3. Recombinant viral proteins and synthetic peptide production

TGEV structural proteins were expressed in *Escherichia coli* using the pMAL vectors (New England Biolabs) which yield cytoplasmic proteins fused to maltose-binding protein (MBP). N gene was cloned into pMALcRI vector and S and M genes were cloned into pMALc-2 vector. MBP, used as control antigen, was obtained from a modified pMALc vector (kindly provided by P. L. Rodríguez, CNB, Madrid), in which a stop codon was introduced after mal E gene to prevent β-galactosidase-α expression. All plasmids were replicated in *E. coli* strain DH5 except pMALcRI-N, which was maintained in *E. coli* strain JM109. Recombinant proteins were solubilized after cell disruption by sonication and freeze-thawing, and were clarified by centrifugation at 9000*g*. Fusion proteins were purified by amylose resin affinity chromatography following the manufacturer's instructions (New England Biolabs) with the following modifications: after binding of induced bacterial extracts, column was washed with four

Table 1
Blastogenic response to infectious or inactivated purified TGEV

Virus[a]	Inactivation[b] procedure	Infectivity[c] (PFU/ml)	^{3}H-thymidine incorporation (cpm $\pm \sigma_{\bar{x}}$)[d]
Control	(no virus)	—	424 ± 46
MHV	—	N.D.	479 ± 97
TGEV	—	2×10^9	67.819 ± 12.489
	95°C	2×10^2	53.292 ± 9.160
	US	4 ± 10^7	68.999 ± 10.566
	UV	2 ± 10^5	47.239 ± 9.209
	US+UV	$<2 \times 10^2$	71.791 ± 11.704

[a] Proliferation assays were performed with final vims concentrations of 5 μg/ml.
[b] US, ultrasounds; UV, ultraviolet light.
[c] N.D., not determined. MHV does not infect porcine cells; $<2 \times 10^2$, infectivity was less than the detection limit of the titration assay (200 PFU/ml).
[d] $\sigma_{\bar{x}}$ standard error, defined as $\sigma/\sqrt{n}$, where σ is standard deviation and n the number of values.

column volumes of 20 mM Tris–HCl (pH 7.4), 500 mM NaCl, 1 mM EDTA, 4 column volumes of column buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA), and four column volumes of 0.1 M phosphate buffer pH 7.0, 30 mM NaCl. Elution buffer was 0.1 M phosphate buffer pH 7.0, 30 mM NaCl, 10 mM maltose. Purified fusion proteins were analyzed by SDS–PAGE (Laemmli, 1970) and Western blot (Towbin et al., 1979).

Fifteen-mer N_{321} peptide was synthesized and assayed as previously described (Antón et al., 1995). Based on the sequence of the S-protein, a complete set of overlapping 15-mer peptides (1433 peptides) was synthesized on polyethylene pins according to standard PEPSCAN procedures (Geysen et al., 1984), but with modifications to release them in free form (Valero et al., 1993). The 15-mer S-protein peptides were acetylated at their amino-terminus, and contained an amidated alpha-alanine at their C-terminus.

2.4. Proliferation assays

One month old, haplotype defined NIH-miniswine (Lunney et al., 1986; Sachs et al., 1976) were intragastrically inoculated with 2×10^8 plaque forming units (PFU) of virulent MAD88 virus and boosted 2 weeks later with the same dose of virulent MAD88 virus, followed by an intra-muscular (i.m.) and a intra-peritoneal (i.p.) injection of 90 μg of purified PUR46-MAD virus in phosphate buffered saline (PBS). Mesenteric lymph nodes (MLN) were collected 5 days after the last booster dose. In vitro proliferation assays were performed using nylon wool enriched T-cells as previously described (Antón et al., 1995). MLN cells were pulse-labeled with [^{3}H]thymidine (Amersham, TRA 310, 1 μCi per well) for 18 h, harvested, and evaluated for incorporation of radiolabeled precursor using a liquid scintillation spectrometer. The standard errors of triplicate determinations were less than 20% of the mean and are not shown. Results were expressed as stimulation index (SI): mean counts per minute (cpm) incorporated in the presence of antigen divided by mean counts per minute incorporated in the absence of antigen.

