EXPRESSION OF TGEV STRUCTURAL GENES IN VIRUS VECTORS

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INTRODUCTION

TGEV is a coronavirus that causes gastroenteritis in pigs, resulting in a high mortality of neonates. The TGEV virion contains three major structural polypeptides; a surface glycoprotein (spike or peplomer protein) with a monomeric M_r 200000, a glycosylated integral membrane protein observed as a series of polypeptides of M_r 28000-31000 and a basic phosphorylated protein (the nucleoprotein) of M_r 47000 associated with the viral genomic RNA (1). The genes encoding TGEV nucleoprotein (NP), integral membrane protein (E1) and peplomer (E2) have been cloned and sequenced from an avirulent laboratory strain, Purdue (2, 3, 4, 5) and a virulent British field isolate, FS772/70 (6, 7).

TGEV antigens may be produced by gene expression in a eukaryotic vector system. Vaccinia viruses (VV) and baculoviruses have the potential for transporting, processing and folding foreign eukaryotic gene products correctly. Many heterologous genes have been expressed in these viral vector systems and are antigenically and functionally identical to their original gene product (8, 9). VV is a poxvirus, a large DNA (186 kpb) pleomorphic virus capable of carrying a 25 kb insert of foreign DNA, providing the potential to express all three coronavirus structural genes simultaneously. Wild-type and recombinant vaccinia viruses (RVV) have a wide host range, capable of infecting most mammalian species and inducing humoral and cell mediated immune responses. Utilisation of the VV $P_{7.5K}$ promoter enables modest expression of foreign genes during early and late infection. The insect baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV) has a large DNA genome 126-129 kbp that can incorporate relatively large segments of foreign DNA. The virus is non-pathogenic and incapable of replication in vertebrate cells, making the vector inherently safe, but limiting its potential for expressing antigen to the immune system. The polyhedrin gene is a nonessential segment of the viral genome regulated by a strong late P_{33K} promoter. There is no evidence for cryptic RNA splicing and by utilising the strong late P_{33K}, very large amounts of a foreign gene product can be expressed with most post-translational modifications.

In this paper we describe the construction of recombinant viruses containing the TGEV NP, E1 and E2 genes and compare the products expressed with TGEV structural proteins to evaluate a suitable TGEV protein candidate for providing immunity.

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METHODS AND MATERIALS

Viruses and cells

Transfections and growth of wild-type VV (WR strain) and recombinant vaccinia viruses (RVV) were performed as in Mackett et al. (10). AcNPV and recombinant baculovirus stocks were grown and assayed according to the procedures of Brown and Faulkner (11) on Spodoptera frugiperda cells (Sf9) at 28°C with Graces Insect medium (Flow Labs.) containing 10% FCS. TGEV (FS772/70) was grown on adult pig thyroid (APT/2) cell monolayers (12) for neutralisation assays. TGEV antigens were isolated from infected LLC-PK1 cells (13).

Construction of plasmid insertion vectors

Recombinant plasmids pGSN1, pGSIM1 and PGSP1 were constructed by ligating the NP, E1 and E2 BamHI gene cassettes from plasmids pBNP5 (6), pBIM3 (7), and pPBP1 respectively into the BamHI site of pGS20 (14). The E1 BamHI gene cassette was ligated into the baculovirus insertion vector pAcRP23 BamHI site to generate pAcI6.

Production of recombinant viruses

RVV containing only one TGEV gene cassette were constructed by the methods of Mackett et al. (10, 14). Recombinant baculoviruses were generated by cotransfecting pAcI6 plasmid DNA with infectious AcNPV DNA (15) on Sf9 cells (16). Plaques not containing visible occlusion bodies were recovered from agarose overlays of infected monolayers.

Extraction and characterisation of viral DNA

Wild-type and recombinant vaccinia virus DNA was prepared as by Merchlinsky and Moss (17). Recombinant and wild-type AcNPV virions were isolated from infected cell culture medium as in Matsuura et al. (16) and DNA prepared as by Merchlinsky and Moss (17).

