Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Virology 410 (2011) 107–118

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Transmissible gastroenteritis virus (TGEV)-based vectors with engineered murine tropism express the rotavirus VP7 protein and immunize mice against rotavirus

Juan Manuel Ribes [a], Javier Ortego [b,c], Juan Ceriani [b], Rebeca Montava [a], Luis Enjuanes [b], Javier Buesa [a,*]

[a] Department of Microbiology and Ecology, School of Medicine, University of Valencia, Avda. Blasco Ibáñez, 17, 46010 Valencia, Spain
[b] Department of Molecular and Cell Biology, Centro Nacional de Biotecnología, CSIC, Cantoblanco, 28049 Madrid, Spain
[c] CISA-INIA, Valdeolmos, 28130 Madrid, Spain

ARTICLE INFO

Article history:
Received 14 July 2010
Returned to author for revision
18 August 2010
Accepted 27 October 2010
Available online 21 November 2010

Keywords:
Transmissible gastroenteritis virus
Viral vectors
Tropism
Rotavirus
Diarrhea
Protection

ABSTRACT

A coronavirus vector based on the genome of the porcine transmissible gastroenteritis virus (TGEV) expressing the rotavirus VP7 protein was constructed to immunize and protect against rotavirus infections in a murine model. The tropism of this TGEV-derived vector was modified by replacing the spike S protein with the homologous protein from mouse hepatitis virus (MHV). The rotavirus gene encoding the VP7 protein was cloned into the coronavirus cDNA. BALB/c and STAT1-deficient mice were inoculated with the recombinant viral vector rTGEV$_{S\text{-}MHV}$–VP7, which replicates in the intestine and spreads to other organs such as liver, spleen and lungs. TGEV-specific antibodies were detected in all the inoculated BALB/c mice, while rotavirus-specific antibodies were found only after immunization by the intraperitoneal route. Partial protection against rotavirus-induced diarrhea was achieved in suckling BALB/c mice born to dams immunized with the recombinant virus expressing VP7 when they were orally challenged with the homotypic rotavirus strain.

Introduction

Transmissible gastroenteritis virus (TGEV) is a member of the *Coronaviridae* family within the *Nidovirales* order (Enjuanes et al., 2000). TGEV has a single-stranded, plus sense 28.5-kb RNA genome (Penzes et al., 2001). Open reading frames (ORFs) 1a and 1b, which encode the replicase (rep) are located at the 5′-end of the genome and comprise approximately two-thirds of the entire RNA. The 3′-end of the genome includes the genes encoding structural and nonstructural proteins, in the order 5′-S-3a-3b-E-M-N-7-3′ and occupy one-third of the genome (Enjuanes et al., 2000; de Groot et al., 2010). TGEVs are enveloped spherical viruses containing four essential structural proteins: the membrane (M), the small envelope (E), the spike (S), and the nucleocapsid (N) proteins. The M protein spans the membrane three or four times and interacts with the nucleocapsid (N) and spike (S) proteins during assembly (Escors et al., 2001). The S protein, a large type I transmembrane glycoprotein, forms peplomers and is responsible for viral attachment to specific host receptors and membrane fusion (Lewicki and Gallagher, 2002; Sune et al., 1990). The small envelope (E) protein is a transmembrane protein that is present as a minor structural component. The N protein wraps the genomic RNA into a nucleocapsid (Escors et al., 2001; Kapke and Brian, 1986).

Coronaviruses have several advantages as vectors over other viral expression systems: (i) they are single-stranded RNA viruses that replicate in the cytoplasm without a DNA intermediary, making integration of the virus genome into the host cell chromosome unlikely (Lai and Cavanagh, 1997); (ii) these viruses have the largest RNA virus genome and, in principle, have room for the insertion of large foreign genes (Enjuanes et al., 2001); (iii) a pleiotropic secretory immune response is best induced by the stimulation of gut-associated lymphoid tissues. Since coronaviruses generally infect mucosal surfaces, both respiratory and enteric, they could be used to target the antigen to the enteric and respiratory areas to induce a strong secretory immune response; (iv) the tropism of coronaviruses may be engineered by modifying the S gene (Haijema et al., 2003; Kuo et al., 2000; Sanchez et al., 1999); (v) nonpathogenic coronavirus strains infecting most species of interest (human, porcine, bovine, canine, feline, and avian) are available and therefore suitable to develop safe vectors; and (vi) infectious coronavirus cDNA clones (Almazan et al., 2000; Casais et al., 2001; Thiel et al., 2001; Yount et al., 2003) and coronavirus-derived replicons (Almazan et al., 2004, 2006) are available to design expression systems.

Rotavirus is the main cause of diarrhea-related illness and death in children worldwide. It is associated with 25 million outpatient visits, more than 2.3 million hospitalizations and up to 600,000 deaths in children <5 years old (Parashar et al., 2006). Rotavirus infection

* Corresponding author. Department of Microbiology, School of Medicine, University of Valencia, Avda. Blasco Ibáñez, 17, 46010 Valencia, Spain. Fax: +34 96 386 4960.
E-mail address: javier.buesa@uv.es (J. Buesa).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.10.036

usually results in acute gastroenteritis characterized by vomiting and severe watery diarrhea, often leading to dehydration (Kapikian et al., 2001). Children usually become infected in the first 3–5 years of life, with the highest incidence in those aged <2 years. Naturally acquired rotavirus infection protects against subsequent severe disease, particularly following repeated exposures (Velazquez et al., 1996).

Rotavirus is a member of the *Reoviridae* family. The virion is a nonenveloped particle and possesses a triple layered capsid structure that surrounds the genome composed of 11 segments of double-stranded RNA. There are six structural proteins and six nonstructural proteins, each protein being encoded by a unique genome segment except for two nonstructural proteins (NSP5 and NSP6), which are encoded by an overlapping reading frame of a single segment. The antigenic properties of group, subgroup and serotype/genotype of rotaviruses are determined by the viral capsid proteins (VP), named VP6, VP4 and VP7. VP4 and VP7 (the outer capsid proteins) contain epitopes that elicit neutralizing antibodies (Kapikian et al., 2001).

The use of recombinant viruses is a very promising approach in the design of new vaccines against rotavirus. In this study, a viral vector has been developed based on porcine transmissible gastroenteritis coronavirus (TGEV) full-length genome sequence expressing the rotavirus VP7 protein. Genetic engineering techniques were used to generate this recombinant vector from a cDNA fragment containing the full-length genome sequence of TGEV. Thereafter, the tropism of the TGEV-derived vector was modified by replacing the spike S protein with the homologous protein from mouse hepatitis virus (MHV-A59 strain). The recombinant vector replicated stably *in vitro* in murine cell cultures producing the rotavirus VP7 protein. The vector was inoculated to BALB/c and STAT1−/− mice and *in vivo* replication was confirmed in different organs by RT-PCR and immunofluorescence of histological sections. BALB/c mice immunized with the vector produced rotavirus-specific IgG and IgA class antibodies, as determined by enzyme-linked immunosorbent assay. Cytokine production in serum samples and cultivated splenocytes of inoculated mice was determined by analysing protein levels of 10 cytokines using a multiplex bead-based flow cytometry assay. The protection study in the newborn mouse model demonstrated partial protection (62% of the challenged animals) against rotavirus-induced diarrhea by the homotypic rotavirus RF strain.

Results

Generation of rTGEV$_{S\text{-}MHV/C11}$

Recombinant rTGEV$_{S\text{-}MHV/C11}$ was obtained by using the strategy described to generate a cDNA encoding a full-length TGEV RNA (Almazan et al., 2000). The plasmid pBAC-TGEV-S$_{MHV}$-C11 was transfected into BHK-21 cells that 6 h later were overlaid into monolayers of murine LR7 or porcine ST cells to select for recombinants that had acquired the ability to infect murine cells and had simultaneously lost the ability to infect porcine cells (Fig. 1). LR7 monolayers cocultured with BHK-21 transfected cells exhibited syncytia formation 48 h posttransfection. By contrast, ST monolayers cocultured with BHK-21 transfected cells did not show detectable cytopathic effect, even up to 96 h posttransfection (data not shown). The growth kinetics of the rTGEV$_{S\text{-}MHV/C11}$, TGEV, and MHV-A59 in porcine ST and murine LR7 cells were compared (Fig. 2). The growth rate of MHV-A59 and rTGEV$_{S\text{-}MHV/C11}$ was quite similar; however, MHV-A59 reached a peak titer (3×10^6 PFU/mL) sooner than rTGEV$_{S\text{-}MHV/C11}$ (5×10^5 PFU/mL), at 12 and 24 h postinfection, respectively. As expected, TGEV replicated

