

SHORT COMMUNICATION

A Transmissible Gastroenteritis Coronavirus Nucleoprotein Epitope Elicits T Helper Cells That Collaborate in the *in Vitro* Antibody Synthesis to the Three Major Structural Viral Proteins

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Received July 6, 1995; accepted August 3, 1995

Four strong T cell epitopes have been identified studying the blastogenic response of lymphocytes from haplotype-defined transmissible gastroenteritis virus (TGEV) immune miniswine to sixty-one 15-mer synthetic peptides. Three of these epitopes are located on the nucleoprotein (N₄₆, amino acids 46 to 60; N₂₇₂, amino acids 272 to 286; and N₃₂₁, amino acids 321 to 335), and one on the membrane protein (M₁₉₆, amino acids 196 to 210). N₃₂₁ peptide induced the highest T cell response and was recognized by immune miniswine lymphocytes with haplotypes dd, aa, and cc. T lymphocytes from peptide N₃₂₁-immune miniswine reconstituted the *in vitro* synthesis of TGEV-specific antibodies by complementing CD4⁺ TGEV-immune cells. This response was directed at least against the three major structural proteins. The synthesized antibodies specific for S protein preferentially recognized discontinuous epitopes and neutralized TGEV infectivity. These results show that peptide N₃₂₁ defines a functional T helper epitope eliciting T cells capable of collaborating with B cells specific for different proteins of TGEV. © 1995 Academic Press, Inc.

Porcine transmissible gastroenteritis (TGE) is a highly contagious enteric disease of swine caused by a coronavirus (TGEV). The disease is a major cause of death in piglets under 2 weeks of age (1). The viral RNA genome encodes three major structural proteins: S, M, and N proteins (2, 3) and a fourth structural protein that has recently been described, the small membrane (sM) protein (4). Protein S is the major inducer of TGEV neutralizing antibodies (5, 6).

Lactogenic immunity induced in sows after natural infection or oral immunization appears to be the most important mechanism protecting newborn piglets against TGEV infection (7). Lactogenic immunity can be stimulated by antigen presentation to gut-associated lymphoid tissues (8, 9). Newborn animals can be protected by oral administration of TGEV-specific antibodies with IgG and sIgA isotypes isolated from immune colostrum or serum (10, 11). Since TGEV is a T-cell-dependent antigen (12), for effective activation of the humoral immune response determinants recognized by both B- and T-helper (Th) cells are required.

New vaccines tend to include only those antigenic domains that induce a protective immune response despite antigenic variability, underscoring the need to identify essential B and T cell epitopes. 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) and three antigenic subsites on site A, have

been identified (5, 13-15). TGEV induces in swine antibodies binding to all these antigenic sites (14). By contrast, very little information is available on the T cell epitopes of TGEV. The response of Th cells is restricted to a limited number of antigenic sites, presented by major histocompatibility complex (MHC) class II molecules, and requires antigen processing (16). T cell epitopes often present particular physicochemical or sequential characteristics (17-19). S, N, and M proteins from coronavirus are relevant targets for cellular immune recognition (20-24). The T cell response to sM and nonstructural proteins is unknown.

A model to study basic swine immune responses to infection was made available when inbred miniature swine were developed (NIH minipigs) and their MHC, termed the swine lymphocyte antigen (SLA) complex, was defined (25, 26). Using this animal model, previous studies in our laboratory showed that intragastric infection of haplotype-defined miniswine with a virulent TGEV strain induced optimum blastogenic responses to purified TGEV of T cells from mesenteric lymph nodes (MLN), and weaker responses of peripheral blood leukocytes (PBL) or T cells from Peyer's patches (27), as expected for infections preferentially located in the gastrointestinal tract. The specific blastogenic response was directed to the three major structural proteins with similar optimum stimulation index (SI), ranging from 40 to 60. These SI were lower than the proliferation elicited by the whole