Immunofluorescence

Cultures of HTK^- cells on glass coverslips were infected with recombinant vaccinia viruses at m.o.i. = 1 and incubated for 24 h. The infected cells were fixed in cold 80% acetone and probed with mouse monoclonal antibodies (mAbs) DA3 (18) and 3BB3 (19) specific to TGEV NP and E1 respectively, and a cocktail of mouse anti-TGEV E2 mAbs, including 1B6, 5A5, 6A6, 8A4, 3C1 and 6D4 (20) all diluted 1/500. Bound antibody was detected with fluorescein-conjugated rabbit anti-mouse IgG antiserum (Nordic Immunology).

Immunoblot analysis

Infected cell cultures were lysed in 0.1M Tris-HCl pH 8.0, 0.1M NaCl, 0.5% Nonidet P40, 0.1% Aprotinin (Sigma), and proteins transferred onto nitrocellulose membrane (BA85, Schleicher and Schuell) using a Biorad Dot Blot apparatus or after SDS polyacrylamide gel electrophoresis.

Immunoprecipitations

Infected cells were lysed with RIPA buffer as described by Garwes et al. (20) and supernatants containing TGEV polypeptides were preabsorbed with formalin-fixed Staphylococcus aureus (Sac) cells (Cowan strain, Immunoprecipitin, BRL) before incubating with antisera for 1 h and precipitating with washed Sac cells and analysed as in Britton et al. (21).

Animal inoculations

Partially purified recombinant and wild-type VV were prepared by pelleting stock virus through an equal volume of 36% w/v sucrose in 10 mM Tris-HCl, 1 mM EDTA pH 8.8 in a 55.5 swing-out rotor (Kontron Instruments) at 25,000 x g for 80 min at 4°C. Pelleted virus was resuspended in phosphate buffered saline (PBS 10 mM potassium phosphate, 150 mM NaCl, pH 7.2) for inoculation into animals.

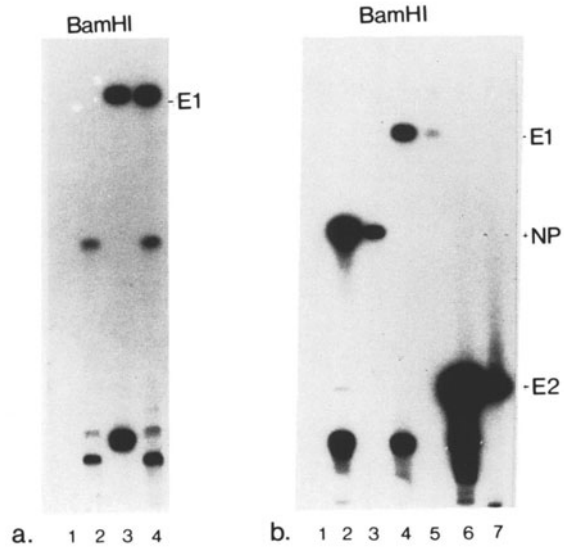

Fig. 1. Identification of TGEV genes in recombinant virus genomes by Southern blot with high molecular weight fragments at the bottom. Plasmid and viral DNA was digested with BamHI for 4 h and DNA fragments separated by electrophoresis on a 0.8% agarose gel. Denatured DNA was transferred onto a Biodyne filter and probed. Fig. 1a Lane (1) contained pAcRP23, (2) vAcNPV, (3) pAcI6, (4) vAcI2 DNA probed with ^{32}P-labelled E1 BamHI gene cassette. Fig. 1b Lane (1) contained wt VV, (2) pGSN1, (3) vTN1, (4) pGSI1, (5) vTI1, (6) pGSP1, (7) vTP1 DNA probed with ^{32}P-labelled NP, E1 and E2 BamHI gene cassettes.

