Immunogenicity of the S protein of transmissible gastroenteritis virus expressed in baculovirus

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Summary. Seven fragments of the spike (S) gene cDNA of transmissible gastroenteritis virus (TGEV), as well as the full length cDNA, were cloned and expressed in baculovirus vectors. Piglets were immunized with cells infected with the recombinant viruses. Each of the recombinants induced TGEV-specific antibodies detected in a fixed cell enzyme immunoassay. The amino terminal half of the S protein, containing all four major antigenic sites (A, B, C and D), and encoded by a 2.2 kb fragment of the S gene, induced virus neutralizing (VN) antibody titers comparable with those induced by the complete S protein. Recombinant proteins lacking the A antigenic site, or with a deletion including the putative receptor binding sites and the D antigenic site, were not capable of inducing levels of VN antibodies similar to those induced by the whole S protein.

Introduction

Transmissible gastroenteritis (TGE) is an important disease of swine, character-ized by severe diarrhoea and vomiting, often fatal to piglets under two weeks of age. The disease is caused by a porcine coronavirus which, like other members of the family *Coronaviridae*, is an enveloped, pleomorphic virus, with a single stranded RNA genome of positive polarity. The virions are constructed of three major structural proteins: a phosphorylated nucleocapsid protein (N) and two glycoproteins, the membrane (M) and the large spike (S) protein. The mature S protein, after cleavage, which forms the characteristic club-shaped peplomers, contains 1431 amino acids and is the major inducer of virus neutralizing (VN) antibodies [13, 18].

Studies of the antigenic structure of the S protein by monoclonal antibodies (Mabs) revealed at least four major epitope groups, all of them located on the amino-terminal half of the polypeptide [4, 18]. Those antigenic determinants that are involved in neutralization were highly conserved among different

TGEV strains [12] and appeared to be conformation- and glycosylation dependent[3, 10].

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) is widely used for the high level expression of foreign genes [20] and the complete S protein of TGEV (together with a carboxy-terminal truncated version) has been expressed in a recombinant baculovirus [15]. The complete S protein induced VN antibodies in rats. The complete and a truncated form of the TGEV S protein have also been expressed in recombinant vaccinia viruses, and they were tested in mice, where both were shown to induce TGEV neutralizing antibodies [17, 25].

Passive protection of neonatal piglets is of primary importance in the control of TGE and the only efficient way to achieve this is by TGEV specific immuno-globulins (Igs) primarily of the secretory (s) IgA class in the colostrum and milk of the sow. TGEV strains that are capable of generating sufficient sIgA response in the sow may also cause the disease in newborn piglets. An alternative approach to vaccination could be the use of a helper independent porcine adenovirus vector expressing the S gene and replicating in the intestinal tract, but the size of the S gene (4341 bases) probably exceeds the predicted packaging capabilities of such a vector, based on studies with human adenovirus vectors [1].

The objective of our study was to select the shortest piece of the S protein capable of inducing a VN antibody response similar to the response to the whole S protein.

Materials and methods

Cells, viruses and control sera

AcNPV and the recombinant baculoviruses were grown and assayed in monolayers of *Spodoptera frugiperda* cells (Sf21) maintained in Grace's medium supplemented with 0.3% yeastolate, 0.3% lactalbumin hydrolysate and 10% fetal bovine serum (FBS, SIGMA) at 28 °C [30]. The origin of the AcNPV has been previously described [22]. The tissue culture adapted Purdue-115 (P-115) strain of TGEV, kindly provided by Dr Linda Saif (Ohio Agricultural Research and Development Centre, Wooster OH, U.S.A.), was propagated in confluent swine testis (ST) cells [21]. TGEV specific hyperimmune serum was generated in pigs as described by Welch and Saif [32] with an initial intramuscular dose of the P-115 strain and subsequent oral dosing with the virulent Purdue strain. The source of AcNPV specific serum has been described earlier [31].

cDNA synthesis and cloning

The viral genomic RNA was extracted from concentrated TGEV P-115 as described by Wesley et al. [33]. PolyA mRNA was purified by oligo (dT) spun-column chromatography (QuickPrep Micro mRNA Purification Kit, Pharmacia) according to the manufacturers' instructions, from ST cells 6 h after infection with TGEV P-115 at a m.o.i. of 10.