virus (SI 95). To identify strong T-cell epitopes in TGEV proteins, nylon wool-fractionated MLN cells from *dd*, *aa*, and *cc* haplotype inbred TGEV-immune miniswine were stimulated with each of 61 synthetic peptides that encompassed sequences from TGEV S, M, and N proteins (Fig. 1). Ten to thirteen-day-old piglets were intragastrically inoculated with 2×10^8 PFU of virulent MAD88 virus (28) and boosted 2 weeks later with the same dose of virulent MAD88 virus, followed by an intramuscularly and intraperitoneally administered dose of purified PUR46-MAD virus (90 μ g). After nylon wool fractionation, unbound T cells were cultured in triplicate, in round-bottom 96-well plates with antigen, at 4×10^5 viable cells per well in supplemented Iscove's medium and 10% nonimmune porcine serum. Four days after incubation in presence of the antigen, MLN cells were pulse-labeled with [3 H]-thymidine (1 μ Ci per well) for 18 hr, harvested, and evaluated for incorporation of radiolabeled precursor. Synthetic peptides were selected based on the presence of T-cell motif predicted according to TSites program (18) and those peptides showing high scores as potential T cell recognition epitopes by both the AMPHI (29) and the Rothbard and Taylor (19) methods were synthesized. Blastogenic responses of cells from miniswine with haplotypes *dd* and *aa* showed strong response to three peptides, two (N_{272} and N_{321}) located on N protein and one (M_{196}) on M protein. The highest responses were induced by peptides N_{321} and M_{196} . These peptides induced weak response in cells from haplotype *cc* animals. The specificity of the response was confirmed by the lack of proliferation by nonimmune cells (data not shown) and the absence of stimulation of immune cells by other peptides. M_{191} peptide, although partially overlapping with M_{196} peptide, induced responses only in *dd* miniswine, suggesting that M_{196} peptide might contain two different T-cell epitopes, one of which only induced responses in cells with haplotype *dd*. The amino-terminal N protein peptide N_{46} was a strong epitope for immune T lymphocytes from *cc* miniswine but did not induce a significant response in cells with *aa* or *dd* haplotype. Small differences in responses to other peptides were also seen among animals with the same haplotype. In summary, haplotype *dd* miniswine gave the highest response to selected peptides, and haplotype *cc* gave the lowest (Fig. 1), following the same gradation as the response to the whole virus previously described (27). Although in a multiantigenic 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 is influenced by genetic factors, possibly SLA-linked genes (25, 30).

In the blastogenic response to S protein, minor responses to S protein peptides were observed which were not coincident among the three haplotypes. The response to the complete spike protein (27) seems to be the result of a response to many weak T-cell sites on S protein. Since not all peptides spanning the S protein have been tested, we cannot exclude the presence of a strong T site on the S protein.

Four peptides containing good T-cell epitopes have been identified, three in the N protein, N_{321} , N_{272} , and N_{46} , and one in M protein, M_{196} . The two stronger T-cell peptides (N_{321} and M_{196}) elicited high responses on cells from immune swine of haplotypes *dd* and *aa*, and responses with SI 2.7, which are just above the limit of significant responses, on *cc* cells. Considering that optimum *cc* haplotype responses to TGEV are weaker, these results suggest that these two epitopes will be recognized with different affinity by cells with most haplotypes (31, 32).

In order to characterize the epitope located in N_{321} peptide, nylon wool fractionated MLN cells from TGEV-immune *dd* miniswine were exposed to increasing concentrations of N_{321} peptide. The blastogenic response was dose-dependent and peptide specific, since control peptides unrelated to TGEV sequences (data not shown) or several peptides derived from TGEV (Fig. 1) did not induce immune cells to proliferate. To more precisely map the minimum sequence in the N_{321} peptide, seven nested 15-mer peptides scanning the entire length of the N_{321} peptide were synthesized and the proliferative response of immune lymphocytes to these peptides was examined (Fig. 2A). Peptide N_{321} elicited responses significantly higher than the other overlapping peptides, indicating that for an optimal response full-length N_{321} peptide is required. Similar results have been obtained with T cells from N_{321} peptide-immune animals (I. M. Antón and L. Enjuanes, unpublished results).