TGEV neutralisation assay

Mouse and pig anti-recombinant vaccinia sera were heat treated at 55°C for 30 mins to destroy any complement activity before being serially diluted and mixed with 100 pfu of TGEV in an equal volume of M199 medium (Medium 199 (Flow Laboratories), supplemented with 50 mM HEPES, 0.14% sodium bicarbonate, 2.5% calf serum and antibiotics) for 1 h at 37°C. Residual virus activity was assayed in triplicate by plaque reduction using APT/2 cells incubated with a M199/0.6% agarose overlay. After 3 days the plaques were detected with a neutral red overlay and the neutralising titres were expressed as the reciprocal of the serum dilution that reduced 50% of the plaques.

RESULTS AND DISCUSSION

Construction and DNA analysis of recombinant viruses

All three complete TGEV structural protein genes were inserted downstream of the vaccinia early/late $P_{7.5K}$ promoter in the plasmid insertion vector pGS20. The TGEV NP gene was contained in a BamHI cassette (6), which also included a second small open reading frame (ORF4), and was used to generate RVV vTN1. The TGEV E1 gene was inserted into vaccinia and baculovirus genomes as a BamHI gene cassette (7). The E1 gene was regulated by the early/late $P_{7.5K}$ promoter in RVV vTI1 and by the strong late P_{33K} polyhedrin promoter in vAcI2. The E2 gene was constructed as a 4657 bp BamHI gene cassette (unpublished result) and used to generate a recombinant vaccinia virus vTP1. The recombinant viral genomes were analysed by Southern blot to confirm the integration of the TGEV structural genes (Fig. 1).

Recombinant virus expression of nucleoprotein

The gene products have been characterised by immune blotting and immunoprecipitation of recombinant VV infected cell lysates. vTN1 infected cell lysates contained a polypeptide M_r 47000, recognised by DA3 and polyclonal cat anti-feline infectious peritonitis virus (FIPV) serum, a serologically related coronavirus. The recombinant NP comigrated with the TGEV nucleoprotein (Fig. 2) implying that it was the same as the TGEV product. The last 20 residues of the NP carboxyl-terminus have been identified as containing the epitope site for the DA3 mAb (J. M. Alonso Martin, personal communication). A smaller NP species of M_r 42000 has been observed in TGEV infected cells using polyclonal antiserum (13), but is not detected with DA3. A potential trypsin cleavage site, KRK, has been identified at the carboxyl-terminus of the nucleoprotein that could result in the deletion of the last 39 amino acids, containing the DA3 epitope,

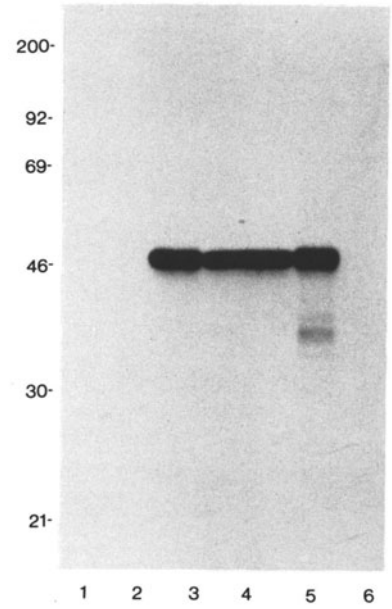

Fig. 2. Cell lysates from uninfected (1) WR infected (2) vTN1 24 h (3) and 48 h (4) post infected HTK^- cell lysates; TGEV infected (5) and uninfected (6) LLC PK1 cells were separated by electrophoresis on a 10% SDS PAGE gel, transferred onto nitrocellulose and probed with DA3.

resulting in the truncated form of the nucleoprotein. Immunoprecipitation of radiolabelled TGEV antigen with DA3 can co-precipitate the M_r 42000 species with the M_r 47000 species, indicating that these two species of NP can associate. Smaller species of NP from vTN1 infected cells have also been detected with FIPV polyclonal antiserum, but not with DA3, and may be progressive degradation products. Breakdown products are found in TGEV infected cells when using DA3 (Fig. 2, lane 5) indicating that degradation may also occur from the N-terminus.

By indirect immunofluorescence (IIF), NP appeared to aggregate into complexes in the cell cytoplasm, at 24 h post infection with vTN1 (Fig. 4a) and by 8 h in TGEV infected cells. No recombinant or TGEV NP has been seen at the cell surface of unfixed infected cells.