The cDNA synthesis was carried out with the RiboClone cDNA Synthesis System (Promega) using the TGEV genomic RNA and mRNA with three different oligonucleotide primers (Regional DNA Synthesis Laboratory, Univ. of Calgary) indicated in Fig. 1
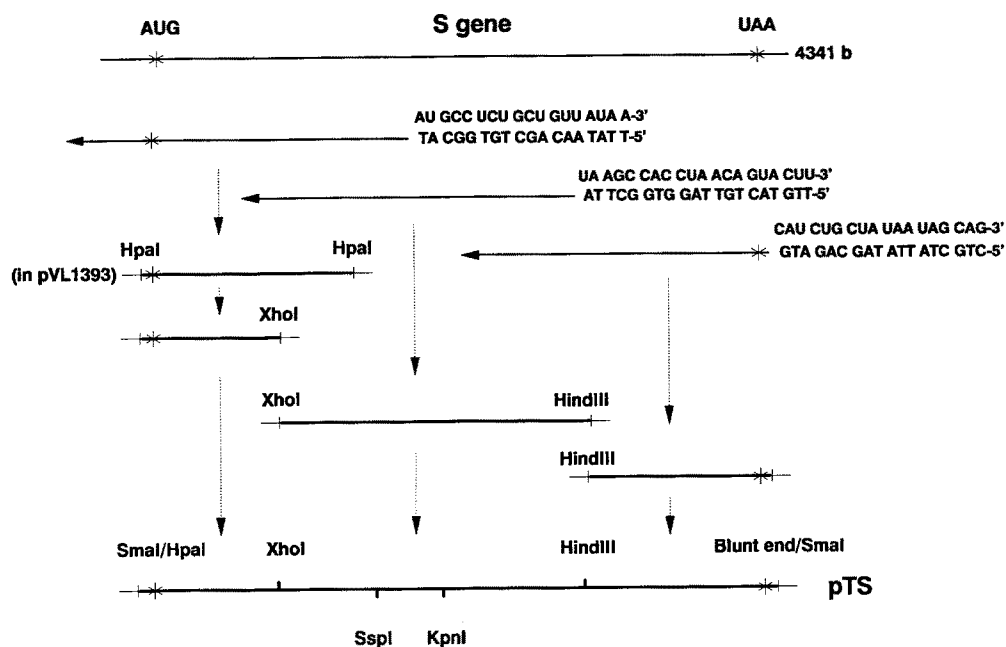
Fig. 1. Synthesis of the TGEV S gene cDNA. The generation of the cDNA fragments with the primers binding to the indicated bases is shown by the horizontal arrows. The lines represent the intermediate DNA fragments used for the construction of the full length S gene cDNA cloned into the pGEM-7 vector, designated pTS. The restriction enzyme sites used for the generation of pTS and the recombinant baculovirus transfer vectors are indicated. The initiation and stop sites are marked with asterisks

(5'-TTATAACAGCTGTGGCAT-3', 5'-TTGTACTGTTAGGTGGCTTA-3', 5'-CTGCTA-TTATAGCAGATG-3") complementary to the genome [26] downstream of the initiation site at 1793–1810, 3335–3354 and 4365–4382 bases respectively. The resulting overlapping cDNA fragments were blunt ended with T4 DNA polymerase, cloned into the SmaI site of the pGEM-7Zf(+) vector in both orientations: BamHI-EcoRI(+), EcoRI-BamHI(−). The clones were propagated in DH5α E. coli cells and analyzed by restriction enzyme (RE) digestions. All REs were obtained from BRL (Life Technologies, Gaithersburg, MD, U.S.A.).