In order to determine the immunogenicity of the identified epitope and its ability to prime the immune system, *dd* miniswine were immunized with purified peptide N_{321} in the absence of carrier proteins. PBL from peptide N_{321} -immune swine proliferated in the presence of peptide N_{321} (SI 49), of purified recombinant TGEV N protein (SI 47), and of purified TGEV (SI 27) (Fig. 2B). The N protein used was expressed fused to maltose binding protein (MBP), affinity chromatography purified, and analyzed by SDS-PAGE and Western blot (27). The results indicate that peptide N_{321} represents an immunogenic T cell epitope which elicits porcine cellular responses against TGEV.

CD4⁺ lymphocyte subset from TGEV-immune miniswine is the major responder to TGEV antigens (27), suggesting the helper nature of the T cell response. A system for TGEV-specific antibody synthesis described previously (12, 33) was modified in order to determine if peptide N_{321} induced functional Th cells. Miniature swine

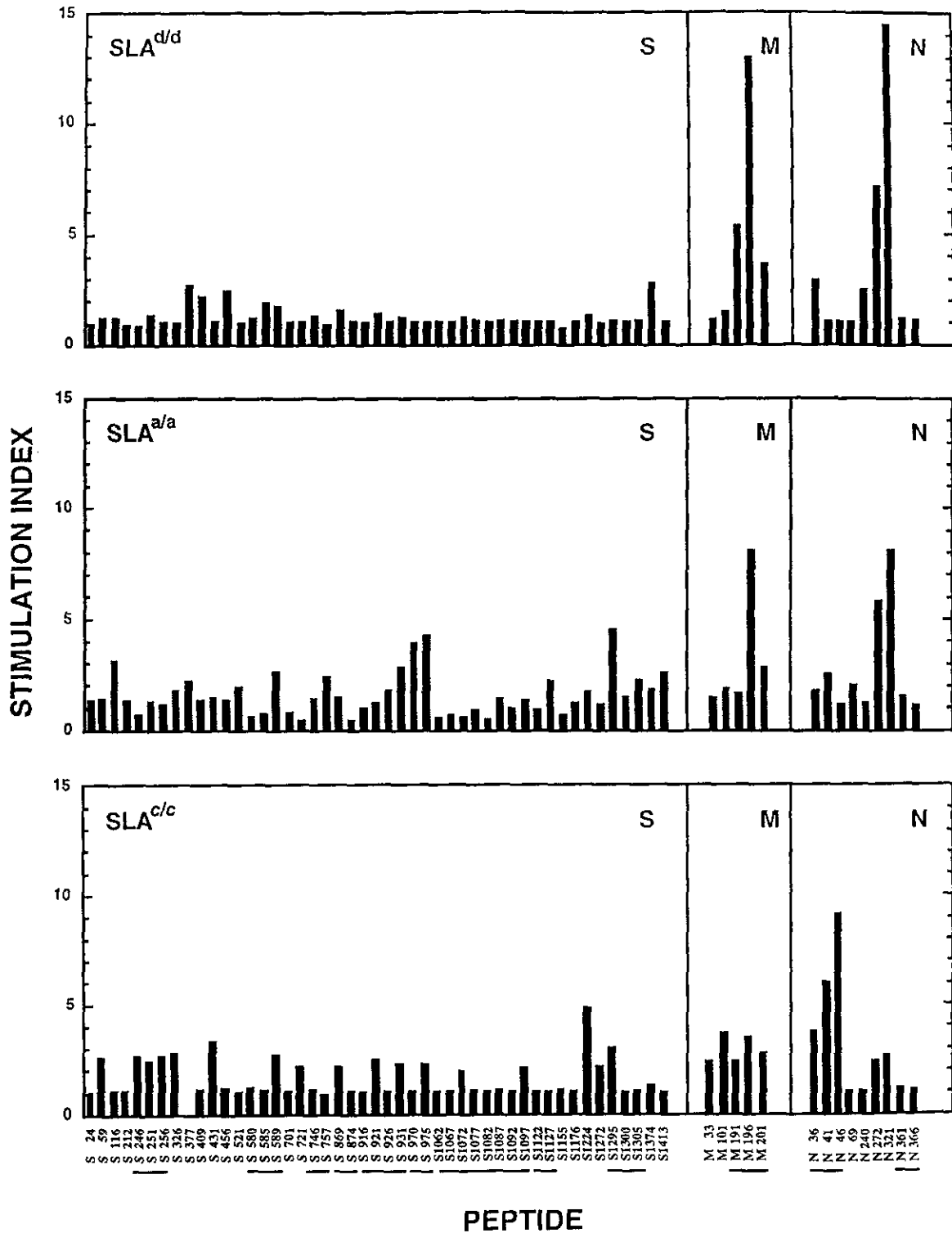


FIG. 1. Blastogenic responses of *dd*, *aa*, and *cc* TGEV-immune miniswine lymphocytes to synthetic peptides modeled on S, M, and N proteins of TGEV. MLN cells from 9 TGEV-immune miniature swine (5 *dd*, 2 *aa*, and 2 *cc* animals) were stimulated *in vitro* with individual synthetic peptides containing sequences from S, M, and N proteins of TGEV. Data represent the mean stimulation index to each peptide of animals with the same haplotype. In the different experiments the background was around 1000 cpm or lower and the standard errors of triplicate