Recombinant virus expression of integral membrane protein (E1)

Sequence analysis of the protein has identified three potential N-glycosylation sites at Asn [32], [55] and [251] each potentially adding 2000 daltons onto the M_r 29459 polypeptide (7). vTI1 infected cells produce a single species polypeptide of M_r 30000 but vAcI2 infected Sf9 cells produce two species of E1, an abundant M_r 30000 protein and a less common M_r 28000 species (Fig. 3). Cells infected with either vTI1 or vAcI2 in the presence of tunicamycin only express the M_r 28000 species. These observations support the theory that only one E1 N-glycosylation site, Asn [32], is occupied. The other two potential N-glycosylation sites occur in hydrophobic areas of the molecule and are thought to be hidden in the viral membrane. Larger but minor species of E1 have been observed in TGEV

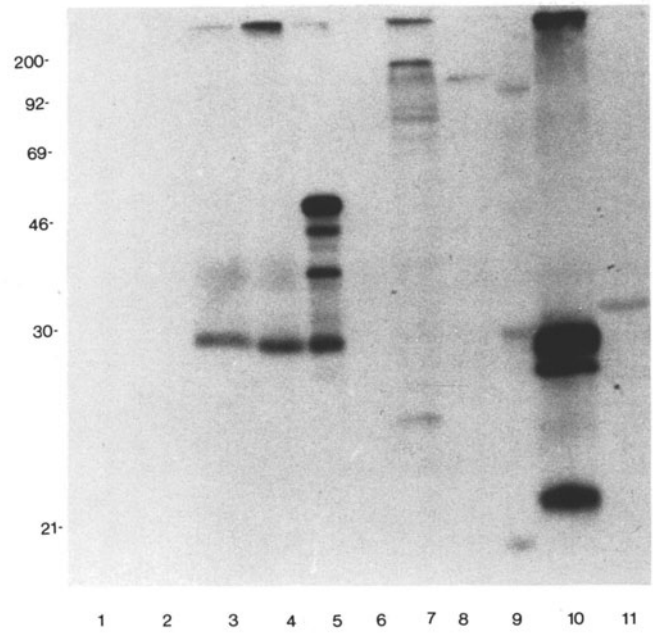

Fig. 3. Cell lysates from uninfected (1), WR infected (2), vTI1 24 h (3) and 48 h (4) post infected HTK$^-$ cell lysates; TGEV infected (5) and uninfected (6) LLC PK1 cells; pURIM2 transfected (7) and pUR290 (8) transfected E. coli JM101 cell lysates [7, 21]; protein high molecular weight markers (9); vAcI2 (10) and vAcNPV (11) infected cell lysates were electrophoresed on a 10% SDS PAGE gel, transferred onto nitrocellulose and probed with cat anti-FIPV serum.

infected cells (1) but have not been detected in either recombinant virus infections. The immunogenicity of the unglycosylated form of the E1 molecule appears unaffected as it is recognised by both FIPV polyclonal and 3BB3 monoclonal antiserum.

By IIF, E1 usually has a polar distribution within the cytoplasm of TGEV and vTI1 infected cells (Fig. 4b), consistent with the hypothesis that E1 is associated with the endoplasmic reticulum (ER) and golgi apparatus. The distribution of E1 in Sf9 cells was generally cytoplasmic (Fig. 4d). Contrary to earlier observations, TGEV E1 was not found on the cell surface of TGEV or recombinant virus infected cells, supporting the evidence that the E1 molecule has its own targeting and anchoring transport signals which restrict it to the endoplasmic reticulum and golgi apparatus.