2.5. Inhibition of blastogenesis by MAb specific for cell surface markers

Murine hybridoma cell culture supernatant fluid containing MAbs to porcine surface leukocyte antigens [anti-CD4 (74-12-4), anti-CD8 (76-2-11), anti-CD1 (76-7-4), anti-resting lymphocyte marker (76-6-7), or anti-macrophage marker (76-5-28) (Pescovitz et al., 1984)] were added at 10% (v/v) to virus-stimulated MLN cell cultures at the time of initiation of the culture. MAbs were kindly provided by Joan Lunney, Beltsville, MD.

2.6. Purification of micellar aggregates of S-protein (rosettes)

S-rosettes were purified in sucrose gradients. PUR46-MAD virus purified as previously described (Jiménez et al., 1986) was dissociated with NP-40 (200 μg of purified virus in 200 μl of PBS were mixed with 200 μl of 1% NP-40 and incubated for 20 min at room temperature). Dissociated virions were centrifuged 45 min through a 4.6 ml linear 15–40% sucrose gradient, at 27 000 rpm in a Beckman SW 55Ti rotor at 4°C. The gradients were fractionated into 24 aliquots of 200 μl which were diluted 3-fold in PBS and centrifuged 8 min at 90 000 rpm in a Beckman TLA 120.1 rotor. S-rosettes were negatively stained with 2% sodium phosphotungstate and analyzed by electron microscopy following standard procedures (Risco et al., 1996).

2.7. In vitro antibody synthesis

In vitro antibody synthesis was performed with macrophage-depleted peripheral blood leukocyte (PBL) cells obtained from animals hyperimmunized with purified PUR46-MAD inactivated with 0.5% formaldehyde. Miniature swine of *dd* haplotype were immunized by the i.m. and i.p. routes with four doses of 35 μg of PUR46-MAD virus at 2-week intervals, and the in vitro antibody synthesis was performed as previously described (Antón et al., 1995). Briefly, 4×10^5 cell/well in complete RPMI medium with 10% fetal bovine serum (FBS) were incubated in flat-bottomed microtest II plates with purified virus, purified recombinant viral proteins, N_{321} peptide, or S-rosettes. Supernatants were harvested after 7 days. Specific antibodies were determined by solid-phase radioimmunoassay (RIA) (Jiménez et al., 1986). TGEV neutralization assay was performed by incubating 50 μl of two-fold dilutions of the antibody supernatants with 50 μl of PBS 2% FBS containing 10, 10^2, 10^3, or 10^4 PFU of TGEV at 37°C for 30 min. The mixture was plated on confluent ST-cells, allowed adsorption for 1 h and incubated for 48 h. Neutralization indices were calculated as the log of the ratio between the virus titer in the presence of control supernatant or in the presence of antibody-containing medium.

3. Results

3.1. In vitro proliferative response of T-cells from immune haplotype-defined miniswine to TGEV

Immune leukocytes from the three tested haplotypes strongly responded to in vitro TGEV stimulation in a dose-dependent manner (Fig. 1A). The optimum stimulation indices ranged from 45 to 95, and optimal stimulating antigen concentrations ranged from 2 to 20 μg/ml in different swine. Miniswine of *dd* haplotype elicited the