determinations were less than 20% of the mean. Results are expressed as SI: mean counts per minute incorporated in the presence of antigen divided by mean counts per minute incorporated in the absence of antigen. The optimal stimulating peptide concentration for each case (50 or 10 $\mu\text{g/ml}$) has been shown. Peptide designation indicates the viral protein (capital letter) and the position in the protein of the first residue of the synthetic peptide. Underlined peptides are partially overlapping. Note that only the blastogenic response to a selected panel of peptides is shown.

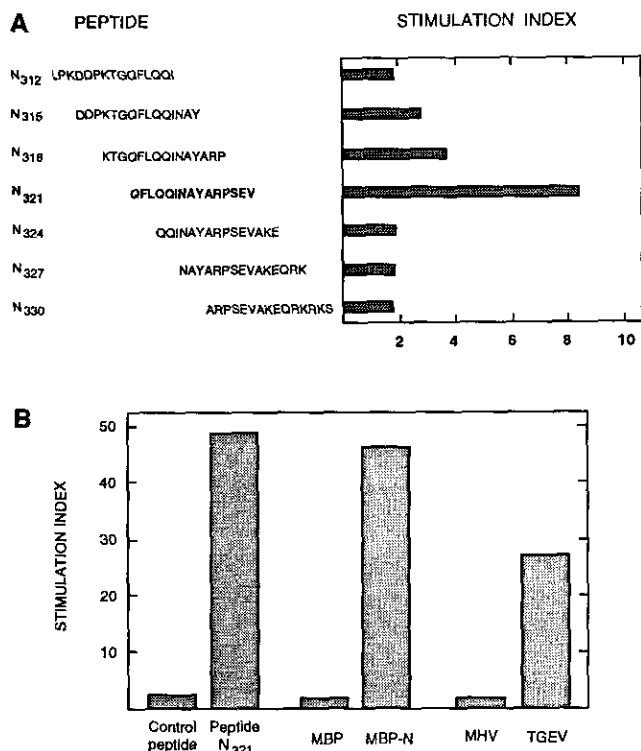


FIG. 2. Antigenicity and immunogenicity of N₃₂₁ peptide. (A) Antigenicity of nested peptides for TGEV-immune porcine *dd* lymphocytes. A nested set of partially overlapping peptides which encompass N₃₂₁ peptide adjacent sequence was used to stimulate immune MLN cells for 4 days. Average values from four experiments are represented. (B) The blastogenic response of peptide N₃₂₁-immune porcine *dd* T lymphocytes to the peptide, TGEV N protein, and TGEV. PBL from peptide N₃₂₁-immune *dd* miniswine were stimulated *in vitro* with peptide N₃₂₁ (75 μ g/ml), purified recombinant TGEV N protein fused to MBP (90 μ g/ml), or purified TGEV (5 μ g/ml). The concentration of control antigens (control peptide, MBP, or MHV) was the same as that of the respective antigen. A representative result of three experiments is shown. In the different experiments the background was around 1000 cpm or lower and the standard errors of triplicate determinations were less than 20% of the mean.