Sequence analysis of the TGEV peplomer

The E2 gene is an ORF of 4341 bp coding for 1447 amino acids contained within the TGEV 8.4 kb mRNA subgenomic species. The sequence context, (AC)ACCATGA, of the peplomer gene is favourable for initiation by eukaryotic ribosomes ((CC)ACCATGG) (22, 23). The peplomer gene is terminated by the codon TAA which is also the terminator of the nucleoprotein and integral membrane protein genes. The first 16 amino acids of the peplomer polypeptide fulfil the criteria of being a eukaryotic signal sequence, with the potential cleavage site between the glycine [16] and aspartic acid [17] residues. The cleavage site of the peplomer signal sequence has been confirmed (5), from the avirulent Purdue strain of TGEV, as being between residues 16 and 17 by N-terminal amino acid sequencing of the peplomer isolated from virions. Following cleavage of the signal sequence the

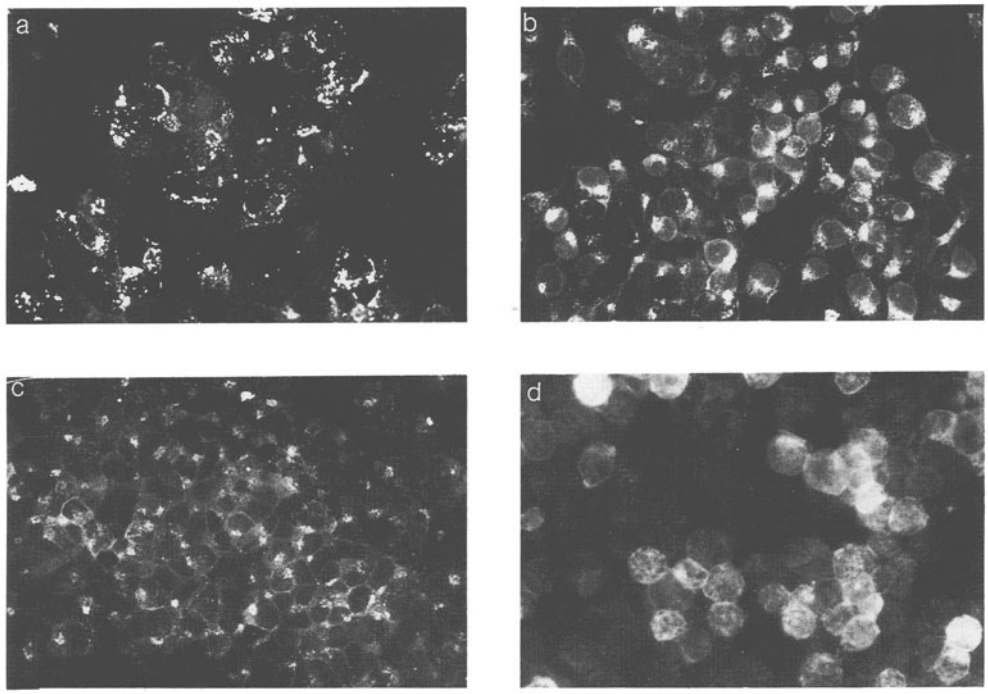

Fig. 4. Cellular location of TGEV structural proteins in recombinant virus infected cells by indirect immunofluorescence. NP was detected using DA3 (a), E1 using 3BB3 (b), and E2 using a mAb cocktail (c). E1 was also detected in fixed vAcI2 infected Sf9 cells with 3BB3 at 48 h post infection (d).