Generation and analysis of the recombinant baculoviruses

The 1.6 kb HpaI cDNA fragment was cloned into the SmaI site of the pVL1393 baculovirus transfer vector (kindly provided by Dr. C. Richardson. Biotechnology Research Institute, Montreal, Canada) in (+) orientation and the 1.1 kb BamHI-XhoI subfragment of this clone was used together with the cDNA generated by the other two primers for the construction of the complete TGEV S cDNA (pTS. Fig. 1). Figure 2 shows the size of the cDNA fragments selected for the generation of the listed recombinant viruses and their relation to the positions of the major antigenic sites [3,9]. The truncated cDNA fragments in the recombinant baculoviruses expressing the TGEV S gene (rBTS) 1.8, 2.2 and 3.3 were generated by double RE digestions of pTS (+) with BamHI-SspI, BamHI-KpnI or BamHI-HindIII and cloned into the BamHI-SmaI (rBTS 1.8), BamHI-KpnI (rBTS 2.2) and BamHI-HindIII (rBTS 3.3) sites of pGEM-7 respectively. The in phase cDNAs of rBTS 2.2D, 3.3D and 4.4D were generated by deleting the two HaeIII fragments between 529
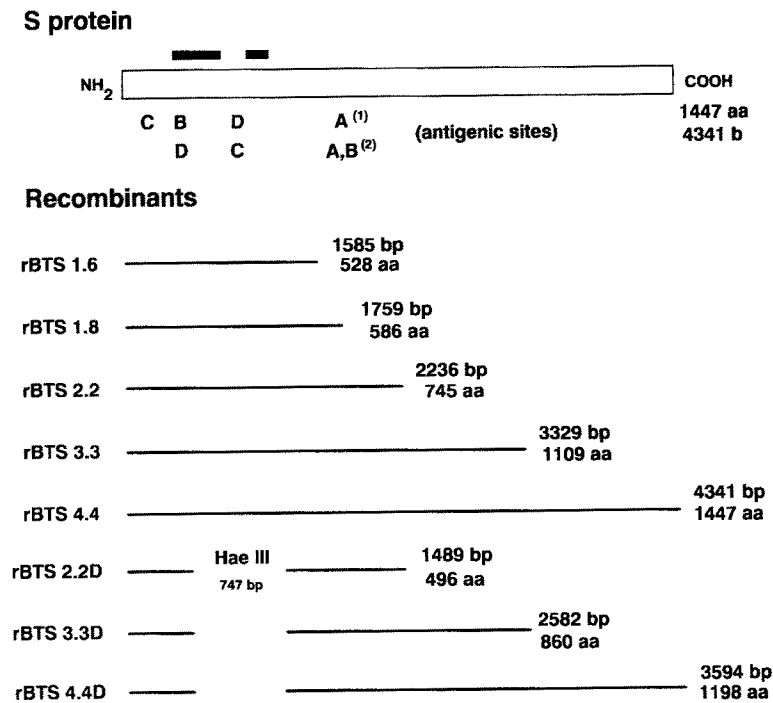
S protein

Recombinants

Fig. 2. The S gene fragments of the recombinant baculoviruses and their relation to the S protein. The open bar indicates the complete S glycoprotein of the TGEV P-115 strain with the approximate location of the major antigenic sites (*A* 538–591aa, *B* 97–144aa, *C* 49–52aa and *D* 378–395aa) [1]followed throughout this study, according to Correa et al. [3] and [2]after Delmas et al. [9]. The putative receptor binding sites are shown by the solid bars (92–219aa and 405–465aa). The designations of the recombinant baculoviruses are indicated on the left. The size of the cDNA fragment (represented by the lines) in each recombinant virus is given in basepairs together with the number of amino acids encoded by these fragments. The dotted line indicates the deletion of the *Hae*III DNA fragments

and 1276 bp and religating the cohesive ends. These cloned fragments were released from pGEM-7 by *Bam*HI-*Eco*RI digestions and cloned into the identical unique sites of the pVL1393 transfer vector behind the polyhedrin gene promoter. The resulting constructs were cotransfected with linearized AcNPV DNA into Sf21 cells using cationic liposomes (Ac*M*NPV Linear DNA Transfection Module, Invitrogen). The recombinant viruses were selected by plaque morphology and dot blot hybridization probing with [32P]dCTP (3000 Ci/mmol, ICN) labelled S gene cDNA [30]). Three times plaque purified viruses were propagated and the extracellular virus was used in further studies.

Analysis of the recombinant virus DNA

The recombinant and wild type virus DNAs were extracted from purified virions [5], digested with REs and analyzed on 0.8% agarose gel in the presence of 0.25 µg/ml ethidium bromide [27]. The DNA was transferred onto Nytran membranes (Schleicher and Schuell) and probed with the 32P labelled S gene cDNA [22].

Radiolabelling and immunoprecipitation

The viral proteins were radiolabelled as described [15]. Briefly, monolayers of Sf21 cells were infected with AcNPV or the recombinant viruses at a m.o.i. of 10 and incubated at 28 °C for 36 h. The medium was then replaced by methionine free Grace's medium (BRL) supplemented with 2% FBS and 100 μCi of [^{35}S] methionine (1143 Ci/mmol, TRAN^{35}S-Label, ICN) per ml. After incubation for 4 h at 28 °C, the cells were washed with PBS, lysed with RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulphate, 10 mM Tris-HCl pH 7.2) and analyzed directly, or after immunoprecipitation with TGEV specific hyperimmune pig serum and *Staphylococcus aureus* protein A (Pansorbin cells, Calbiochem) on 8% SDS-polyacrylamide gels [19]. Labelled uninfected and TGEV infected ST cell lysates were prepared as described by De Diego et al. [7].