of *dd* haplotype were immunized by the intramuscular and intraperitoneal routes with four doses of 35 μ g of formaldehyde-inactivated PUR46-MAD virus (33) at 2-week intervals. Sephadex G25-purified N₃₂₁ peptide or a control peptide (CGFPIEGYSFFSSDS) representing a sequence from S protein of TGEV were used to immunize *dd* miniature pigs that were inoculated intraperitoneally and intramuscularly with 400 μ g of peptide on Days 1, 20, 38, and 63. T lymphocytes from PBL were selected by removal of adherent cells incubating at 37° for 1 hr on petri dishes and on nylon wool columns (34). T cell depletion was based on the removal of the helper subset of T cells by complement-mediated cytotoxicity with monoclonal antibody (MAb) 74-12-4 (specific for porcine CD4⁺ cells). The cooperation of T lymphocytes from N₃₂₁ peptide-immune cells from haplotype *dd* swine, with B cells from TGEV-immune syngenic swine, was evaluated in an *in vitro* antibody synthesis assay (33). Briefly, 4 \times

10⁵ cells were incubated in complete RPMI medium with 10% heat-inactivated fetal bovine serum in flat-bottomed microtest II plates with 0.5 μ g/ml purified virus for 7 days. Supernatants were harvested and specific antibodies were determined by solid-phase radioimmunoassay (RIA), neutralization of TGEV, and Western blot analysis. The origin of the different cell types used in the *in vitro* antibody synthesis is described in Fig. 3A. T lymphocytes from N₃₂₁ peptide-immune swine (T_p) were cultured with CD4⁻ PBL from TGEV-immune miniswine (B_v), and the production of TGEV-specific antibodies in the supernatants was determined. Peptide N₃₂₁-specific T lymphocytes reconstituted the response of CD4⁻ TGEV-immune PBL (Fig. 3B). By contrast, similar numbers of control-peptide immune T cells (T_n), which induced neither antibody nor proliferative response to TGEV or to the peptide, did not restore the antibody synthesis by CD4-depleted PBL (B_v) (Fig. 3B, T_n + B_v). The specificity of the response is demonstrated by the lack of antibody production by T cells from TGEV-immune animals (T_v), T cells (T_p) or M ϕ -depleted PBLs (PBL-M ϕ _v) from peptide-immune miniswine, and B cells depleted of CD4⁺ lymphocytes from virus (B_v) immune animals. Combinations of immune with nonimmune counterparts (T_n + B_v, T_v + B_n, and T_p + B_n) also did not produce antibodies (Fig. 3B). In addition, virus-specific antibodies were generated only by TGEV-stimulated cells. Culture supernatants containing TGEV-specific antibodies demonstrated TGEV-neutralizing activity (Table 1). These antibodies were TGEV specific, since they did not neutralize vesicular stomatitis virus. N₃₂₁ peptide-specific T lymphocytes cooperated in the synthesis of antibodies specific for the three main structural proteins, as determined by Western blot analysis after TGEV protein fractionation in reducing and nonreducing conditions (results not shown). The binding to N and M proteins both in the presence and in the absence of 2-mercaptoethanol was almost identical, suggesting that the N- and M-specific antibodies *in vitro* synthesized bind to continuous epitopes. Under nonreducing conditions, most of the antibody binding activity is directed to S protein, strongly suggesting that *in vitro* the S protein must be the dominant antigen. Under reducing conditions, most of the binding to S protein disappeared, indicating that most S protein-elicited antibodies were directed against discontinuous determinants, mimicking the *in vivo* immune response (15, 35). The specificity of the synthesized antibodies was determined by competitive RIA (cRIA) using ¹²⁵I-labeled MAb specific for antigenic sites A, B, and D of S protein, for site B of N protein, and for M protein (Fig. 4). cRIA was performed as previously described (14) with slight modifications. Briefly, purified TGEV (0.1 μ g/well) was plated, the remaining binding sites saturated with 5% BSA in PBS, and ¹²⁵I-labeled MAbs (s.a. 1.7 \times 10⁷ cpm/ μ g; 4 \times 10⁵ cpm/well) were added and incubated for 2 hr at 37° in the presence of competitor supernatant. Microplates were washed six