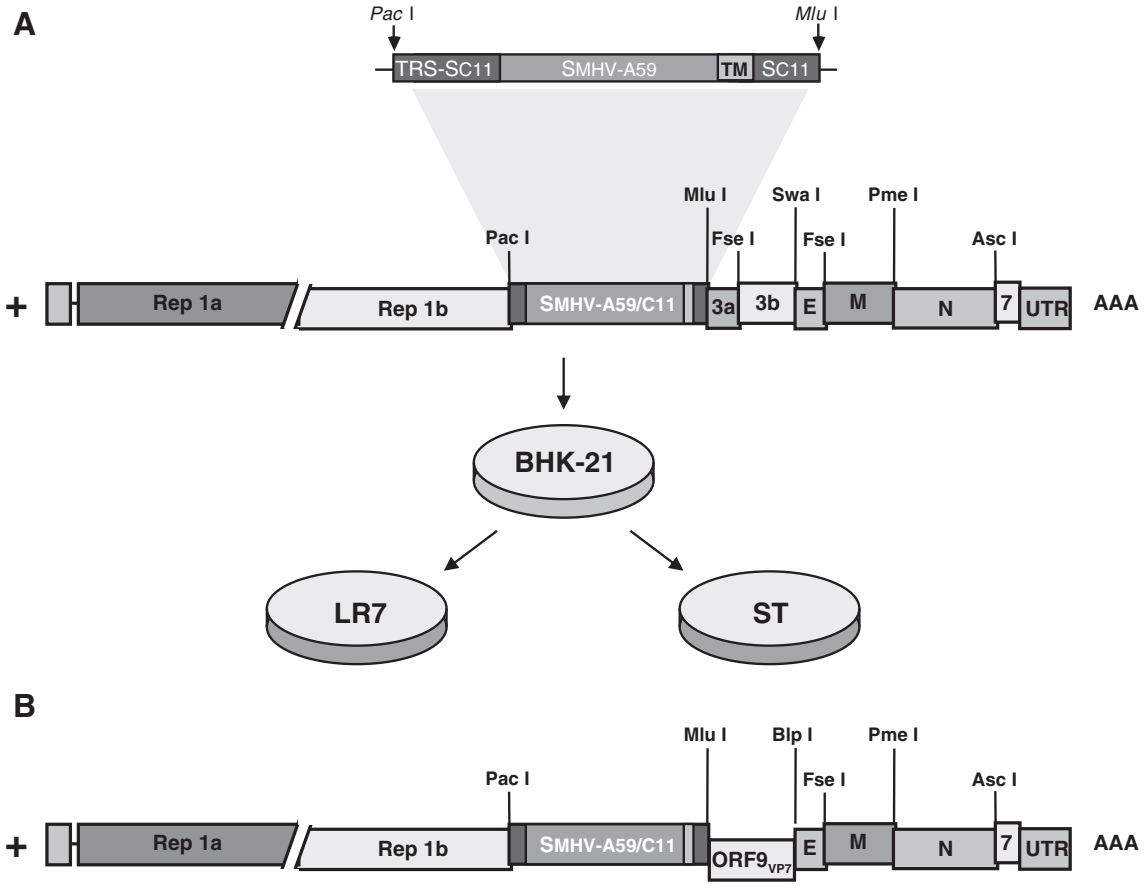

Fig. 1. Rescue of rTGEV$_{S\text{-}MHV/C11}$ from cDNA in LR7 cells. (A) Genetic organization of the rTGEV$_{S\text{-}MHV/C11}$ virus. Restriction sites Pac I, Mlu I, Fse I, Pme I, and Asc I are indicated in the genome. Letters and numbers indicate the viral genes. TRS-SC11, transcription regulating sequence of TGEV-C11 S gene; SMHV-A59, MHV-A59 S gene; TM, transmembrane domain; SC11, TGEV S gene. (B) Genetic organization of the rTGEV$_{S\text{-}MHV/C11}$-VP7 virus. Restriction sites Pac I, Mlu I, Blp I, FseI, Pme I, and Asc I are indicated in the genome.

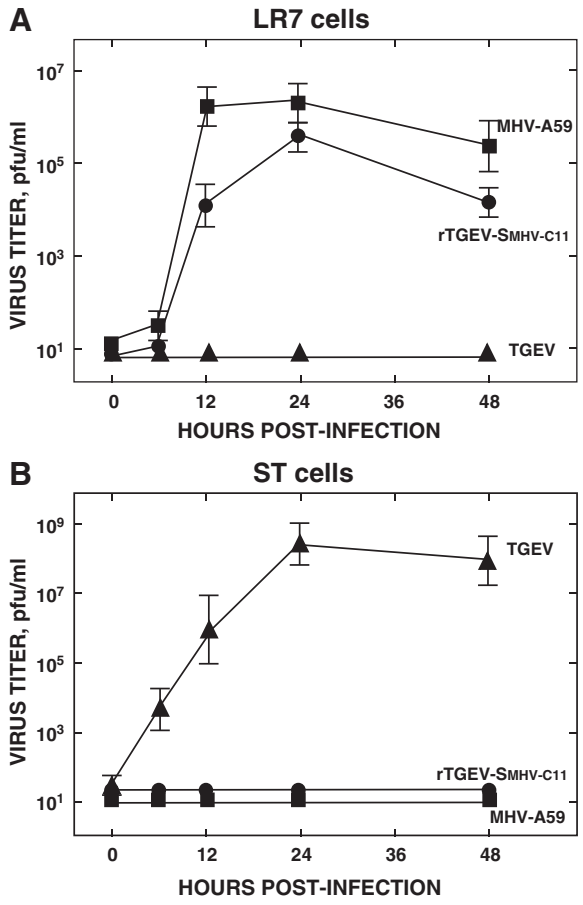

Fig. 2. Growth kinetics of $rTGEV_{S\text{-}MHV/C11}$ in murine and porcine cells. The viral infectivity of $rTGEV_{S\text{-}MHV/C11}$, TGEV, and MHV-A59 in murine LR7 (A) and porcine ST cells (B) was determined at different times postinfection by plaque assay. Mean values from four experiments are represented, and standard deviations are shown as error bars.

only in porcine ST cells, in which MHV-A59 and $rTGEV_{S\text{-}MHV/C11}$ failed to grow, thus indicating that TGEV S protein replacement with the chimerical TGEV-MHV-A59 S protein had altered the host species specificity of the virus (Fig. 2).

Tissue culture growth phenotype of $rTGEV_{S\text{-}MHV/C11}$

To analyze the growth characteristics of the recombinant viruses, murine LR7 and porcine ST cells were infected with $rTGEV_{S\text{-}MHV/C11}$, as well as with the parental viruses TGEV and MHV-A59. Consistently, recombinant viruses were unable to produce syncytia or cytopathic effects in porcine ST cells. As shown in Fig. 3A, no plaques of any size were evident on ST cell monolayers at 72 h postinoculation with recombinant viruses, in contrast to the plaques generated by wild-type TGEV on the same cells. In the same postinfection period, however, smaller plaques were obvious on LR7 cells infected with $rTGEV_{S\text{-}MHV/C11}$, while wild-type TGEV was unable to form plaques on these cells. These results confirmed that TGEV S protein replacement with chimerical TGEV/MHV S protein was sufficient to change the species tropism of the virus. The $rTGEV_{S\text{-}MHV/C11}$ virus grew successfully in LR7 cells, causing an extensive syncytia and cytopathic effect comparable to that of MHV-A59. This indicated that $rTGEV_{S\text{-}MHV/C11}$ had acquired the capacity to generate syncytia, which is typical of MHV-A59 but not of TGEV, due to the expression of the MHV-A59 S protein (Fig. 3B). These results were further confirmed by infecting CMR cells (Caco-2 transfected with the MHV receptor) with $rTGEV_{S\text{-}MHV/C11}$ (results not shown). The growth kinetics of the recombinant $rTGEV_{S\text{-}MHV/C11}$ vector in different murine cells lines showed that LR7 was the most productive cell line, reaching a titer of 6×10^7 FFU/mL). The CMR cells yielded titers of 10^6 FFU/mL, whereas other cell lines (3T3, MC57 and BALB Cl.7) yielded lower titers (less than 10^4 FFU/mL) (results not shown).

Analysis of the $rTGEV_{S\text{-}MHV/C11}$ proteins

To verify the identity of $rTGEV_{S\text{-}MHV/C11}$, we characterized the recombinant virus at the protein level, particularly its S protein. Murine LR7 and porcine ST cells were infected with $rTGEV_{S\text{-}MHV/C11}$. As controls, both cell lines were infected with TGEV and MHV-A59 and analyzed in parallel. The infected cells were incubated with MAbs specific for the MHV-A59 S protein (α-S-MHV) and TGEV M (α-M-TGEV) protein. In TGEV-infected ST cells, a specific immunofluorescence signal was only observed when probed with α-M-TGEV antibodies but not with α-S-MHV. The MHV-infected LR7 cells emitted a specific signal only when treated with α-S-MHV antibodies but not with α-M-TGEV (Fig. 3B). The analysis of the $rTGEV_{S\text{-}MHV/C11}$-infected LR7 cells revealed a specific immunofluorescence signal after treatment with antibodies α-M-TGEV and α-S-MHV, demonstrating that $rTGEV_{S\text{-}MHV/C11}$ expressed TGEV proteins, but also the MHV-A59 S protein.

Generation of recombinant $rTGEV_{S\text{-}MHV/C11}$ vector expressing rotavirus VP7

To recover infectious $rTGEV_{S\text{-}MHV/C11}$-VP7 from pBAC-TGEV-S_{MHV}-C11-VP7 plasmid, BHK-21 cells were grown to 95% confluence and transfected with the cDNA clones. At 6 h posttransfection, cells were trypsinized, plated over a confluent monolayer of LR7, and incubated at 37 °C for 72 h. After two passages in LR7, the recovered virus was cloned by three rounds of plaque purification. The expression of heterologous VP7 protein in murine cells was verified by indirect immunofluorescence on $rTGEV_{S\text{-}MHV/C11}$-VP7-infected LR7 cells (Fig. 4). In contrast to cells that were infected with $rTGEV_{S\text{-}MHV/C11}$, a strong fluorescence was shown by $rTGEV_{S\text{-}MHV/C11}$-VP7-infected cells, indicating the successful expression of the VP7 protein. VP7 was detected primarily in the cytoplasm. We also performed confocal fluorescent microscopy on cells infected with $rTGEV_{S\text{-}MHV/C11}$-VP7 and found that the VP7 protein was distributed evenly throughout the cytoplasm but not in the nucleus. To study the expression kinetics of VP7 protein following $rTGEV_{S\text{-}MHV/C11}$-VP7 infection, LR7 cells were infected and aliquots were taken at different times. The virus growth kinetics showed that the maximum virus titer was reached at 20 h.p.i., whereas maximum protein expression was at 22 h.p.i. (data not shown).

To check the stability of the recombinant virus several rounds of purification were performed by limited dilution in 96-well plates to isolate a clone of $rTGEV_{S\text{-}MHV/C11}$-VP7 that expresses the heterologous protein. The isolated clones were tested to confirm the presence of the ORF9 gene in the viral genome through RNA isolation and RT-PCR. The expression of the heterologous VP7 protein was confirmed by immunofluorescence. After five purification steps, virus clone 12A was obtained. When this clone was amplified, VP7 was expressed in at least 20% of the infected cells, and the expression was stable.