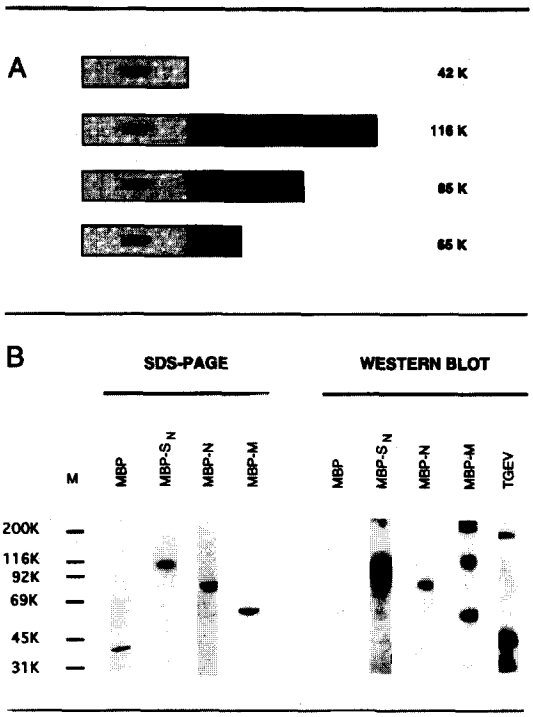

Fig. 2. Purification of TGEV recombinant antigens used in the blastogenic assays. (A) Schematic diagram of the fusion proteins and their expected M_r. MBP, maltose binding protein. S_N, N, and M, represent the amino terminus of S-protein (amino acids 1–746), N, and M-proteins, respectively. (B) SDS–PAGE and Western blot analysis of affinity-purified fusion proteins performed as described in Section 2. The western blot was probed with TGEV-immune rabbit serum, diluted 1/500, preadsorbed with an *E. coli* lysate.

highest responses with mean SI values of 95. Lymphoproliferative responses were TGEV specific, since MLN cells from TGEV-immune *dd* animals proliferated to TGEV but not to MHV, a serologically unrelated coronavirus (Fig. 1A). Moreover, MLN cells from non-immune animals did not proliferate in response to TGEV (data not shown). Lymphocytes from MLN gave the strongest response after intra-gastric and i.p. inoculation of TGEV when compared with PBL or Peyer's patches cells (data not shown).

In infected animals, TGEV can be recovered from macrophages and other cells of the reticuloendothelial system (Underdahl et al., 1974). TGEV infects alveolar macrophages but not porcine blood monocytes (Laude et al., 1984). Blastogenic responses could be increased by infection of macrophages or MLN lymphocytes due to the synthesis of viral antigens, or could be decreased by either altering antigen presentation or the responder cells. Thus, we studied the lymphoproliferative responses upon in vitro stimulation with live or inactivated TGEV. Antigen pool aliquots were inactivated by heat, UV light, or ultrasonic (US) treatment. Residual infectivity was evaluated in ST-cell culture. The lymphoproliferative response to TGEV was independent of viral infectivity and the method of virus inactivation used (Table 1).

3.2. Inhibition of the blastogenic response to TGEV by MAb specific for porcine leukocyte surface markers

To assess the contribution of the different leukocyte populations to TGEV-specific blastogenesis, T-cell-enriched lymphocytes from immune swine were incubated with TGEV in the presence of MAbs specific for porcine CD4, CD8, CD1, macrophages, or resting lymphocytes marker. $CD4^+$ and $CD8^+$ specific MAbs inhibited the blastogenic response by 80 and 11%, respectively (Fig. 1B). Antibodies specific for resting lymphocytes marker inhibited the response by 75%. MAb to macrophages and to CD1 thymocyte antigen either did not inhibit the response or caused a 12% reduction, respectively. These data indicate that the $CD4^+$ lymphocyte subset is the major responder to TGEV antigens.