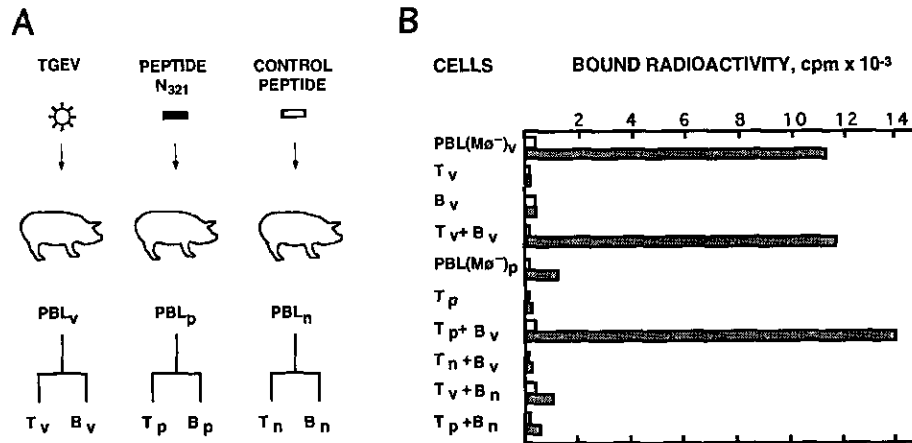


FIG. 3. *In vitro* synthesis of TGEV-specific antibodies with the cooperation of N_{321} peptide-immune Th cells. (A) Scheme of the origin of the different cell types used for the *in vitro* antibody synthesis assay. Swine were immunized with purified virus, with peptide N_{321} , or with a control peptide as described in the text and the corresponding immune PBL (PBL_v, PBL_p, and PBL_n, respectively) were collected from these animals and used as a source to purify the indicated T and B cell populations. (B) The reconstitution of TGEV-specific antibody synthesis by the supplementation of Th cell-depleted TGEV-immune PBL with N_{321} peptide-immune Th cells is shown. TGEV-immune PBL were depleted of CD4⁺ cells (B_v , 2×10^5 cells/well) by antibody-mediated cytotoxicity (using MAb 74-12-4 (39) kindly provided by Dr. Joan Lunney, Beltsville, MD) or T cell-enriched by using nylon wool columns (T_v , 2×10^5 cells/well). The same treatments were applied to nonimmune cells (T_n and B_n , 2×10^5 cells/well) used as a negative control. N_{321} peptide-immune T cells (T_p , 2×10^5 cells/well) were obtained after nylon wool fractionation. TGEV-immune M ϕ -depleted PBL (PBL-M ϕ _v, 4×10^5 cells/well) were used as a positive control. The indicated cells were incubated in the absence (open bars) or presence (closed bars) of TGEV. Antibody synthesis was evaluated by RIA using purified TGEV as antigen as described in the text.

times with 0.1% BSA and 0.1% Tween-20 in PBS and bound radioactivity was determined in a gamma-counter. The percentage of radioactivity bound was calculated in relationship to the radioactivity bound in the presence of control supernatant. Purified homologous MAbs were used as positive controls in the cRIA. The *in vitro*-synthesized antibodies with the cooperation of peptide N_{321} -specific T lymphocytes bound to epitopes located on all the antigenic sites tested (Fig. 4).

N_{321} peptide-induced functional Th cells, since T cells from swine immunized with the purified peptide help CD4-depleted lymphocytes from TGEV-immune swine in the *in vitro* antibody synthesis. Furthermore, peptide N_{321} -specific Th cells provided *in vitro* help for synthesis of antibodies to at least the three major structural proteins, i.e., collaborating with B cells of several specificities, an activity similar to the one described previously in the

TABLE 1
Neutralization of TGEV by Antibodies Synthesized *In Vitro* with the Cooperation of Peptide N_{321} -Specific Th Cells

Cells or serum	Titer, RIA ^a	Neutralization index ^b for		
		TGEV		
		-2nd Ab ^c	+2nd Ab	VSV
B_v	0	<0.1	<0.1	<0.1
$T_p + B_v$	160	2.5	2.5	<0.1
$T_v + B_v$	377	3.5	3.5	<0.1
immune serum ^d	> 10^5	>6	N.T.	N.T.