'In vivo' replication of $rTGEV_{S\text{-}MHV/C11}$

The ability of $rTGEV_{S\text{-}MHV/C11}$-VP7 virus to infect mice was assayed. Eight BALB/c and 12 STAT1−/− mice were inoculated with 2×10^6 FFU/mice by the oro-nasal route. On specified days, the mice were sacrificed and spleen, liver, intestine, lungs and sera were extracted. Subgenomic viral RNA was detected with RNA extraction and RT-PCR of the N viral gene. The results revealed the presence of viral RNA in different organs of BALB/c mice (intestine, liver, spleen and lungs) and in STAT1-deficient mice in the small and large intestine for a 6-day period. These results were confirmed by the isolation of infectious virus in cell culture. To verify virus active replication, two STAT1-deficient mice were inoculated. The mice were sacrificed at 48 and

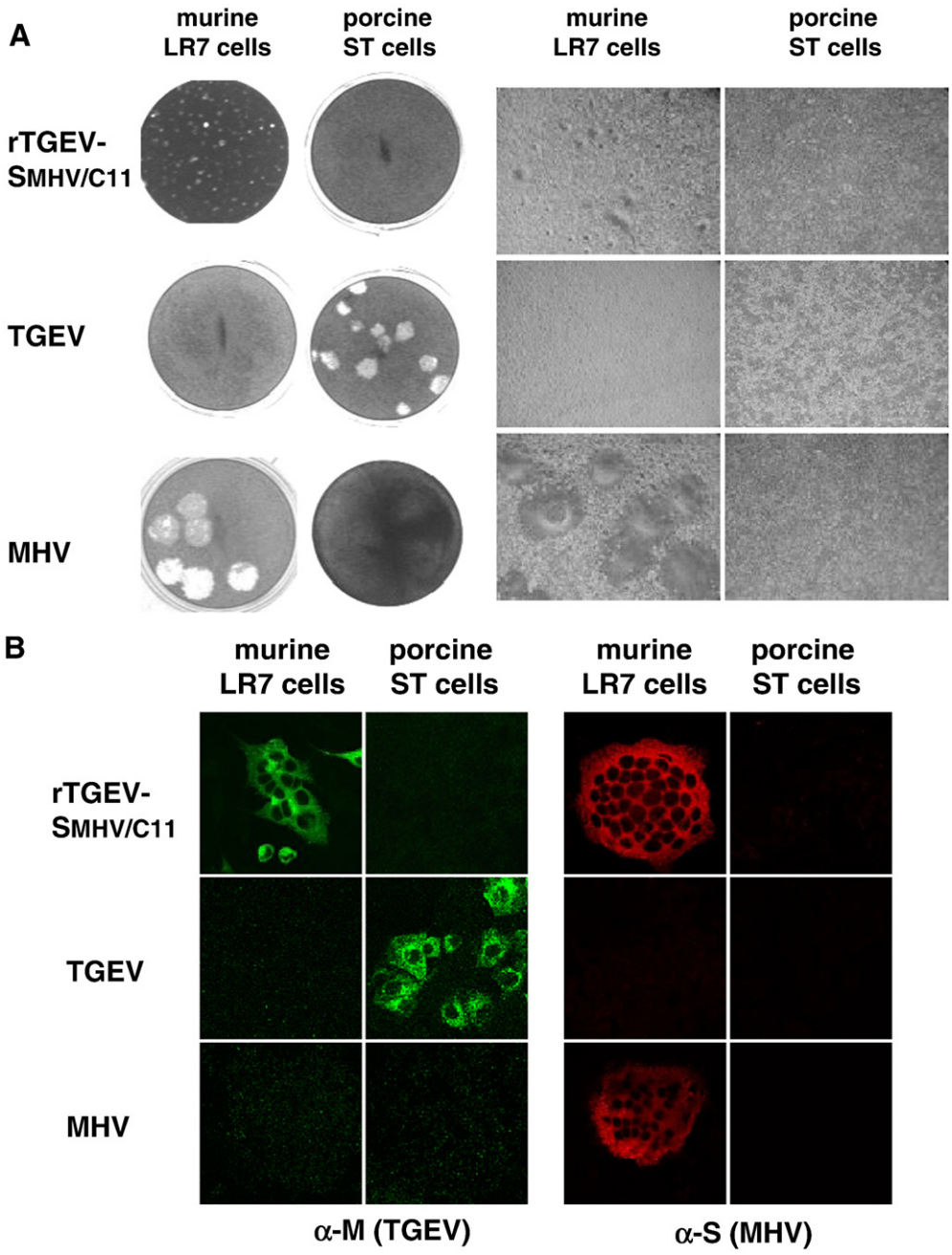

Fig. 3. Growth phenotype of $rTGEV_{S\text{-}MHV/C11}$ in LR7 murine and ST porcine cells. (A) Monolayers of murine LR7 and porcine ST cells were infected with $rTGEV_{S\text{-}MHV/C11}$, TGEV or MHV-A59. Cytopathic effect was analyzed by plaque assay and optical microscopy and visualized at 72 h postinfection. (B) Immunofluorescence analysis of $rTGEV_{S\text{-}MHV/C11}$-infected cells. LR7 and ST cells were infected with $rTGEV_{S\text{-}MHV/C11}$, TGEV or MHV-A59. Infections were visualized at 24 h.p.i. by immunofluorescence microscopy on permeabilized cells with specific MAbs against TGEV M protein (α-M-TGEV) and MHV-A59 S protein (α-S-MHV).

72 h and the intestine, spleen, liver and lungs were collected. Viral antigens were found in the submucosa of the small intestine, in spleen, liver and lungs, confirming viral replication in these organs (Table 1 and Fig. 5).

Antibody responses to $rTGEV_{S\text{-}MHV/C11}$ and $rTGEV_{S\text{-}MHV/C11}$-VP7 recombinant viruses in immunized mice

TGEV and rotavirus-specific serum IgA and IgG antibody responses were evaluated in BALB/c mice inoculated by intraperitoneal, intragastric or intranasal routes with $rTGEV_{S\text{-}MHV/C11}$-VP7. One control group was inoculated intragastrically with $rTGEV_{S\text{-}MHV/C11}$. Anti-TGEV serum IgG antibody responses were detected at high titers in the mice inoculated intranasally and intraperitoneally (1/2500) ($p<0.05$), and at lower titers in mice inoculated intragastrically (1/500) ($p<0.05$) (Fig. 6A). We examined whether intranasal inoculation of rTGEV produces antigen-specific IgA in the mucosal compartment. To test whether a mucosal response was induced, intestinal contents as well as serum samples from immunized mice were analyzed by ELISA for TGEV intestinal IgA. The second inoculation of rTGEV led to a significant increase ($p<0.05$) of detectable serum IgA, which rose slightly after the third inoculation (Fig. 7). IgA levels in the intestinal contents varied between animals, with detection of IgA production in the intestinal lumen in two out of six inoculated mice (results not shown). The $rTGEV_{S\text{-}MHV/C11}$-VP7 vector induced specific antibodies against rotavirus in mice. A single dose of $rTGEV_{S\text{-}MHV/C11}$-VP7 by the intraperitoneal route induced rotavirus-specific antibodies in immunized mice ($p<0.05$), with a further increase after the second immunization. The

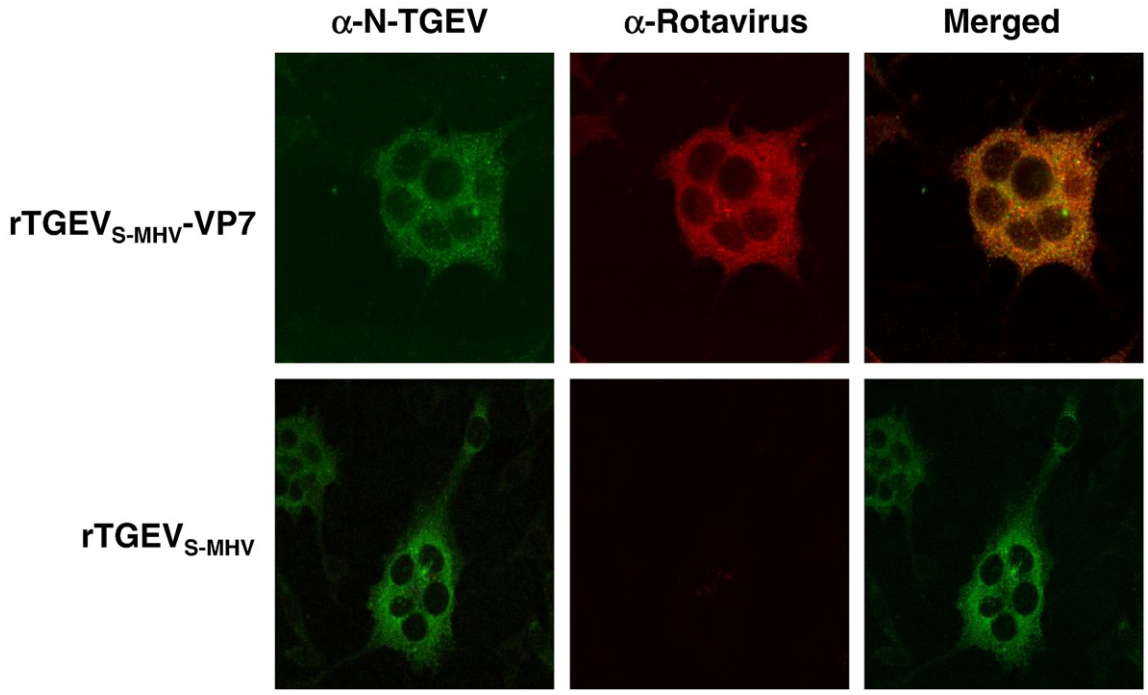

Fig. 4. Expression of VP7 protein in LR7 cells infected with rTGEV$_{S\text{-}MHV/C11}$-VP7 detected by immunofluorescence assay. LR7 cells were infected with rTGEV$_{S\text{-}MHV/C11}$-VP7 (MOI = 1), at 24 h.p.i. cells were fixed in metanol/acetone for 10 min and stained with a sheep polyclonal anti-rotavirus antibody (Silenus) and 3D.C10 MAb to recognize N protein of TGEV. An appropriate secondary fluorescent-conjugated antibody was applied (Cy5-labeled anti-sheep IgG to detect rotavirus protein and FITC-labeled anti-mouse IgG to reveal TGEV protein).

specificity of the antibody assays for rTGEV$_{S\text{-}MHV/C11}$-VP7 was shown by the absence of an anti-VP7 response in the rTGEV$_{S\text{-}MHV/C11}$-immunized mice (Fig. 6B). No fecal IgA against rotavirus was detected in any group. The neutralizing activity of these IgG antibodies was also assessed, and neutralizing antibody levels increased in the rTGEV$_{S\text{-}MHV/C11}$-VP7 mice inoculated intraperitoneally compared to the preimmune mice, with differences near the confidence level of 5% ($p = 0.079$).