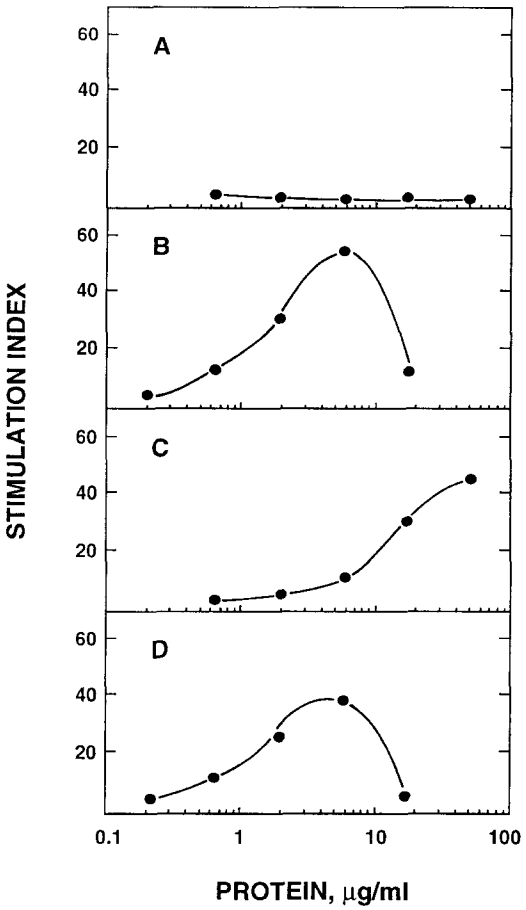

Fig. 3. Proliferative responses to TGEV structural proteins S, N, and M. TGEV-immune *dd* T-lymphocytes were cultured with affinity-purified viral recombinant fusion proteins: MBP-S_N (B), MBP-N (C), and MBP-M (D) for 4 days and then pulsed and harvested as described. As a control, MBP alone (A) was used. The mean of four experiments performed with cells from two animals is reported. Protein concentration refers to the amount of viral recombinant protein, except for MBP where total protein is represented. Average background levels were 550 cpm.

3.3. Recombinant protein production and purification

To determine the contribution of different virus structural proteins to the in vitro proliferative response of cells from the infected animals, TGEV S, N, and M genes were cloned in pMAL vectors, expressed in *E. coli* as MBP-fusion proteins and

purified by affinity chromatography as described in Methods. The S gene was divided into two fragments, the amino half (S_N) fragment spanned from nt 1 to 2239, and the carboxy-half (S_C) from nt 2240 to 4341. A schematic representation of fusion proteins and their M_r is provided (Fig. 2A). Since MBP-S_C was expressed only at very low levels, its purification was not attempted. Fusion proteins eluted from the affinity columns were analyzed by SDS–PAGE under reducing conditions and silver stained (Fig. 2B). MBP, MBP-N, and MBP-M migrated as major bands of M_r 42, 85 and 65 K, respectively. Viral proteins were stained in the Western blot with TGEV-specific antibodies. In addition, MBP-M-protein regularly showed products of high molecular weight binding M-specific MAbs in the Western-blot analysis, which probably correspond to aggregates of this protein. MBP-S_N migrated as a main band of 116 kD accompanied by other minor bands, presumably degradation products or aggregated forms as shown by Western-blot analysis probed with 1/500 dilution of rabbit anti-TGEV serum (Fig. 2B) or rabbit anti-MBP serum (data not shown).

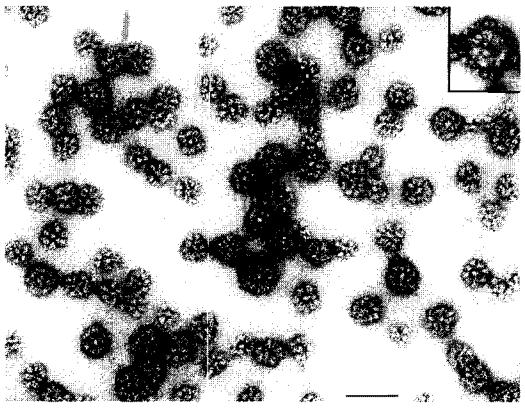

Fig. 4. Purified micellar S-rosette oligomers. TGEV purified in sucrose gradients (Jiménez et al., 1986) was dissociated with NP-40 and the micellar S-rosettes were formed during detergent removal by centrifugation on a sucrose gradient. S-rosettes were negatively stained with 2% sodium phosphotungstate and studied by electron microscopy. The large figure shows that S-rosettes formed micellar aggregates with a variable number of S peplomers, but the aggregates usually included more then ten S-peplomers. A TGEV particle has been included in the inset for size comparative purposes. Bar length represents 100 nm.