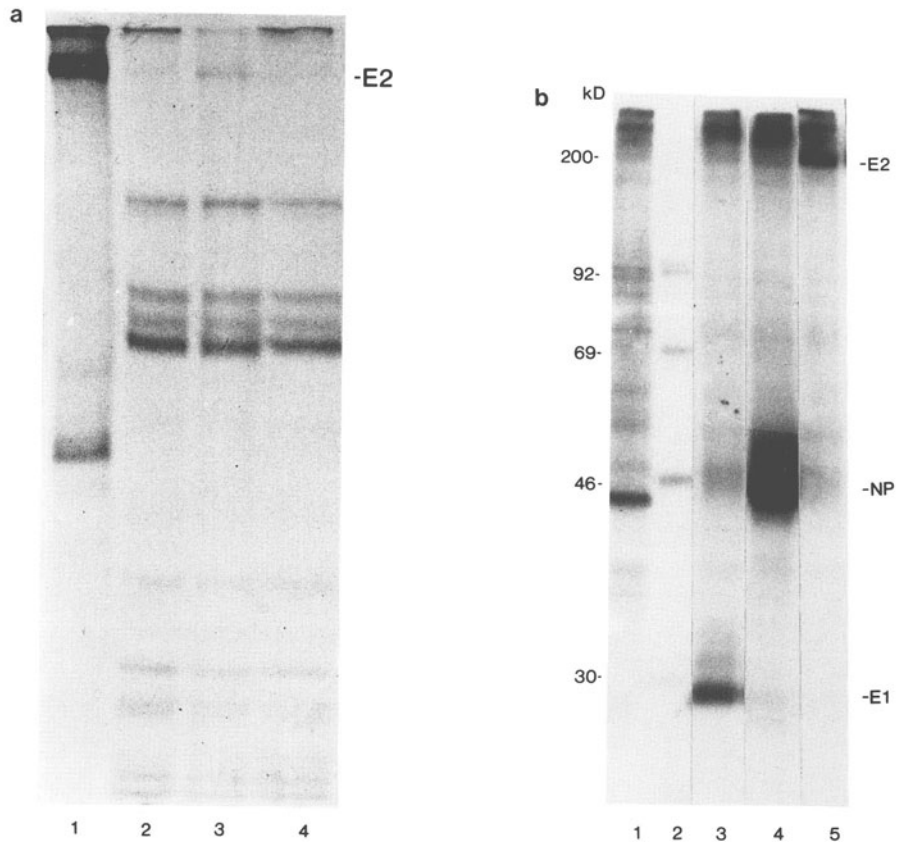

Fig. 5a. Immunoprecipitation of [^{35}S]-methionine labelled E2 protein from TGEV infected LLC PK1 cells (1); vTP1 (2), (3) and WR (4) infected HTK$^-$ cells with cat anti-FIPV serum (1), (3), (4), and 3C1 (2) a major neutralising E2 monoclonal antibody.

Fig. 5b. Immunoprecipitation of [^{35}S]-methionine labelled TGEV polypeptides with mouse anti-vTI1 (3), anti-vTN1 (4) and anti-vTP1 (5) antiserum. Lane (1) contains whole cell lysate diluted 1:100 and (2) protein high molecular weight markers.

primary structure of the peplomer would be 1433 amino acids with a M_r 157891. There are 33 potential N-linked glycosylation sites providing the TGEV peplomer monomer with an overall M_r 226000. The monomeric molecular weight of the TGEV peplomer, by SDS PAGE, is M_r 200000 (1) implying that most of the potential N-linked glycosylation sites must be occupied.

Recombinant virus expression of peplomer (E2)

The E2 antigen is sensitive to reduction by 2-mercaptoethanol. E2 expressed by RVV can be detected by immunoblotting in non-reducing conditions, but is best observed by immunoprecipitation (Fig. 5a), where it comigrated with the peplomer protein of TGEV M_r 200000. The amount of RVV expressed E2 is poor compared with TGEV. A recombinant vaccinia virus expressing TGEV E2 cloned from the Miller strain has been previously published (24). This E2 gene construct had a deletion at the 3' end which resulted in the loss of the last 190 amino acids, which included part of the stalk and all of the anchor region. This observation was obtained from

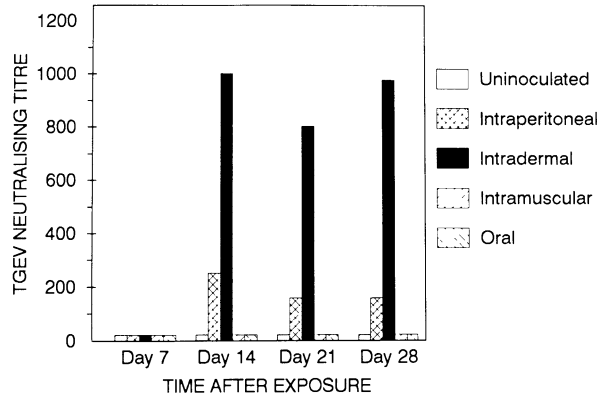

Fig. 6. The effect of inoculation route at inducing TGEV neutralising antibodies in vTP1 infected gnotobiotic piglets.

our sequencing analysis and was confirmed by other data (5) for the Purdue strain of TGEV. The truncated form of E2 was capable of inducing low level TGEV neutralising antibody, but could not be detected on the cell surface by IIF probably due to the deletion of the anchor region.