Table 1
TGEV RNA detection by RT-PCR in rTGEV$_{S\text{-}MHV/C11}$-inoculated BALB/c and STAT1−/− mice. Eight BALB/c and 12 STAT1−/− mice were oro-nasally inoculated with 2×10^6 FFU of rTGEV$_{S\text{-}MHV/C11}$. Mice were euthanized on the days indicated, and their organs were removed, weighed, and resuspended as 10% (w/v) tissue homogenate in PBS. RNA from the resulting homogenates was extracted and RT-PCR with specific primers for the N viral gene was performed. GAPDH gene was also investigated by RT-PCR as an RNA extraction control. Results corresponding to the organs obtained from BALB/c (A) and STAT1−/− (B) mice are shown.

A) BALB/c mice

	Spleen	Liver	Intestine	Lung	Serum	Stools
Day 1	++	−	−	+	++	+
Day 2	−	++	++	+	+	+
Day 3	−	+	−	−	+	−
Day 4	−	+	−	−	−	−

B) STAT1−/− mice

	Spleen	Liver	Small intestine	Large intestine	Serum	Stools
Day 1	−	−	+	+	−	+
Day 2	++	++	++	+	−	+
Day 3	++	++	++	+	−	−
Day 4	++	−	++	+	−	−
Day 5	+	−	++	+	ND	ND
Day 6	−	−	+	+	ND	ND

+, positive result by RT-PCR for TGEV N gene.
++, very intense amplicon by RT-PCR for TGEV N gene.
ND, not determined.

Cytokine analyses

Cytokine levels in the serum samples and splenocytes stimulated in vitro were studied in 6 BALB/c mice inoculated three times at 2-week intervals with rTGEV$_{S\text{-}MHV/C11}$-VP7 and six mice with rTGEV$_{S\text{-}MHV/C11}$. GM-CSF levels were significantly increased (41 pg/mL) in the sera from mice 48 h postinoculation. Also, increased levels of IL-2 (30 pg/mL) were present in the sera at 48 h postinoculation ($p>0.05$). After the last inoculation there was a similar result in the sera of rTGEV$_{S\text{-}MHV/C11}$-VP7-inoculated mice (37 pg/mL) for GM-CSF ($p<0.05$) and 22 pg/mL of IL-2 ($p>0.05$). In serum samples from rTGEV$_{S\text{-}MHV/C11}$-inoculated mice, both levels of GM-CSF (47 pg/mL; $p<0.05$) and INF-γ (6 pg/mL; $p>0.05$) increased (Fig. 8A).

At 5-day poststimulation cultures of splenocytes revealed significant increases of IL-10 (108 pg/mL) in rTGEV$_{S\text{-}MHV/C11}$-stimulated splenocytes from rTGEV$_{S\text{-}MHV/C11}$-VP7-inoculated mice and in rTGEV$_{S\text{-}MHV/C11}$ (300 pg/mL; $p>0.05$) and RF (63 pg/mL; $p>0.05$)-stimulated splenocytes from rTGEV$_{S\text{-}MHV/C11}$-inoculated mice. No significant increases of GM-CSF or IFN-γ were found (Fig. 8B).

Protection against diarrhea induced by rotavirus of pups born to dams immunized with rTGEV$_{S\text{-}MHV/C11}$-VP7

We tested whether mothers immunized with rTGEV$_{S\text{-}MHV/C11}$-VP7 can transfer antibodies to their newborns and thus protect them against diarrhea induced by rotavirus challenge. Four-day-old newborns from female BALB/c mice intragastrically or intreperitoneally inoculated with rTGEV$_{S\text{-}MHV/C11}$-VP7 were challenged with an oral inoculum of 2×10^6 FFU of bovine rotavirus strain RF or 10 DD$_{50}$ of murine rotavirus strain EDIM. To assess infection, we evaluated diarrhea during the 4-day period following viral challenge. A passive protection against rotavirus-induced diarrhea was transferred to the newborn pups (Fig. 9). Sixty-two percent of pups born to dams inoculated intraperitoneally were protected against RF rotavirus strain diarrhea. Fisher's exact test did not show any significant difference in protection levels between this group and the RF strain-challenged newborn offspring of RF strain-immunized

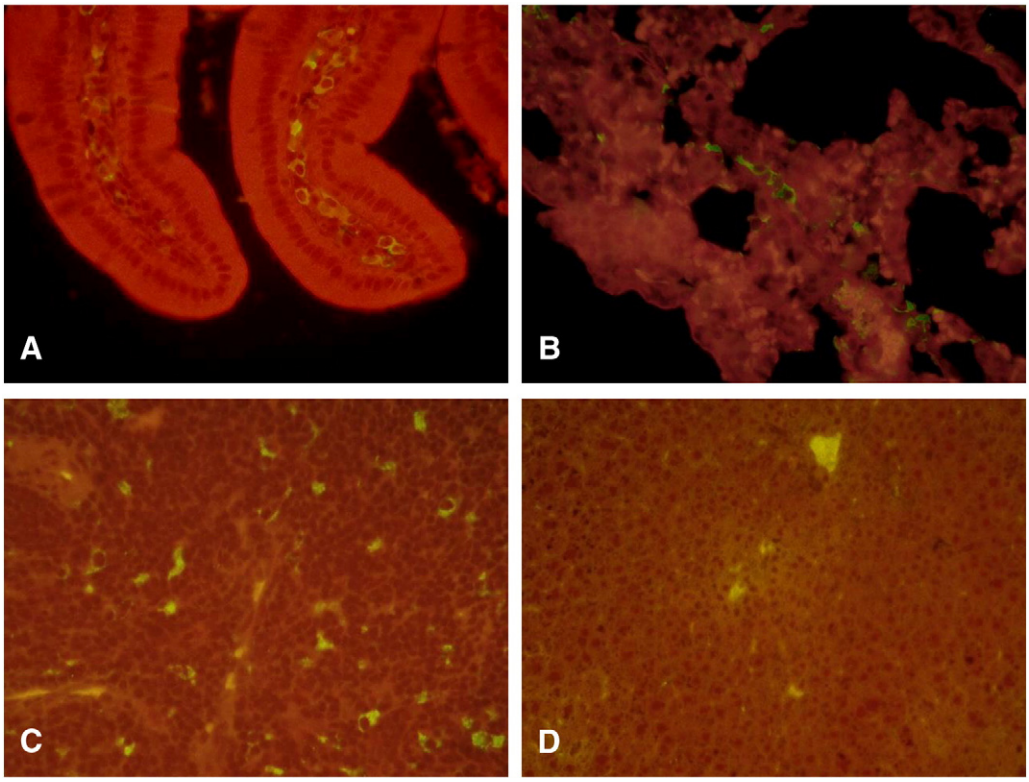

Fig. 5. Detection of rTGEV$_{S\text{-}MHV/C11}$ antigen in histological sections of tissue samples of rTGEV$_{S\text{-}MHV/C1}$-inoculated STAT1−/− mice. Mice were inoculated with 2×10^6 FFU of rTGEV$_{S\text{-}MHV/C11}$. Two days later intestine (A), lungs (B), liver (C) and spleen (D) tissue samples were collected. Histological sections of tissues were prepared and stained with Mab 3D.C10 raised against the N protein. FITC-conjugated secondary antibody was then used to visualize viral protein by immunofluorescence.

dams ($p=0.603$). By contrast, only 13% of RF strain-inoculated offspring of intragastrically inoculated dams were protected against diarrhea. Significant differences were found between the proportion of protected pups in this group and the corresponding control group ($p=0.013$). Eighteen percent of newborn offspring of intraperitoneally immunized dams inoculated with EDIM rotavirus did not develop diarrhea. Differences between this group and EDIM-challenged control mice were statistically significant ($p=0.001$).

Discussion

This study confirms that coronavirus TGEV genomes can incorporate heterologous gene sequences and express them and supports their potential use as vaccine vectors. An infectious cDNA clone of TGEV has been genetically engineered to incorporate the rotavirus VP7 gene, replacing nonessential 3a and 3b genes, and a monocistronic vector expressing the VP7 protein stably *in vitro* for at least five passages has been developed.

Two attenuated human oral rotavirus vaccines are currently available worldwide. Rotarix (Glaxo-Smith-Kline), a human attenuated rotavirus vaccine, and RotaTeq (Merck), a human-bovine reassortant rotavirus vaccine, were shown to be efficacious against severe rotavirus disease in many countries (Dennehy, 2008; Parashar and Glass, 2009) but in Malawi and South Africa the efficacy was only 49.4% and 76.9%, respectively (Madhi et al., 2010). Other live, attenuated oral vaccines such as vaccine 116E, based on rotaviruses obtained from asymptomatically infected newborns in Delhi, India, are also undergoing clinical trials (Bhandari et al., 2009). Improved vaccines are still needed, particularly in developing countries, and also for veterinarian applications.

In recent years, different heterologous genes have been cloned and expressed in coronavirus (de Haan et al., 2005; Fischer et al., 1998; Sola et al., 2003). This makes them attractive candidates for the development of genetically modified live vaccines or for use in gene therapy. The identification of nonessential genes in the viral genome represents an important step to identify viral genes involved in virulence and to incorporate foreign genes in coronavirus vectors, as accessory genes may be deleted leading to attenuated viruses and providing appropriate insertion targets for heterologous genes. In TGEV, 3a and 3b are genes encoding viral nonstructural proteins whose function is not essential for *in vitro* or *in vivo* virus replication and can be deleted (Ortego et al., 2002; Sola et al., 2003). The gene encoding GFP protein has been cloned in the position occupied by genes 3a and 3b in the TGEV genome, achieving high expression levels of this protein (Sola et al., 2003).