Immunizations

Sf21 cells infected at a m.o.i. of 10 were collected between 60 and 72 h p.i., a time frame providing for similar concentrations of the recombinant proteins (judged by SDS-PAGE), washed and resuspended in PBS. The samples were aliquoted into 3×10^7 cells/dose and stored at −70 °C. Four week old crossbred Yorkshire piglets from a TGEV seronegative herd (confirmed by VN assay) were immunized in groups of three by the intraperitoneal route with one dose of infected cells emulsified in an oil adjuvant [2] and two doses without adjuvant at two and four weeks after the first injection. Blood samples were taken at weekly intervals and the sera were tested after heat inactivation at 56 °C for 30 min for the presence of TGEV specific antibodies.

Serology

VN antibodies were assayed by standard methods in 96 well microtiter plates of ST cells using 100 TCID$_{50}$ of the TGEV P-115 strain. The sera were also tested in a TGEV specific fixed cell enzyme immunoassay (FCEIA) as described by Cubero et al. [6] after an overnight preincubation of each sample with uninfected ST cells at 4 °C. The horseradish peroxidase labelled anti-swine IgG used in this assay was purchased from Kirkegaard and Perry Laboratories.

Results

Construction of the recombinants

Eight baculovirus recombinants were generated by the cotransfection of AcNPV DNA and the pVL1393 transfer vector carrying different fragments of the TGEV S gene cDNA. The construction of the recombinant transfer vectors resulted in an identical 8 basepair 5' leader sequence from the TGEV genome upstream of the S gene initiation codon.

*Bam*HI-*Eco*RI and *Sal*I generated DNA fragment patterns of the purified wild type and recombinant virus DNAs and the corresponding Southern blot hybridization are shown in Fig. 3. The *Bam*HI-*Eco*RI double digest confirmed the size of the foreign gene in the recombinant viruses and the *Sal*I RE pattern indicated that these cDNA fragments were within the polyhedrin gene as the size of the 3.07 kb AcNPV *Sal*I band was increased in each recombinant by
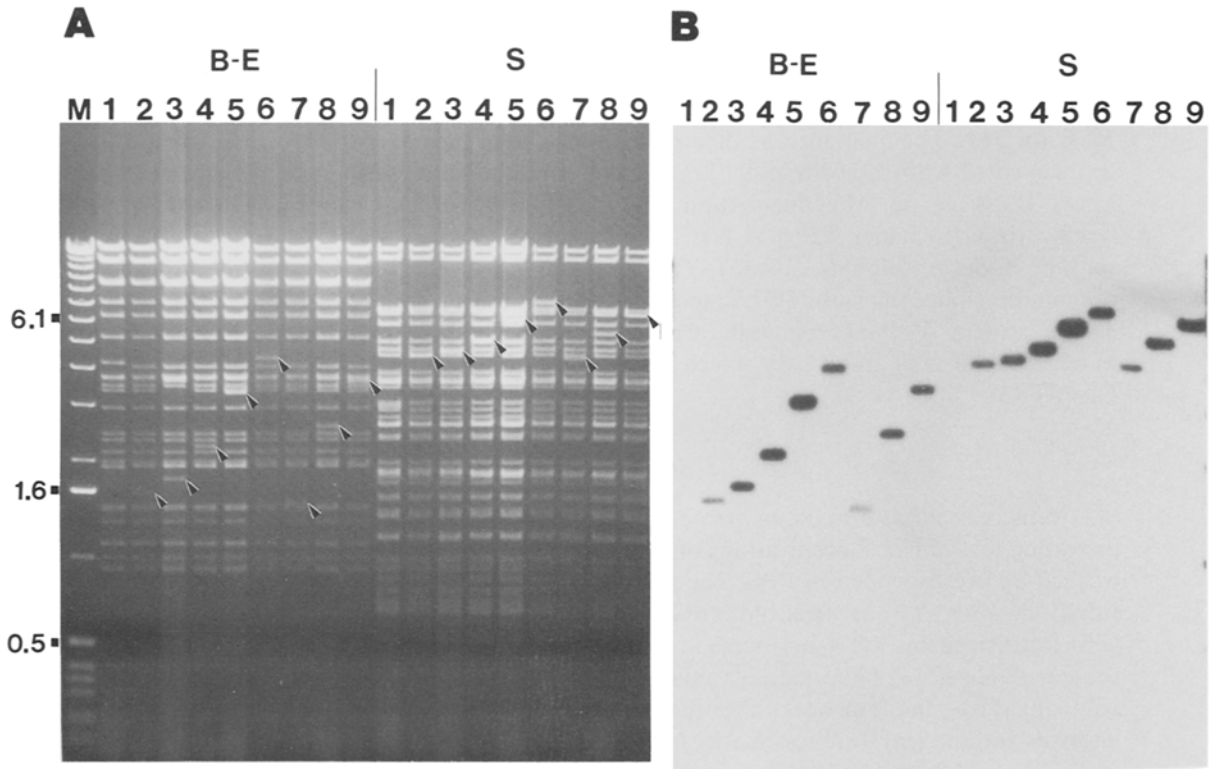
Fig. 3. Analysis of the DNA extracted from the recombinant baculoviruses. **A** The *Bam*HI-*Eco*RI (*B-E*) and *Sal*I (*S*) generated DNA fragment patterns of the wild type and the recombinant baculoviruses. Arrows show the different S gene cDNA bands. The size of some of the DNA markers (1 kb DNA ladder) in *M* is indicated on the left of the gel. *1* is the wild type AcNPV, *2* rBTS 1.6 *3* rBTS 1.8, *4* rBTS 2.2, *5* rBTS 3.3, *6* rBTS 4.4, *7* rBTS 2.2D, *8* rBTS 3.3D, *9* rBTS 4.4D. **B** Southern blot hybridization of the same gel with the cloned S gene cDNA