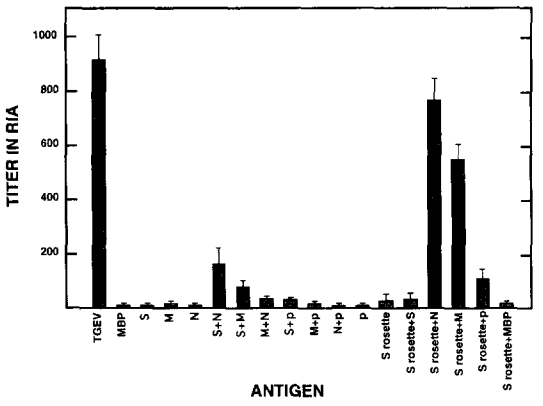

Fig. 5. In vitro synthesis of TGEV-specific antibodies by immune cells stimulated with combinations of viral derived antigens. TGEV-immune macrophage-depleted PBL (4×10^5 cells/well) (Antón et al., 1995) were incubated in the presence of the indicated antigens. After culture for 7 days antibody synthesis was evaluated by RIA using purified TGEV as antigen. Titer was defined as the maximum supernatant dilution that bound three-fold the background radioactivity. Medium values of four experiment are shown. Bar represents standard error of the mean. MBP, maltose binding protein. S, M, and N represent purified MBP fusion proteins: spike, membrane, and nucleoprotein, respectively. p, N-protein derived peptide containing a T helper epitope (N_{321}) (Antón et al., 1995). S-rosette, micellar S-oligomers.

3.4. Specific proliferation of T-cells from TGEV immune miniswine to the three major structural viral proteins

Purified MBP-β-galactosidase-α (MBP-β-galα) expressed from pMALcRI induced a significant T-cell response which obscured the response to the fusion partner (data not shown). Therefore, MBP constructions with a stop codon after the coding sequence of MBP were used and purified MBP assayed for lymphoproliferation. Blastogenic responses to MBP were similar to that of medium alone (Fig. 3A). A stop codon was created after the coding sequence of the S_N fragment to prevent β-galα expression and nonspecific stimulation.

Dose response curves of the blastogenic response of nylon wool-purified MLN cells from TGEV-immune *dd* miniswine to the three main

structural proteins of TGEV are presented in Fig. 3B–D. MBP-S_N induced the strongest response (SI 56) at an optimal concentration of 6 μg/ml (Fig. 3B). In addition, strong responses against MBP-N (SI 43) and MBP-M (SI 37) were detected (Fig. 3C and 3D, respectively). Antigen concentrations required for maximal proliferation were 50 μg/ml for MBP-N and 6 μg/ml for MBP-M. The response against the recombinant viral proteins was specific, since proliferation of immune cells to purified MBP (Fig. 3A) and proliferation of lymphocytes from uninfected miniswine to fusion proteins were not detected (data not shown).

S-protein is the major inducer of neutralizing antibodies. Thus it was interesting to locate T epitopes on S-protein using the pMAL vectors and to relate them with previously described B epitopes from S-protein. We expressed and purified fragments of TGEV S-protein containing antigenic sites C and B; C, B, and D; C, B, D, and A; or A alone (Gebauer et al., 1991) and analyzed their ability to induce proliferation of immune cells (data not shown). The four S-protein fragments elicited responses with stimulation indices that were not significantly different.

The proliferation induced by 120 pools of twelve 15-mer overlapping peptides staggered by 1 aa, covering the entire sequence of S-protein using immune cells from three NIH-miniswine with *dd* haplotype was determined (data not shown). None of the pools comprising 1433 peptides elicited significant T-cell responses, in contrast to the clear response shown by the same cells in response to the N-protein derived peptide N_{321} which contains a functional T-cell epitope (Antón et al., 1995).

3.5. Purification of micellar S-protein oligomers

S-rosettes were purified from NP-40 dissociated TGEV in sucrose gradients and visualized by negative staining (Fig. 4). S-rosettes were usually formed by more than 10 peplomers. These structures were found in the top part of the gradient (fractions 6–10), but were present in highest concentration in fraction 6. This fraction only contained S-protein as determined by PAGE in the presence of SDS (results not shown).