The natural host of TGEV, the pig, is a suitable model for studying the immune response and the expression of heterologous recombinant proteins, although it is expensive and difficult to manage and assess. Fortunately, coronavirus tropism can be genetically engineered to adapt porcine specificity of TGEV to the murine model, which is an affordable, measurable and widely used animal model. Attenuation of the virus with modified tropism when it replicates in other host-cell machinery is yet another advantage for its use in vaccine design.

The S glycoprotein is responsible for virus binding to specific cell receptors present on the membrane of susceptible cells, besides inducing the fusion of viral and cellular membranes during viral entry. This gene is a determinant of viral tropism and pathogenesis in coronavirus (Casais et al., 2003; Navas et al., 2001; Sanchez et al., 1999), but not the only one (Casais et al., 2003; Ortego et al., 2003). In this study we produced TGEV virus with a chimeric S protein derived from TGEV and MHV S proteins. In order to assemble a functional S protein it was important to incorporate into the recombinant S gene the two HR domains involved in S protein supercoiling (HR-1 and HR-2) from the same viral strain (Godeke et al., 2000). In our construct HR-1 and HR-2 domains from MHV were used to generate a chimeric protein. These domains have been identified in all coronavirus S proteins.

The results reported in this manuscript show that, in the case of TGEV, species tropism was controlled mainly at the level of viral entry

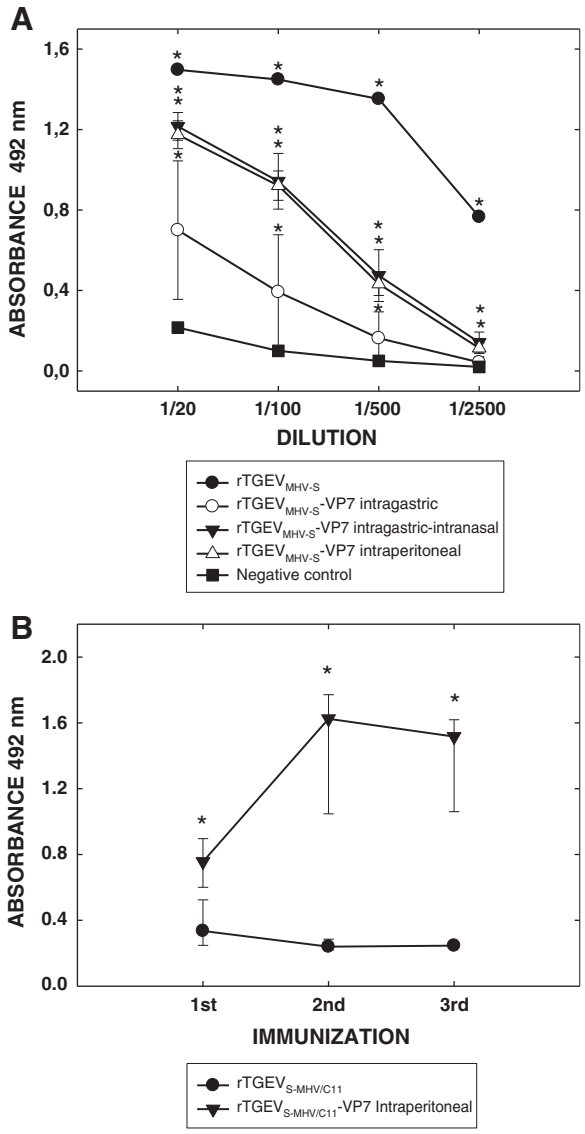

Fig. 6. IgG antibody response elicited by immunization with rTGEV$_{S\text{-}MHV/C11}$-VP7 vector. Groups of five female BALB/c mice were immunized with three doses of 10^6 FFU of rTGEV$_{S\text{-}MHV/C11}$-VP7 by either the intragastric, intranasal, or intraperitoneal routes three times at two-weekly intervals. Mice in the control group received three doses of 2×10^6 FFU of rTGEV$_{S\text{-}MHV/C11}$ (void vector) by the intragastric route with the same schedule. Mice were bled at the indicated points. TGEV (A) and rotavirus (B)-specific serum IgG antibodies were determined by ELISA. *Significantly higher than levels of the negative control sera ($p<0.05$ by the Mann–Whitney test).

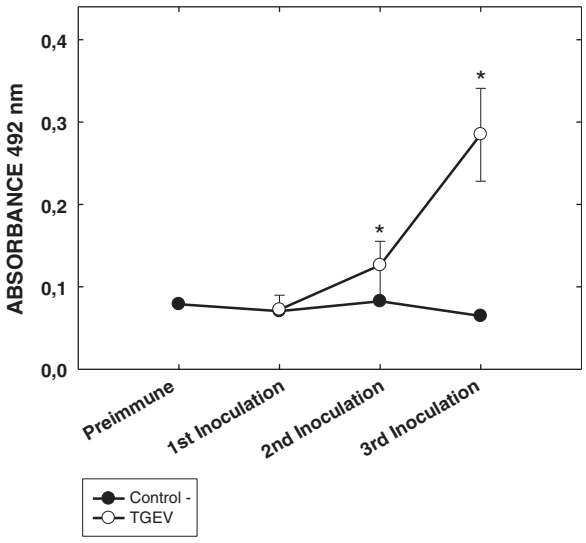

Fig. 7. Serum IgA response to TGEV. Mice were immunized three times at two-weekly intervals with 2×10^6 FFU/mL of rTGEV$_{S\text{-}MHV/C11}$ or LR7 cells lysates (negative control). Serum samples were collected from all mice at the indicated time points. Serum IgA to TGEV were assayed by ELISA. Data are means ± SD (six mice per group). *Significantly higher than levels of the preimmune sera ($p<0.05$ by the Rang Wilcoxon test).

into the cell. However, although the engineered TGEV with murine tropism replicated in murine cells, it reached titers of at least 1 log lower than the parental virus MHV. This suggests that the replication machinery of TGEV is slightly less efficient in mouse cells, and that specific cell factors present in porcine cells may be required for optimum virus replication. In other reports in which S chimeric proteins were assembled between MHV (murine tropism) and FIPV (feline tropism) the viral titer decreased from 10 to 100 times, providing additional support for the requirement of species-specific cell factors for optimum coronavirus replication (Kuo et al., 2000).

BALB/c and STAT1−/− mice were inoculated to determine the *in vivo* response to the infection with the recombinant rTGEV$_{S\text{-}MHV/C11}$ murine virus. MHV usually causes subclinical infection, producing significant mortality in immunodeficient mice only (Compton et al., 1993). There are two major disease patterns of MHV based on the tropism of the viral strain, the respiratory and the enteric pattern, which have been identified by intranasal inoculation of mice with different MHV strains (Barthold and Smith, 1984). In the enteric pattern (mainly produced by JHM strain), the virus infects intestinal mucosa and spreads to the organs and bloodstream. Meanwhile, in the respiratory pattern (represented by A-59 strain) the virus primarily replicates in the nasal cavity and infects the olfactory bulb, thus entering the brain through the olfactory nerve. The inhaled virus invades the lungs, with subsequent viremia and dissemination to other organs through the bloodstream. Our data suggest that the recombinant rTGEV$_{S\text{-}MHV/C11}$ virus follows a respiratory rather than an enteric pattern. The virus infected both strains of mice evaluated in this study, BALB/c and immunocompromised STAT1−/− mice. In addition to having changed species tropism, a modification in tissue tropism was also observed. The recombinant virus replicated in the lungs, showing a respiratory pattern similar to the parental virus MHV-A59, infecting the same organs but less efficiently. Viral RNA was detected in different organs, and replication was confirmed by immunofluorescence assays on tissue sections. In fact, infectious virus was recovered by cell culture. These results were confirmed by analyzing the kinetics of viral RNA detection. Our data suggest that the recombinant virus was attenuated in mice, particularly if its virulence is compared with that of MHV, which is lethal for STAT1−/− mice (Durbin et al., 1996). These authors showed that litters of this knockout mouse died of fulminant hepatitis. The engineered rTGEV$_{S\text{-}MHV/C11}$ vector was attenuated in STAT1−/− mice as no deaths were recorded in this mouse strain.

The results of this study show that the engineered rTGEV vector promoted an immune response in BALB/c mice to the vector itself and to the rotavirus VP7 protein. The recombinant virus triggered a humoral response in mice via systemic (serum IgG and IgA) and mucosal (intestinal IgA) antibodies. Nevertheless, only partial protection was achieved. This could be due to the limited stability of the heterologous gene within the vector. The stability of heterologous genes within viral vectors is associated to virus recombination and mutation rates. Insertion of foreign genes in coronavirus may result in large deletions rather than point mutations (de Haan et al., 2005), and these deletions can be caused by intra and inter-molecular recombination events (Lai, 1996). The emergence of deletion variants without foreign genes may led to viruses with higher fitness. Probably this is the main drawback that compromises coronaviruses efficacy as vectors. Besides, recombinant proteins may also