the size of the cloned DNA (Fig. 3A). The Southern blot hybridization with the S gene cDNA showed reactions only with the DNA bands which were expected to carry the homologous S sequences (Fig. 3B). The orientation of the foreign gene in the recombinant viruses was determined by extensive RE analysis (data not shown).

Expression of TGEV S polypeptides in Sf21 cells

The recombinant S polypeptide expression was monitored by SDS-PAGE using [^{35}S] methionine labelled lysed infected Sf21 cells (Fig. 4A), or the proteins immunoprecipitated by TGEV specific hyperimmune serum (Fig. 4B), and these gels were used for the estimation of the size of the recombinant polypeptides. The glycosylated proteins expressed by rBTS 1.6, 2.2 and 2.2D were larger (99.4 kD, 130 kD and 97.4 kD respectively) than the size predicted by the length of the cloned fragments, as these coding sequences were in frame with the downstream polyhedrin gene sequences. The size of the glycoproteins expressed
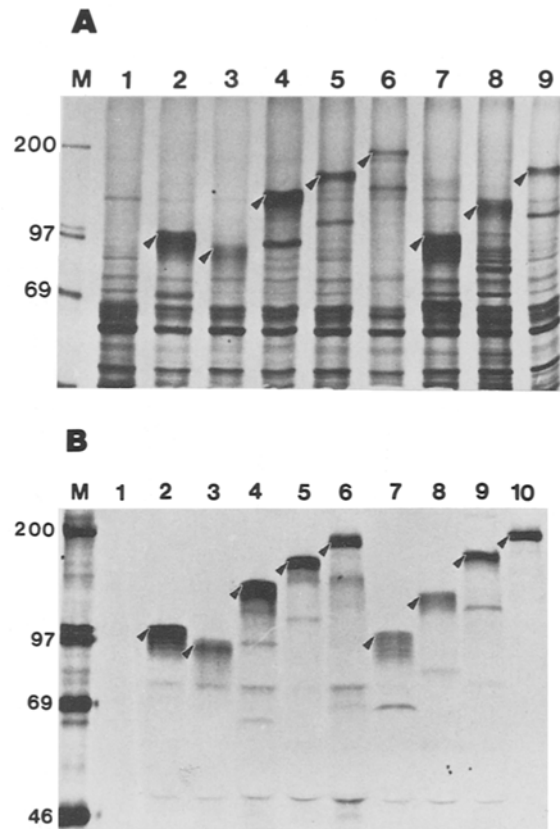
Fig. 4. A Autoradiogram of the [^{35}S] methionine labelled recombinant proteins analyzed by 8% SDS-PAGE. Each lane represents a lysate of 1×10^4 infected Sf21 cells. Arrows indicate the glycosylated S proteins expressed by the different constructs. **B** Autoradigram of the labelled recombinant proteins of 3×10^4 infected Sf21 cells, immunoprecipitated with TGEV specific pig antiserum. The size of the ^{14}C-labelled protein markers in *M* is indicated on the left. *1* AcNPV, *2* rBTS 1.6, *3* rBTS 1.8, *4* rBTS 2.2, *5* rBTS 3.3, *6* rBTS 4.4, *7* rBTS 2.2D, *8* rBTS 3.3D, *9* rBTS 4.4D, *10* TGEV infected ST cells