^a Titer in RIA was defined as the maximum antibody dilution that bound threefold the background radioactivity.

^b The neutralization index was determined by dividing the number of PFU of virus per milliliter mixed with control supernatant by the number of PFU of virus per milliliter in the presence of culture supernatant. The index is expressed as the \log_{10} of this ratio.

^c Rabbit anti-swine was used as second antibody; N.T., not tested

^d Immune serum was obtained from a *dd* miniswine immunized four times with inactivated purified TGEV as described in the text.

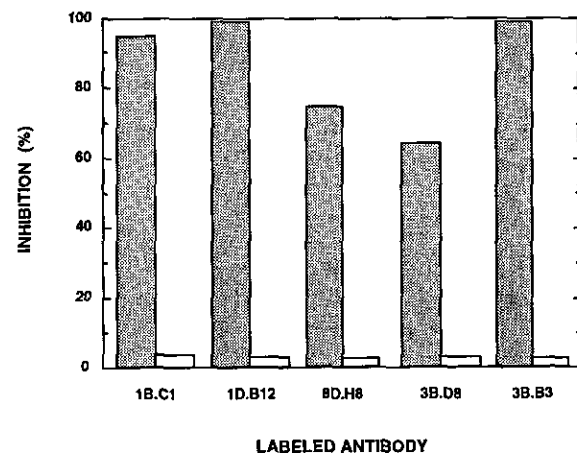


FIG. 4. Inhibition of TGEV-specific MAbs binding to TGEV by porcine antibodies synthesized *in vitro* with the help of peptide N_{321} -specific T lymphocytes. MAbs 1B.C1, 1D.B12, and 8D.H8 specific for sites A, B, and D of S protein, respectively, MAb 3B.D8 specific for site B of N protein, and MAb 3B.B3 specific for M protein were [¹²⁵I]-labeled and their binding to plated TGEV inhibited by *in vitro*-synthesized antibodies present in the supernatants from B_v (open bars) and $T_p + B_v$ (closed bars) cultures. The percentage of radioactivity bound was determined in relation to the radioactivity bound in presence of control supernatant.

response to Hepatitis B (36, 37), which is described for the first time in coronaviruses. In addition, N₃₂₁-specific Th cells (N protein specific) collaborate in the synthesis of TGEV neutralizing antibodies which are S protein specific. These data indicate that recruitment of Th cells is antigen-specific but that effector function might be polyvalent.

The blastogenic response elicited by the N protein and peptide N₃₂₁ were 45% (27) and 15% of the response elicited by the whole virus, respectively. The response induced by peptide N₃₂₁-specific Th cells is relevant since these cells reconstituted up to 45% of the *in vitro* antibody synthesis by TGEV-immune B cells elicited by the whole virus, according to RIA antibody titers (Table 1). The *in vitro*-synthesized antibodies generated with the help of N₃₂₁ peptide-specific T cells recognized the three major viral proteins with the same pattern observed with those generated with the help of Th cells from TGEV-immune miniswine (data not shown). Furthermore, these antibodies bound the antigenic sites (A, B, and D) previously described on S protein (15, 38), as determined by cRIA (Fig. 4). These data illustrate the strong potential of the identified Th epitope. Our study has been performed in the most relevant animal system available so our results may find practical applications in the design of subunit vaccines. Actually we are testing the immune response induced in swine by recombinant adenovirus expressing B epitopes from S protein fused to several copies of the N₃₂₁ peptide sequence.

ACKNOWLEDGMENTS

We are grateful to J. Palacín and J. C. Martín for their excellent technical assistance and to J. E. Domínguez for help in peptide purification. We thank Granja Cantoblanco de Animales de Laboratorio (Hospital General Gregorio Marañón, Comunidad de Madrid and CSIC) and Instituto Nacional de Investigaciones Agrarias for the use of animal facilities. I.M.A. and C.S. received fellowships from the Spanish Ministry of Education and Science. This investigation was founded by grants from the Consejo Superior de Investigaciones Científicas, Comisión Interministerial de Ciencia y Tecnología, Consejería de Cultura de la Comunidad de Madrid, and European Communities (Science and Biotech Projects).

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