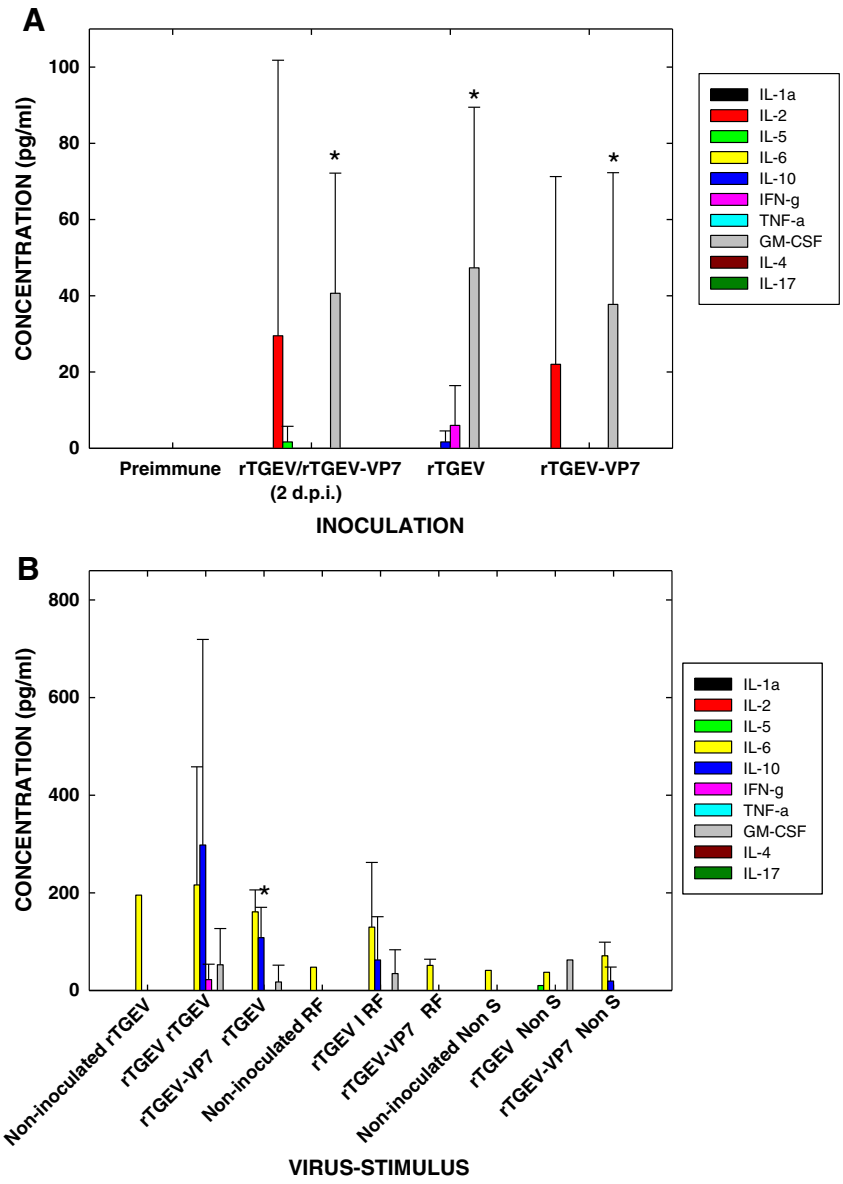

Fig. 8. Cytokine responses in BALB/c mice after immunization with $rTGEV_{S\text{-}MHV/C11}$ and $rTGEV_{S\text{-}MHV/C11}$-VP7. Mice were immunized intragastrically three times at two-weekly intervals with 2×10^6 PFU of $rTGEV_{S\text{-}MHV/C11}$ or 1×10^6 FFU of $rTGEV_{S\text{-}MHV/C11}$-VP7, and serum was obtained 15 days after the last inoculation. Serum samples were also obtained 2 days after the first inoculation from both mice groups (A). Two weeks after the last immunization, mice were sacrificed and their spleens were collected. After erythrocyte depletion, splenocytes were cultured in 24-well plates at a concentration of 2×10^6 cells/mL. The cells were either stimulated with the mitogen ConA (5 μg/mL), TGEV coronavirus (2×10^6 FFU), RF rotavirus (5×10^6 FFU) or cultured without stimulation. Cytokine levels were measured in the culture supernatants at the 5th day poststimulation (B). The levels of cytokines were evaluated by a multiplex fluorescent bead immunoassay for quantitative detection of 10 mouse cytokines (FlowCytomix mouse Th1/Th2 10plex, Bender Medsystem) and flow cytometry (Beckman Coulter). Data are mean values ± SD. *Significantly higher than cytokine levels produced by preimmune sera or not stimulated splenocytes ($p<0.05$ by the Mann–Whitney test or Wilcoxon test). Non S, nonstimulated.

contain toxic domains that can affect the level of heterologous gene expression. In principle, this limitation can be overcome by identification of these toxic domains and their elimination by site-directed mutagenesis.

Previous attempts to develop a rotavirus vaccine through the expression of cloned genes encoding the outer capsid proteins VP4 or VP7 have achieved diverse results. In most studies, trials immunizing animals against rotavirus by VP7 protein expression have been of limited success due to the complexity and instability of neutralization domains of the expressed VP7 (McGonigal et al., 1992; Salas-Vidal et al., 1990). In other studies, only antibodies to denatured VP7 were induced, indicating that the expressed protein was immunogenic; however, it did not elicit the formation of antibodies that neutralized virus (Johnson et al., 1989; Salas-Vidal et al., 1990). Some authors suggest that calcium binding is necessary to form the neutralization domain on expressed VP7 (Dormitzer et al., 1994). Studies of VP7 expression in prokaryotic vectors (*E. coli* and attenuated strains of *S. typhimurium*) obtained disappointing results (Arias et al., 1986; McCrae and McCorquodale, 1987). Expression of rotavirus in vaccinia virus vector gave good results when VP7 was expressed on the plasma membrane of infected cells, which was achieved by adding the membrane anchor and cytoplasmatic domain from influenza virus hemagglutinin (Andrew et al., 1990). Similar results were reported for the same VP7 construct expressed in a recombinant type 5 adenovirus (Both et al., 1993).

The immunization of BALB/c mice with $rTGEV_{S\text{-}MHV/C11}$-VP7 vector stimulated an anti-rotavirus antibody response. The intraperitoneal route was more effective in inducing a significant response after the first

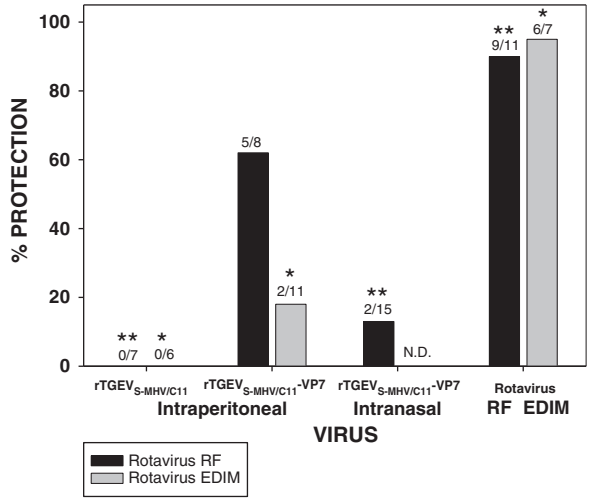

Fig. 9. Passive protection of newborns against rotavirus-induced diarrhea. Female mice were immunized three times intragastrically or intraperitoneally with $rTGEV_{S\text{-}MHV/C11}$-VP7. Immunized females were mated with males. Four-day-old newborns were orally infected with 2×10^6 ffu/mice of RF or 10 DD_{50} of EDIM virus. Control groups of females were inoculated with RF and EDIM, and the offspring was challenged with RF or EDIM strains, respectively. Pups presenting diarrhea were considered unprotected. Fisher's exact test was used to determine significant differences in protection percentage between rotavirus-challenged pups born to rTGEV-VP7 and to rotavirus-inoculated dams. The symbols * and ** indicate significant differences with respect to the EDIM and RF control groups ($p<0.05$), respectively. N.D., determined.

inoculation than the intragastric or intranasal routes. This antibody response was enhanced after the second inoculation and was maintained after the third inoculation. Specific antibody response to the vector itself was detected in mice inoculated by all three routes. The virus may infect organs through mucosal surfaces at low efficiency, whereas a direct route of inoculation, such as the intraperitoneal route, probably led to an increased viral replication allowing a wider dissemination of the vector to other tissues, and thus a stronger immune response.

Production of mucosal IgA antibodies against VP7 was not detected in BALB/c mice inoculated intragastrically with $rTGEV_{S\text{-}MHV/C11}$-VP7. In contrast, anti-TGEV intestinal IgA antibodies were induced after intragastric inoculation with $rTGEV_{S\text{-}MHV/C11}$, although in only two out of six inoculated mice. The low replication of the recombinant virus in BALB/c mice probably hampered the antibody IgA response in mucosal areas.

Our results showed a trend towards a Th2-type immune response elicited by $rTGEV_{S\text{-}MHV/C11}$-VP7 vector, as revealed with the production of IL-10 by stimulated splenocytes from inoculated mice. IL-10 was induced only after splenocyte stimulation with $rTGEV_{S\text{-}MHV/C11}$ virus, but not with $rTGEV_{S\text{-}MHV/C11}$-VP7, possibly due to a lower priming by this recombinant. In line with our results, enhanced IL-10 production has also been reported in splenocytes of mice coinfected with MHV and *Trypanosoma cruzi*, and endogenous IL-10 down-regulates IFN-γ production (Torrecilhas et al., 1999). Furthermore, we found an increase in GM-CSF levels in the serum and in stimulated splenocytes of the inoculated mice. GM-CSF strongly influences the biological functions of many different phagocytic and immune cells, stimulating the production of granulocytes and monocytes (Rasko, 1997).

Protection against rotavirus diarrhea by passive maternal transfer of antibodies has been previously reported for mice immunized with live rotavirus vaccines (Offit and Clark, 1985), recombinant adenovirus expressing VP7 protein (Both et al., 1993) or rotavirus VLPs (Coste et al., 2000). We observed that maternal rotavirus-specific antibodies partially protected suckling newborns against rotavirus-induced diarrhea. Suckling pups born to dams inoculated with rTGEV-VP7 by the intragastric or intraperitoneal routes were challenged orally with homotypic (RF) or heterotypic (EDIM) rotavirus. Sixty-two percent of pups from mothers immunized intraperitoneally were protected against rotavirus RF diarrhea, although only 13% of pups from mothers immunized intragastrically were also protected. However, when challenged with the heterotypic murine EDIM strain, only 18% of pups born to intraperitoneally immunized dams were protected against diarrhea. These results are consistent with the differences in VP7 antigenicity between RF (G6 serotype) and EDIM (G3 serotype) rotavirus strains and also coincide with antibody production, because only female mice that produced rotavirus-specific antibodies protected their pups against rotavirus-induced diarrhea. The expression of other VP7 proteins with different antigenic specificities and the simultaneous coexpression of different rotavirus proteins must be addressed in order to achieve more efficient protection against rotavirus infection using TGEV-derived vectors.