by rBTS 1.8, 3.3, 4.4, 3.3D and 4.4D were in agreement with the expected size (86 kD, 154 kD, 184 kD, 121.8 kD and 165.4 kD repectively). The expressed proteins were found mainly intracellularly, however those without the anchor region were also detected in the cell culture supernatants (data not shown). The maximum rate of protein synthesis was detected between 24 and 36 h p.i. but the accumulation of these peptides continued up to 72 h p.i. as measured in Coomassie blue stained polyacrylamide gels (data not shown).

Serological response of pigs immunized with the recombinant S polypeptides

Sera collected weekly from pigs immunized with Sf21 cells infected with the recombinant viruses were examined for the presence of TGEV neutralizing antibodies. Figure 5A summarizes the VN results obtained with the full length
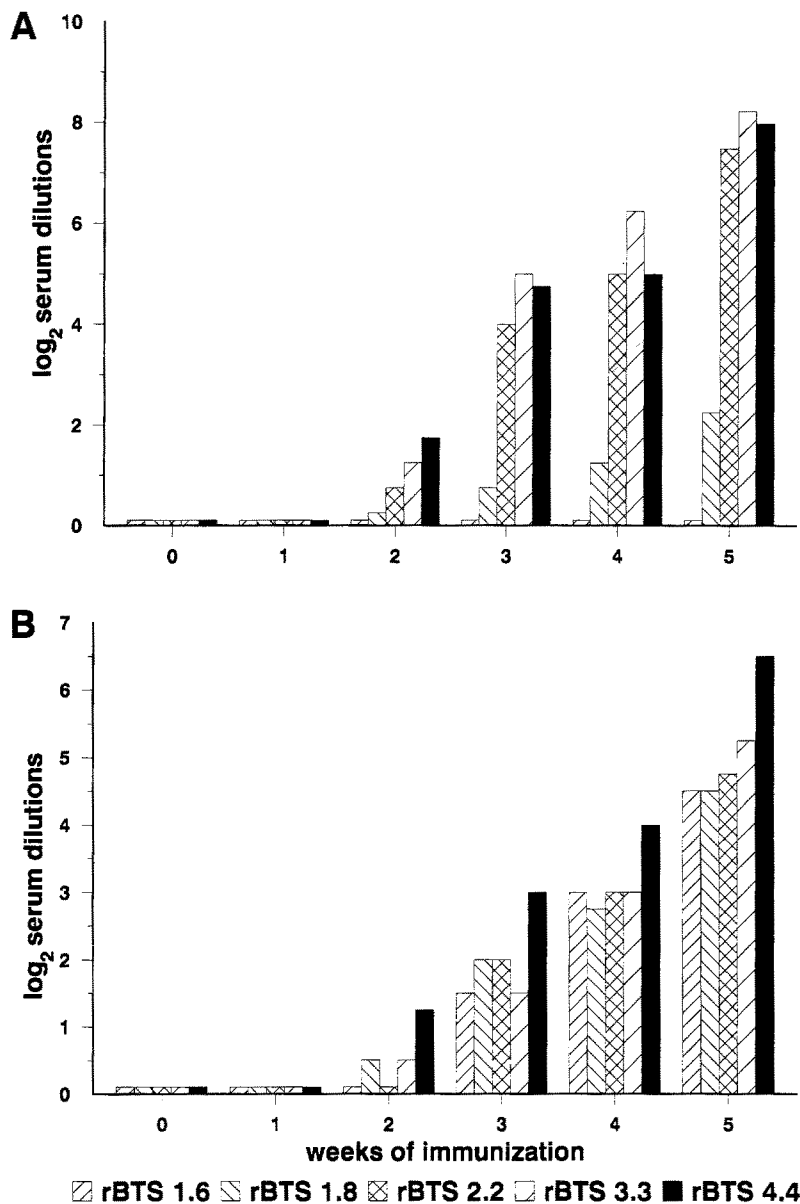
Fig. 5. A Mean TGE VN titers of sera from pigs immunized with the complete recombinant S protein or the recombinant proteins truncated at the carboxy-terminal half. Serum dilutions start at 1:2. **B** The mean antibody titers measured by the TGEV specific FCEIA. Serum dilutions start at 1:25. Standard deviations within groups were less than 5%