3.6. In vitro antibody synthesis of TGEV-specific antibodies after stimulation of lymphocytes from TGEV-immune miniswine with recombinant viral purified proteins

A system for TGEV-specific antibody synthesis described previously (Antón et al., 1995) was used to determine the viral proteins required to induce in vitro antibody synthesis. The helper activity of a single strong T epitope in the presence of several weak T epitopes was also studied. For this purpose, TGEV-immune PBL were depleted of macrophages and cultured in the presence of several antigens (Fig. 5). The specificity of the response was demonstrated because virus-specific antibodies were generated only by TGEV- or TGEV proteins-stimulated cells, while control antigens as MHV or MBP never induced the in vitro synthesis of TGEV-specific antibodies (Fig. 5). Moreover, nonspecific stimulation of T-cells by Concanavalin A did not induce antibody synthesis (data not shown). None of TGEV proteins alone (S, N, or M) induced significant amounts of TGEV-specific antibodies, but 20% of the total antibody synthesis induced by purified TGEV was reconstituted after stimulation with a combination of recombinant purified S and N-proteins. Combined stimulation by S + M-proteins also yielded significant TGEV-specific antibodies as deter-

Table 2
In vitro synthesis of TGEV neutralizing antibodies

Antigen[a]	Neutralization index[b]
TGEV	3.7
MBP	<0.3
S-protein	<0.3
N-protein	<0.3
M-protein	<0.3
S+N	1.7
S+M	1.5
S-rosettes+N	3.0
S-rosettes+M	<0.3
S-rosettes+p	0.6

[a] MBP, maltose binding protein; S, N, M, TGEV proteins; p, N_{321} peptide containing a T helper epitope.
[b] The neutralization index is defined as the ratio between the log of virus titer in the presence of control medium or in the presence of antibody.

mined by RIA (Fig. 5). M + N-proteins gave weak responses above control values. When N-protein was substituted by the 15-mer N_{321} peptide containing a strong T-cell epitope, a weak but significant antibody synthesis was also detected.

Interestingly, cultures of immune lymphocytes stimulated with S-rosettes in collaboration with either N or M-proteins also induced high antibody titers to TGEV (Fig. 5). In lymphocytes cultures stimulated with S-rosettes, the antibody responses were partially elicited if the N-protein was substituted by the N-protein derived T-cell peptide N_{321} containing an strong T-cell epitope.

Culture supernatants from TGEV stimulated cultures neutralized TGEV infectivity (neutralization index (NI) 3.7) (Table 2). Lymphocyte cultures stimulated with either S, N, or M-proteins did not elicit virus neutralizing antibodies, while mixtures of proteins S + N or S + M, and S-rosettes plus N-protein induced high levels of TGEV neutralizing antibodies (NIs of 1.7, 1.5, and 3.0, respectively). Unexpectedly, S-rosettes plus M-protein induced antibodies that bound TGEV in the RIA assay but that did not neutralize its infectivity (Table 2).

4. Discussion

In this report we show that TGEV-immune swine elicited strong T-cell responses to the whole virus and to the three major structural proteins (S, M, and N). In addition, it has been shown that the in vitro induction of TGEV-specific antibodies requires the stimulation by a combination of S and N or M-proteins. Interestingly, micellar S-rosettes in collaboration with N-protein reconstituted 84% of the in vitro antibody synthesis elicited by the whole virus.

New vaccines tend to include only those immunogens that induce a protective immune response despite antigenic variability, underscoring the need to identify essential B and T-cell epitopes. Intragastric infection of swine with a virulent TGEV strain (MAD88) induced strong blastogenic responses preferentially in the MLN. Weaker responses were found in PP and PBL, as expected for infections preferentially localized in the gastro-intestinal tract. Vigorous MLN proliferation responses to virulent TGEV have also been reported (Brim et al., 1994). Analysis of the humoral responses in virulent TGEV-exposed pigs demonstrates the greatest numbers of antibody-secreting cells in the MLN (Berthon et al., 1990; VanCott et al., 1993). In this report, the blastogenic response of lymphocytes from TGEV-infected miniswine, boosted with inactivated virus, is characterized. The blastogenic response was of similar magnitude for S, M, and N-proteins, although optimum responses to N-protein required higher protein concentrations. This effect might be due to a lower number of N-specific T-cell precursors or to a lower density of T-cell epitopes on N-protein. It is not anticipated that T-cells specific for N-protein are of intrinsically lower affinity than S and M-protein-specific T-cells, since three of the four identified strong TGEV T-cell epitopes are located on N-protein (Antón et al., 1995).