Analysis, by IIF, showed that the E2 from RVV vTP1 reacted with all the peplomer competition mAb groups (20) indicating that the immunogenicity of the expressed E2 is preserved. Fixed and unfixed infected cells had a diffuse cytoplasmic staining with occasional polar concentrations on the cell perimeter. Fine points of fluorescence, probably capped protein, appeared around the cell perimeter of unfixed vTP1 infected cells suggesting that the E2 is exported to the cell membrane. No specific staining was observed for unfixed WR infected and uninfected cells using E2 mAbs. Antisera raised in mice to all three RVV were analysed by immunoprecipitation and neutralisation assay. All mouse anti-RVV serum specifically immunoprecipitated a single TGEV structural protein product (Fig. 5b). No

Table 1. TGEV neutralisation assay using mouse anti-RVV serum

Vaccinia virus	Single dose	Hyperimmunisation
vTN1	10	10
vTN2	10	60
vTI1	<10	40
vTI2	<10	<10
vTP1	490	1120
vTP2	160	430
WR	<10	<10

significant TGEV neutralisation was detected with mouse anti-vTN1 or vTI1 serum (Table 1), though it has been reported that anti-E1 mAbs in the presence of guinea pig complement can enhance TGEV neutralisation (25). Only mouse anti-vTP1 serum and hyperimmune vTP-1 serum contained neutralising antibody titred at 490 and 1120 respectively in a 50% TGEV plaque reduction assay, demonstrating that the recombinant product can induce TGEV neutralising activity (Table 1). The efficacy of vTP1 inducing neutralising antibody was studied in gnotobiotic piglets. Animals were given a standard inoculum of vTP1 (2×10^5 pfu) by the intramuscular, intraperitoneal, oral (by feeding through a stomach tube), intradermal (scarification) and control (uninoculated) routes (Fig. 6). Only two routes induced TGEV neutralising serum antibody. Intraperitoneal inoculation induced a titre of 250 at 14 days post infection but diminished by 28 days. Intradermal inoculation induced a neutralising titre of 950 which remained constant up to 28 days post infection. Work is in progress to determine if vTP1 serum antibodies can protect the piglet from a TGEV infection.

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REFERENCES

1. D. J. Garwes and D. H. Pocock, The polypeptide structure of transmissible gastroenteritis virus. J. Gen. Virol., 29:25-34 (1975).
2. P. A. Kapke and D. A. Brian, Sequence analysis of the porcine transmissible gastroenteritis coronavirus nucleocapsid protein gene. Virology, 151:41-49 (1986).
3. P. A. Kapke, F. Y. C. Tung, D. A. Brian, R. D. Woods and R. Wesley, Nucleotide sequence of the porcine transmissible gastroenteritis coronavirus matrix protein, Adv. Exp. Med. Biol., 218: 117-122 (1987).
4. H. Laude, D. Rasschaert and J. C. Huet, Sequence and N-terminal processing of the transmembrane protein E1 of the coronavirus transmissible gastroenteritis virus, J. Gen. Virol., 68:1687-1693 (1987).
5. D. Rasschaert and H. Laude, The predicted primary structure of the peplomer protein E2 of the porcine coronavirus Transmissible Gastroenteritis Virus, J. Gen. Virol., 68:1883-1890 (1987).
6. P. Britton, R. S. Carmenes, K. W. Page, D. J. Garwes and F. Parra, Sequence of the nucleoprotein gene from a virulent British field isolate of transmissible gastroenteritis virus and its expression in Saccharomyces cerevisiae, Mol. Microbiol., 2: 89-99 (1988).
7. P. Britton, R. S. Carmenes, K. W. Page and D. J. Garwes, The integral membrane protein from a virulent isolate of transmissible gastroenteritis virus: Molecular characterisation, sequence and expression in Escherichia coli, Mol. Microbiol., 2:497-505 (1988).
8. B. Moss and C. Flexner, Vaccinia virus expression vectors, Ann. Rev. Immunol., 5:305-324 (1987).