recombinant S protein and the proteins truncated at the carboxy-terminus. rBTS 1.6 did not induce VN antibodies and rBTS 1.8 had only a limited VN inducing capability. Those recombinant viruses which encoded not only the B, C and D antigenic sites but also the entire A region (rBTS 2.2 and 3.3) induced a VN response similar to that induced by the recombinant expressing the complete S polypeptide (rBTS 4.4). The same sera were tested in a FCEIA
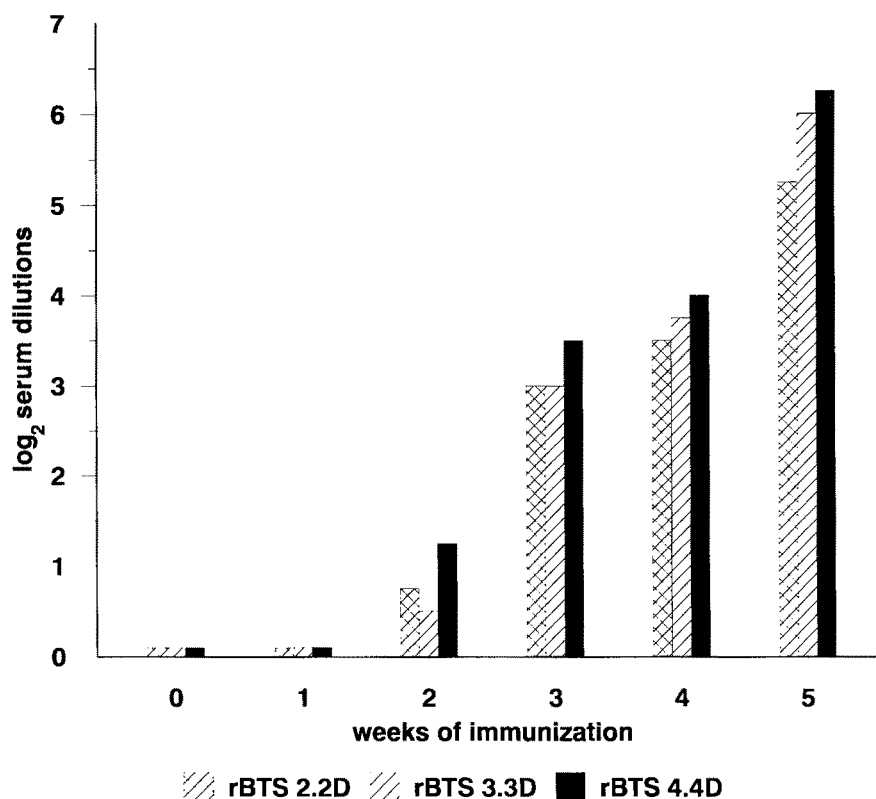
Fig. 6. TGEV specific mean FCEIA titers of the pig sera obtained after immunization with the proteins expressed by recombinant baculoviruses carrying a deletion at the 5' terminal half of the S gene. Dilutions start at 1:25

(Fig. 5B) in which they all reacted with the TGEV antigen by the third week post-vaccination, and the highest titres were generally produced in response to the larger proteins.

The three recombinant viruses with a deletion in the amino-terminal half coding sequences of the S gene did not induce any detectable VN antibodies although they all contained the A site coding sequences. When these serum samples were tested in the FCEIA, the results resembled those obtained by the recombinant viruses without the *Hae*III deletion (Fig. 6).

Sera from the pigs immunized with the wild type baculovirus, used as negative control in both assays, did not show any TGEV specific activity.

Discussion

The antigenic structure of the TGEV S glycoprotein has been well characterized by monoclonal antibodies [3,18] or by expressing fragments of the S gene in prokaryotic systems [4,11] and with the use of synthetic oligopeptides [14,24]. Due to the limitations of the prokaryotic expression system and the use of

nonglycosylated oligopeptides, the immunogenicity of defined regions of the S protein in pigs is unclear.