TGEV may infect some types of macrophage (Enjuanes and Van der Zeijst, 1995; Laude et al., 1984). In addition TGEV may grow in a porcine leukocyte cell line (Wesley et al., 1990). Infection of leukocytes could affect the immune response to the virus. The stimulation of TGEV-immune leukocytes (including macrophages, T and B-cells) by either infectious or inactivated TGEV did not affect the blastogenic response, indicating that either these cells were not infected by the virus or that their infection did not affect their proliferative response.

Most frequently, a Th response is mediated by $CD4^+$ lymphocytes and directed against structural proteins. In our system, the dominant phenotype of the responder cells was $CD4^+$, characteristic of helper T-cells. $CD4^+$, non-adherent, non-T, non-B, MHC class II^+ PBL could also be relevant in protection against TGEV through the production of interferon α (IFNα), since the production of this cytokine precedes the recovery from TGEV infections (La Bonnardière and Laude, 1981). $CD8^+$ cells could also play a role in protection against TGEV, since a small percentage (11%) of the blastogenic response was abrogated by $CD8^+$ specific MAbs. The $CD8^+$ population could come from the $CD2^+$ $CD4^-$

$CD8^+$ subset or from the unusual $CD2^+$ $CD4^+$ $CD8^+$ subpopulation of porcine T-lymphocytes (Saalmüller et al., 1987). This inhibition could also be nonspecific since it is very similar to that mediated by a control antibody specific for CD1, whose presence has not been described in extrathymic lymphocytes.

Haplotype *dd* miniswine gave the highest response to both TGEV and to selected peptides, while haplotype *cc* gave the lowest (Fig. 1; (Antón et al., 1995)). Although in a multi-antigenic system such as a virus, the preferential response of a defined haplotype to the different specificities might be counterbalanced, it is known that certain strains of a given species yield more vigorous immune responses than others. The response of haplotype-defined miniswine to other infectious agents, such as inactivated *Bordetella bronchiseptica*, *Trichinella spiralis*, pseudorabies-modified live virus, and foot-and-mouth-disease virus (FMDV), are influenced by genetic factors, possibly SLA linked genes (Lunney et al., 1986; Rothschild et al., 1984).

TGEV antigens expressed as fusion proteins with MBP were more efficient in the induction of blastogenic responses than similar recombinant products expressed using pEX plasmids (Lenstra et al., 1989), which frequently provided partially soluble or non-soluble antigens (results not shown). The amounts of S_N, N, and M-proteins produced as fusion products in bacteria ranged from 50 to 200 $\mu g/10^9$ cells. In contrast, recombinants expressing whole S-protein or S_C fragment synthesized less than 0.05 $\mu g/10^9$ cells. Similar low protein levels were observed during expression of the carboxyterminus of S-protein in *Salmonella typhimurium* (Smerdou et al., 1996). These data indicate that the half carboxyterminus of S-protein is most likely toxic to the bacteria.

The three major structural proteins of TGEV contain T-cell epitopes and elicited blastogenic responses with similar stimulation index (Fig. 3). In the blastogenic response to S-protein, no dominant T-cell epitope has been identified using lymphocytes from the three haplotypes. All the accumulated data indicate that the clear blastogenic response elicited by S-protein is due to the addition of minor T-cell responses induced by many weak T-cell epitopes distributed along the entire S-protein length. This statement is based on several observations: (i) no proliferative response was observed by stimulating TGEV-immune lymphocytes with overlapping synthetic peptides spanning the full length S-protein; (ii) identical weak stimulation indices were obtained when the immune cells were stimulated with recombinant S-protein fragments spanning the half amino-terminus of S-protein, and no significant increase of the blastogenic response was observed when any combination of two of these recombinant fragments was used; (iii) peptides comprising segments of the S-protein with highest score for T-cell sites (Antón et al., 1995) did not elicit a blastogenic response; (iv) minor non-significant responses to S-protein peptides were observed which were not coincident among the three haplotypes (data not shown). The lack of a blastogenic response to the overlapping S-protein peptides has been the result of stimulating with peptides acetylated in their amino-terminus and containing an amidated α-alanine at their C-terminus. It is very unlikely that these chemical modifications at the ends of the peptides could have inhibited their recognition by immune T-cells, because the peptides were synthesized to a resolution of one amino acid. Nevertheless, we cannot completely rule out this possibility.