In conclusion, a partial protection against rotavirus-induced diarrhea was achieved in suckling BALB/c mice born to dams immunized with the recombinant coronavirus expressing VP7 when they were orally challenged with the homotypic rotavirus strain.

Materials and methods

Cell cultures and viruses

Baby hamster (BHK21), porcine swine testis (ST), human colon adenocarcinoma (Caco-2) and murine fibroblast (LR7, 3T3 and MC57) cells were grown in Dulbecco's modified Eagle's medium (DMEM). Simian kidney (MA104) and murine BALB Cl.7 cells were culture in MEM with glutamine (2 mM). All cell culture media were supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL) and tylosin (100 UI/mL). All reagents were purchased from Gibco-Invitrogen. CMR cells are Caco-2 transfected with the MHV receptor gene and were supplemented with geneticin G418 (500 μg/mL) (Sigma) in order to stably express the recombinant MHV receptor glycoprotein (Rossen et al., 1997). The TGEV strain PUR46-MAD was derived from the Purdue strain by 120 passages on ST cells involving three cloning steps by plaque purification (Sanchez et al., 1990). MHV-A59 was obtained from ATCC. The rotavirus strain RF was a gift from J. Cohen (Laboratoire de Virologie et d'Immunologie Moleculaire, INRA, France). The EDIM strain of murine rotavirus was kindly provided by R. Ward (Children's Hospital, Cincinnati, Ohio).

Viral stocks and standard virus titrations were performed in porcine ST (TGEV) (Correa et al., 1988), murine LR7 (recombinant virus) and MA104 cells (rotavirus) (L'Haridon and Scherrer, 1976). Virus titrations were performed by plaque formation (TGEV and MHV-A59) and immunofluorescence assay (rotavirus and recombinant virus). EDIM rotavirus was replicated by orally infecting 5- to 7-day-old BALB/c suckling mice. The 50% diarrhea dose (DD_{50}) was determined by oral inoculation of pups with serial 10-fold dilutions of the viral stock.

Mice

BALB/c mice were purchased from Charles River Laboratories (Barcelona, Spain) and STAT1−/− mice were kindly provided by M. Mueller (Ludwig Boltzmann Institute for Immunogenetic, Cytogenetic, and Molecular Genetic Research, Vienna, Austria). Inoculated mice were held in an animal storage unit (UA-I, EuroAire, Spain) under negative pressure to prevent the environmental spread of the recombinant viruses. All animals were housed in a conventional animal facility at the University of Valencia and food and water were supplied ad libitum. All experiments were carried out using a protocol approved by the Animal Care and Ethics Committee of the University of Valencia pursuant European Directive 86/609/CEE, amendment 2003/65/CE, and Spanish Act RD1201/2005.

Antibodies

The TGEV M protein-specific monoclonal antibody (MAb) 3B.B3 has been described previously (Risco et al., 1995) and the MAb specific for MHV S protein was a kind gift from J. Fleming (University of Southern California). The MAb 3D.C10 specific for the TGEV N protein was used to detect TGEV antigen in small intestine sections (Jimenez et al., 1986). VP7 rotavirus protein was detected with an anti-VP7 MAb provided by G. Gerna (Policlinico San Matteo, Pavia, Italy) and with a sheep polyclonal anti-rotavirus antibody (Silenus Laboratories, Melbourne, Australia). The anti-VP6 MAb 2F3.E7 that recognizes the rotavirus inner capsid was developed in our laboratory and was used in seroneutralization assays.

Construction of the pBAC-TGEV$_{S\text{-}MHV/C11}$

To generate the chimerical S gene MHV-A59/TGEVC11 the restriction site MluI (3264) was deleted in the gene S of MHV-A59. To generate the deletion, oligonucleotide primers S-MHV-2452-VS (5′-GTTGCTAGTGCAT TAATGC-3′) and S-MHV-3277(C)-RS (5′-GAAATTAATACGGGTGGTTT GGC-3′), inserting a point mutation (G3264→C), were used to generate a PCR product from nucleotides (nt) 2452 to 3277 of the MHV-A59 gene S. The primers S-MHV-3254(C)-VS (5′-GCCAAACCACCCGTATTAATTTC-3′), including the point mutation (G3264→C) and S-MHV-3803-RS (5′-AACATACCAAGGCCATTTC-3′) were used to generate a PCR product from nt 3254 to 3803 of the MHV-A59 gene S. Both overlapping PCR products were used as templates for PCR amplification using the primers S-MHV-2452-VS and MHV-S-3803-RS. The amplified DNA was digested with XhoI and StyI and cloned into the XhoI-StyI-digested pGem4Z-SMHV-A59 to obtain the pGem4Z-SMHV-A59-ΔMluI plasmid. To generate the plasmid pGem4Z-S-A59-C11-PacI-MluI, the unique restriction sites PacI and MluI were inserted into the chimerical gene S. The oligonucleotide primers S-MHV-Hind-Pac-1-VS (5′-CGAAG**CTTAATTAA**GAAGGGTAAGTTGCTCAT-TAGAAATAATGGTAAGTTACTAAACTTTGGTAACCACTTCGTTAACACAC-CATGCTGTTCGTGTTTATTCTATTTTTG-3′), including a HindIII site (underlined), a PacI site (boldface nucleotides), 71 nucleotides from the 5′ transcription regulatory sequence (TRS) of gene S of TGEV, and the first 27 nucleotides of the gene S of MHV-A59, and S-MHV-867-RS (5′-GGTATAACTACTACGACAATC-3′) were used to generate a PCR product comprising from nt 1 to nt 867 of the of the MHV-A59 gene S. PCR product was digested with HindIII and SpeI and cloned into the HindIII-SpeI-digested pGem4Z-SMHV-A59-ΔMluI plasmid, generating pGem4Z-SMHV-A59-ΔMluI-PacI. The primers S-C11-VS (5′-AAATGGCCTTGG-TATGTGTGG), and 3′-S-C11-Mlu-Bam-RS (5′-GCGGATCC**ACGCGT**AAGTT-TAGTTCTTAAAGAC-3′), including a BamHI site (underlined), and a MluI site (boldface nucleotides), were used to generate a PCR product from nt 4158 to 4347 of the TGEV-C11 gene S. PCR product was digested with StyI and BamHI and cloned into the StyI-BamHI-digested pGem4Z-SMHV-A59-ΔMluI-PacI to obtain the pGem4Z-SMHV-A59-ΔMluI-Pac-Mlu plasmid. To assemble pBAC-TGEV-SMHV-C11 encoding the TGEV full-length genome with the chimerical SMHV-C11 gene, the pGem4Z-SMHV-A59-ΔMluI-Pac-Mlu plasmid was digested with PacI and MluI and the chimerical gene SMHV-C11 cloned into PacI-MluI-digested pBAC-TGEV-P-M (Ortego et al., 2003).

Construction of the pBAC-TGEV$_{S\text{-}MHV/C11}$-VP7

Rotavirus VP7 gene was amplified by PCR from the cDNA clone pcDNA-RfORF9 (generously provided by J. Cohen) using oligonucleotides that inserted flanking MluI and BlpI restriction sites at the 5′ end and 3′ end of the PCR product. The oligonucleotide primers 5′-ORF9-VS (5′GCACGCGTCATTACAGGTCCTTGTATGGACTATATAATTTACAGA-TTTCTGCTTATAGTGATCTTGGCC-3′), including a MluI site (underlined), and 3′-*ORF 9*-RS (5′-GCGCGCTAAGCTACACTCTATAGTAGAACGCCGAC-GAAT-3′) including a BlpI site (underlined) were used to amplify VP7. The PCR product was digested with MluI and BlpI and cloned into the MluI-BlpI-digested pACNR1180-TGEV-S$_{PTV}$-*RS* (Ortego et al., 2003) to obtain the pACNR1180-TGEV-S$_{PTV}$-*RS*-Δ3ab-ORF9 plasmid, containing the coding sequence of the rotavirus VP7 gene and the transcription regulating sequence (TRS) of TGEV 3a gene. As previously described, this TRS-3a produces efficient transcription of heterologous sequences (Sola et al., 2003). To generate pBAC-TGEV-SMHV-C11-VP7, the pACNR1180-TGEV-S$_{PTV}$-*RS*-Δ3ab-ORF9 plasmid was digested with MluI and BamHI and the rotavirus VP7, and TGEV E, M, N, and seven genes cloned into MluI-BamHI-digested pBAC-TGEV-SMHV-C11.

Immunofluorescence assay

Cell monolayers grown on glass coverslips were infected at a MOI of 1 in DMEM containing 2% FBS and DEAE-dextran (80 μg/mL). After 90 min of incubation, the extracellular virus was removed, and the cells were further cultured in DMEM 2% FBS. After rinsing with PBS, cells were fixed with methanol/acetone or with 4% paraformaldehyde in PBS for confocal studies. In the latter case the cells were rinsed with PBS 0.1% Triton X-100 in order to permeabilise the cell membrane. For dual-labeling experiments in which the primary antibody was derived from mouse or from rabbit, both antibodies were combined in PBS with 20% FBS. Antibodies were allowed to adsorb for 90 min at room temperature, and washed three times with PBS-2% FBS. The coverslips were mounted on glass slides with glycerol (Light Diagnostics) and observations made with a fluorescence microscope (Nikon Eclipse E600) or by confocal microscopy (Confocal Leica TCS SP analyzed with Leica Lite Software).

RNA extraction and RT-PCR

Viral RNA was extracted with Trizol-LS (Invitrogen) from infected cell culture lysates or tissue homogenates according to the manufacturer's instructions. To obtain tissue homogenates the organs were extracted and triturated using a glass tissue homogenizer and then resuspended in PBS (10% w/v) containing 200 IU penicillin/mL, 200 μg streptomycin, and 10 UI/mL nystatin. The tissue suspensions were clarified by centrifugation at 10,000×*g* for 5 min at 4 °C, and then passed through a 0.2 μm membrane filter and eventually stored at −80 °C.