The baculovirus expression system has, however, not only the advantage of strong foreign gene expression under the control of the polyhedrin gene promoter but also that the recombinant polypeptides are usually expressed in a soluble form allowing the protein to fold into the proper structure. Insect cells also carry most of the enzymes necessary for the posttranslational modifications of such proteins [20].

The approach we used has proven to be useful for the study of the S1 spike glycoprotein subunit of the bovine coronavirus [34] and the TGEV S protein, although Mabs showed a reduced reactivity towards the truncated form of the TGEV S protein [15]. Our results of the pig immunization experiments showed that the baculovirus-expressed S polypeptides were capable of inducing TGEV specific antibodies of the IgG class (measured in the FCEIA, Figs. 5B and 6) and a positive correlation between the size of the recombinant protein and the amount of antibodies could be observed. This phenomenon is probably due to the higher number of antigenic determinants on the larger proteins and not because of differences in the immunogenicity of these antigens, since similar levels of VN activity were found in the sera of pigs immunized with rBTS 2.2, 3.3 and 4.4. Although the S polypeptides of rBTS 1.6, 2.2 and 2.2D were expressed as fusion proteins, their immunogenicity, as judged by the FCEIA, did not seem to be influenced by the coexpressed part of the polyhedrin protein.

Considering that the A region, which has been divided into subsites Aa, Ab and Ac [3] is the immunologically most dominant region, as shown by De Diego et al. [7] the absence of TGE specific VN antibody response to rBTS 1.6 (lacking site A) was not totally unexpected, although sites B and D (encoded by rBTS 1.6) are also thought to be involved in virus neutralization to some extent. The reason for the inability of this recombinant protein to induce a VN response is not clear unless the D site does not generate neutralizing antibodies in pigs, in contrast to mice. This result is in agreement with the observation that not all Mabs binding to the 376 to 389 amino acid sequence of S have neutralizing activity [24] and that pig polyclonal TGEV specific antibodies did not recognize the sequence FFSYGE which is essential in TGEV neutralization with D site specific Mabs [25].

The baculovirus recombinant rBTS 1.8 that carries part of the A site coding sequences, together with B, C, and D, induced only low levels of VN antibodies and the neutralization even at the lowest serum dilution was incomplete (data not shown). Antibodies with complete TGE VN activity were induced by immunization with the recombinant proteins that contained all major antigenic regions (rBTS 2.2, 3.3 and 4.4) and there was no significant difference in the VN titres obtained with these antigens although the titers were considerably lower than those obtained by TGEV immunizations [16]. Based on these results it might be assumed that sites B, C and D are not necessary to generate a VN response in pigs and that expression of the essential A site in a vector vaccine may be sufficient to protect against TGE. However, the data obtained for those

recombinant proteins which were encoded by the recombinant viruses with a 747 bp deletion at the 5'-terminal half of the S gene (rBTS 2.2D, 3.3D, and 4.4D) refute this assumption. None of these recombinants was able to induce a VN response in pigs despite their intact A site coding sequences, and increasing the size of the cloned gene from 1489 bp to 2582 bp or 3594 bp in rBTS 3.3D and 4.4D did not improve their VN generating capability. This result can be explained by the fact that the deletion of these HaeIII DNA fragments not only eliminated the D site coding region but also affected the two putative receptor binding sites of the S protein [28, 29]. This deletion also may have altered the proper folding of the site A epitopes. Our findings do not exclude the possible utility of shorter deletions or deletions of different parts of the 5'-terminal half of the S gene as occurs naturally among strains of the porcine respiratory coronavirus [28] although the effect of such deletions on the globular structure [8] and on the immunogenicity of the amino terminal half of the S protein is difficult to predict.

The results reported in this paper indicate that the amino acid residues encoded by the 3'-terminal half of the S gene do not affect the VN response induced in pigs and that the amino-terminal half of the S protein is sufficient to generate a VN response as effectively as the whole S protein, shown by the comparison of rBTS 2.2 and 4.4. By reducing the size of the S gene from 4.3 kb to 2.2 kb it may be possible to generate a helper independent recombinant porcine adenovirus vaccine expressing the part of the S glycoprotein necessary to induce protective immunity in pigs.

Acknowledgements

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