In TGEV-immune PBL depleted of macrophages and cultured with a mixture of S and N purified recombinant S and N-proteins, 20% of the antibody levels elicited after stimulation with complete virus was recovered, while S and N-proteins alone did not induce significant antibody responses. In contrast, both S or N-proteins induced similar levels of T-cell proliferation. In addition, S-rosettes in collaboration with N-protein elicited in vitro antibody responses accounting for 84% of the response induced by the native virus. This cooperation could be explained on the basis of a B and T-cell collaboration considering that the S-protein provides the B-cell epitopes, and the N-protein elicited the T helper responses. In fact, no dominant T-cell epitopes have been defined in S-protein while strong T-cell epitopes have been identified on N-protein. Co-stimulation by S-rosettes and M-protein induced

the synthesis of TGEV-specific antibodies up to 60% of the antibody levels induced by the native virus, in agreement with the observation that M-protein also contains strong T-cell epitopes (Antón et al., 1995).

The collaboration between S-protein with either N or M-proteins in the in vitro antibody synthesis of TGEV-specific antibodies, as detected by RIA, was also required in the synthesis of TGEV neutralizing antibodies. Optimum TGEV neutralizing antibodies were obtained by the collaboration of S-rosettes and N-protein, leading to levels of neutralizing antibody 50-fold higher that those elicited by non-oligomerized forms of the S-protein. The higher potential of S-rosettes versus the monomeric forms in the induction of virus neutralizing antibodies was expected since polymeric antigens are more efficient in the induction of immune responses.

S-rosettes in collaboration with M-protein did not lead to a significant synthesis of neutralizing antibodies, albeit TGEV specific antibodies were clearly detected by RIA. This unexpected result could be explained in two ways. One is that the levels of the elicited antibodies were not sufficient to provide neutralization, since in our experience the sensitivity of the RIA assay is higher than that of the neutralization assay for TGEV. Alternatively, although both N and M-proteins collaborate with the spike protein, the repertoire of antibody specificities elicited by S-rosettes in collaboration with N-protein may be different from the one elicited by S-rosettes and M-protein, and lower antibody-levels specific for viral epitopes relevant in virus neutralization could have been induced in collaboration with M-protein.

Contact between Th and B-cells is necessary for a T/B-cell cooperation (Noelle and Snow, 1991). However, T/B cooperation can occur in the absence of covalent linkage between T and B epitopes (Bellone et al., 1994; Roehrig et al., 1992; Sarobe et al., 1991). In such cases, preferential pairing would occur between specific T and B-cells for optimal T/B cooperation between non-covalently linked T and B epitopes (Bellone et al., 1994). Yet, though we can not exclude random aggregation of antigens, or non-covalent linkage of a virus-specific nature, this would not be necessary to induce antibody synthesis.

S-rosettes, such as the ones formed by TGEV S-protein, provide non-infectious, multimeric immunocomplexes which can be extremely useful in the induction of protective immunoresponses against viruses. In the case of viruses with enteric tropism these immunocomplexes also might be useful to target other antigenic determinants to the enteric tract, to elicit mucosal immune responses. S-rosettes have been described for coronaviruses MHV and IBV (Sturman et al., 1980; Cavanagh, 1983) and other enveloped viruses (Helenius and Bonsdorff, 1976; Simons et al., 1973). Recently (Heinz et al., 1995), it has been shown that tick-born derived S-rosettes can provide full protection against flaviviruses.

The efficiency in the TGEV-specific antibody induction by co-stimulation with S-rosettes and N-protein may have interesting implications in eliciting protection against coronavirus induced diseases.

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