9. W. Doerfler, Expression of the Autographa californica Nuclear Polyhedrosis virus genome in insect cells: Homologous viral and heterologous vertebrate genes - the Baculovirus vector system, Current Topics in Microbiol. and Immunol., 131:51-65 (1986).
10. M. Mackett, G. L. Smith and B. Moss, The construction and characterisation of vaccinia virus recombinants expressing foreign genes. in: "DNA Cloning, a Practical Approach, Vol. II", D. M. Glover, ed., IRL Press (1985).
11. M. Brown and P. Faulkner, A plaque assay for nuclear polyhedrosis viruses using a solid overlay, J. Gen. Virol., 36:361-364 (1977).
12. D. H. Pocock and D. J. Garwes, The influence of pH on the growth and stability of transmissible gastroenteritis virus in vitro, Arch. Virol., 49:239 (1985).
13. D. J. Garwes, L. Bountiff, G. C. Millson and C. J. Elleman, Defective replication of porcine transmissible gastroenteritis virus in a continuous cell line, Adv. Exp. Med. Biol., 178:79-93 (1984).
14. M. Mackett, G. L. Smith and B. Moss, General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes, J. Virol., 49:857-864 (1984).
15. G. E. Smith and M. D. Summers, Analysis of baculovirus genomes with restriction endonucleases, Virology, 89:517-527 (1978).
16. Y. Matsuura, R. D. Possee, H. A. Overton and D. H. L. Bishop, Baculovirus expression vectors: The requirements for high level expression of proteins, including glycoproteins, J. Gen. Virol., 68:1233-1250 1987).
17. M. Merchlinsky and B. Moss, Resolution of vaccinia virus DNA concatamer junctions requires late-gene expression, J. Virol., 63:1595-1603 (1989).
18. D. J. Garwes, F. Stewart, S. F. Cartwright and I. Brown. Differentiation of porcine coronavirus from transmissible gastroenteritis virus, Vet. Rec., 122:86-87 (1988).
19. G. Jimenez, I. Correa, M. P. Melgosa, M. J. Bullido and L. Enjuanes, Critical epitopes in transmissible gastroenteritis virus neutralisation, J. Virol., 60:131-139 (1986).
20. D. J. Garwes, F. Stewart and C. J. Elleman, Identification of epitopes of immunological importance on the peplomer of porcine transmissible gastroenteritis virus, Adv. Exp. Med. Biol., 218:509-516 (1987).
21. P. Britton, D. J. Garwes, K. W. Page and J. Walmsley, Expression of porcine transmissible gastroenteritis virus genes in E. coli as β-galactosidase chimaeric proteins, Adv. Exp. Med. Biol., 218:55-64 (1987).
22. M. Kozak, Comparison of initiation of protein synthesis in prokaryotes, eukaryotes and organelles, Microb. Rev., 47:1-45 (1983).
23. M. Kozak, Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryote ribosomes, Cell, 44:283-293 (1986).
24. S. Hu, J. Bruszewski and R. Smalling, Infectious vaccinia virus recombinant that expresses the surface antigen of porcine transmissible gastroenteritis virus (TGEV), in: "Vaccinia Viruses as Vectors for Vaccine Antigens," Quinnan, ed., Elsevier Science Publishing Co., the Netherlands (1985).
25. R. D. Woods, R. D. Wesley and P. A. Kapke, Complement-dependent neutralisation of Transmissible Gastroenteritis Virus by monoclonal antibodies, Adv. Exp. Med. Biol., 218:493-500 (1987).