A two-step reverse transcriptase PCR (RT-PCR) was performed with the following procedure. cDNA was synthesized using AMV reverse transcriptase (Promega) for 35 min at 42 °C. cDNA was amplified by PCR using primer pairs targeting the nucleocapsid TGEV sequence gene and the VP7 rotavirus gene. They were primer leader 15 (5' GTGAGTG-TAGCGTGGCTATATCTCTTC 3'; positions 15–41) and TN224 (5' CCTTCACCATGCGATAGC 3'; positions 224–241) for the N gene, and primer leader 15 and 253RF (5' CTGCATACGCAGTATCCATTG 3'; positions 233–253) for rotavirus VP7 gene. Primers designed to amplify a fragment of the RNA encoding mice glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene (GAPDH forward 5′ TGCATCCTGCACCACCAACT 3′, positions 502–520, and reverse 5′ TGCATCCTGCACCACCAACT 3′ positions to 832–850) were used as an internal control for RNA extraction from tissues. The PCR cycling conditions were the following: 1 cycle of 94 °C for 1 min; 40 cycles of 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min, and a final extension step at 72 °C for 10 min.

Detection of rTGEV$_{MHV\text{-}S}$ virus by immunohistochemistry

Small intestine, lung, liver and spleen tissue samples were fixed in 4% buffered formalin and embedded in paraffin (Leica ASP 300 S tissue processor). Serial 2-μm sections (Leica RM 2155 microtome) were mounted on polylysine-coated slides (Polysine, Menzel-Glaser). For the immunofluorescence assay, sections were deparaffinized for 1 h at 65 °C. Antigen retrieval was performed by heating at 98 °C for 40 min in citrate buffer (10 mM sodium citrate, pH 6.0), and slides were

incubated with 10% FBS at room temperature for 30 min. MAb 3D.C10 anti-N of TGEV, diluted 1:20, was applied for 2 h at room temperature. Subsequently, a FITC-labeled anti-mouse IgG secondary antibody was used and eventually the sections were counterstained with Evans blue dye (0.01% in PBS).

Mice immunizations

Six- to eight-week-old female BALB/c mice in groups of five mice were inoculated with $rTGEV_{MHV\text{-}S}$-VP7 propagated in DMEM with 2% FBS containing 1×10^7 fluorescent focus-forming units/mL (FFU/mL). Mice were confirmed by ELISA to have no serum IgG antibody to rotavirus prior to immunization and were inoculated three times at two-weekly intervals by three alternative routes (two groups per each route): (i) intragastrically, by placing 200 μL of viral inoculum containing 2×10^6 FFU/mouse into the lower esophagus using a gavage needle, (ii) oro-nasally, by instilling 10 μL of viral suspension containing 1×10^6 FFU directly in each nostril with a micropipette under isoflurane anesthesia, and (iii) intraperitoneally, by injecting 100 μL of viral inoculum containing 1×10^6 FFU into the abdominal cavity.

Sample collection and processing

Blood samples were collected at 15 days postinoculation by mandibular bleeding and incubated for 4 h at room temperature, centrifuged for 10 min at $3000\times g$, and the sera were stored at −20 °C. Fecal samples were collected from each mouse at 20 days postinoculation and diluted to 1:10 (w/v) in PBS. The suspensions were clarified by centrifugation and supernatants stored at −80 °C.

Analyses of antibodies to rotavirus and coronavirus by ELISA

Antibody production to rotavirus and coronavirus antigens were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (Dormitzer et al., 1994). The levels of neutralizing antibodies against rotaviruses were also determined by a focus reduction neutralization assay. Briefly, serum samples were inactivated for 30 min at 56 °C and serially diluted 4-fold in MEM (Gibco). All dilutions were incubated in the presence of 50–100 peroxidase focus forming-units of rotavirus RF strain for 1 h at 37 °C. Then, each mix was added in duplicate to MA104 cell culture monolayers grown in 96-well microtiter plates (Costar) and incubated at 37 °C. At 18 h.p.i., the cells were fixed with methanol/acetone and an immunoperoxidase assay was performed. Briefly, MAb 2F3E7 against rotavirus VP6 protein was diluted in 1:200 in PBS-BSA and allowed to react with the monolayer for 1 h at 37 °C. After three washes, the secondary antibody HRP-conjugated against mouse IgG (Sigma) diluted 1:2000 in PBS-BSA was added for 1 h at 37 °C. The plates were then washed five times with PBS and stained with DAB (Fast DAB, Sigma). Neutralizing titers were considered as the inverse of the sample dilution that showed 60% reduction of peroxidase focus forming-units.

Cytokine production assay

Five mice per group of 6- to 8-week-old female BALB/c mice were inoculated intragastrically three times with 10^6 FFU/mice of $rTGEV\text{-}_{MHV\text{-}S}$-VP7 or $rTGEV_{MHV\text{-}S}$ viruses. Mice were bled 2 days after the first inoculation and 15 days after the last inoculation, then sacrificed and spleens were extracted. Blood samples were collected by mandibular puncture 2 days postinoculation.

Spleen cell cultures and stimulation

Spleen single-cell suspensions were prepared by mechanical dissociation in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, and 2% FBS. Splenocytes obtained from animals of the same groups were separately pooled. Cells were washed and spleen erythrocytes were removed by osmotic lysis with erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM Na_2EDTA pH 7.3). Splenocytes were cultured in 24-well plates at a cell density of 2.5×10^6 cells/well. Lymphocyte restimulation was performed by supplementing culture medium with TGEV coronavirus (2×10^6 FFU), RF rotavirus (5×10^6 FFU) or concanavalin A (5 μg/mL). After 5 days of incubation at 37 °C supernatants were collected for cytokine detection and quantification. Cytokine levels were determined using the FlowCytomix Mouse Th1/Th2 10plex (Bender MedSystems) following the manufacturer's instructions. Data analyses were performed using MXP Software to generate standard curves for each cytokine and to determine sample cytokine levels.

Mouse protection experiments

Newborn mice were obtained from $rTGEV_{MHV\text{-}S}$-VP7-immunized female BALB/c mice mated with $rTGEV_{MHV\text{-}S}$-VP7-naive male BALB/c mice. Litters of 4-day-old newborns were orally given 10 **μL** of EDIM murine rotavirus (10 DD_{50}) or RF bovine rotavirus (2×10^6 FFU). This inoculum of the RF rotavirus strain induces diarrhea in 90% of challenged animals (Coste et al., 2000). The presence of diarrhea was recorded for 5 days after inoculation. Diarrhea was defined as the production of very fluid bright yellow stools following very gentle abdominal palpation of the mouse, combined with fecal material on the skin and around the anus.

Statistical analysis

In those experiments performed to analyze antibody and cytokine production the nonparametric test was applied to determine the presence or absence of significant differences between groups. Different proportions were compared using the two-tailed Fisher's exact test. In all cases *p*-values below 0.05 were considered statistically significant. The Mann–Whitney test was used to compare independent samples and the Wilcoxon test was applied for dependent sample comparisons. All the analyses were performed by using the SPSS software vs. 14.0 (LEAD Technologies, Inc.).

Acknowledgments

We thank Enrique O'Connor for technical support with the multiplex bead-based flow cytometry assay to analyze cytokines and Alejo Sempere and Cristina Mongort for their help with the immunohistochemistry.

This study was supported by the CICYT grant SAF2003-01911 from the Spanish Ministry for Science and Technology, the CICYT grant Bio2007-60978, and the European Commission grant PoRRSCon EU-245141. The work of J.M.R. was supported by a "V Segles" fellowship from the University of Valencia.

References

Almazan, F., Gonzalez, J.M., Penzes, Z., Izeta, A., Calvo, E., Plana-Duran, J., Enjuanes, L., 2000. Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. Proc. Natl. Acad. Sci. U. S. A. 97 (10), 5516–5521.

Almazan, F., Galan, C., Enjuanes, L., 2004. The nucleoprotein is required for efficient coronavirus genome replication. J. Virol. 78 (22), 12683–12688.

Almazan, F., Dediego, M.L., Galan, C., Escors, D., Alvarez, E., Ortego, J., Sola, I., Zuniga, S., Alonso, S., Moreno, J.L., Nogales, A., Capiscol, C., Enjuanes, L., 2006. Construction of a severe acute respiratory syndrome coronavirus infectious cDNA clone and a replicon to study coronavirus RNA synthesis. J. Virol. 80 (21), 10900–10906.

Andrew, M.E., Boyle, D.B., Whitfeld, P.L., Lockett, L.J., Anthony, I.D., Bellamy, A.R., Both, G.W., 1990. The immunogenicity of VP7, a rotavirus antigen resident in the endoplasmic reticulum, is enhanced by cell surface expression. J. Virol. 64 (10), 4776–4783.

Arias, C.F., Ballado, T., Plebanski, M., 1986. Synthesis of the outer-capsid glycoprotein of the simian rotavirus SA11 in *Escherichia coli*. Gene 47 (2–3), 211–219.

Bhandari, N., Sharma, P., Taneja, S., Kumar, T., Rongsen-Chandola, T., Appaiahgari, M.B., Mishra, A., Singh, S., Vrati, S., 2009. A dose-escalation safety and immunogenicity

study of live attenuated oral rotavirus vaccine 116E in infants: a randomized, double-blind, placebo-controlled trial. J. Infect. Dis. 200 (3), 421–429.
Barthold, S.W., Smith, A.L., 1984. Mouse hepatitis virus strain–related patterns of tissue tropism in suckling mice. Arch. Virol. 81 (1–2), 103–112.
Both, G.W., Lockett, L.J., Janardhana, V., Edwards, S.J., Bellamy, A.R., Graham, F.L., Prevec, L., Andrew, M.E., 1993. Protective immunity to rotavirus-induced diarrhoea is passively transferred to newborn mice from naive dams vaccinated with a single dose of a recombinant adenovirus expressing rotavirus VP7sc. Virology 193 (2), 940–950.
Casais, R., Thiel, V., Siddell, S.G., Cavanagh, D., Britton, P., 2001. Reverse genetics system for the avian coronavirus infectious bronchitis virus. J. Virol. 75 (24), 12359–12369.
Casais, R., Dove, B., Cavanagh, D., Britton, P., 2003. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J. Virol. 77 (16), 9084–9089.
Compton, S.R., Barthold, S.W., Smith, A.L., 1993. The cellular and molecular pathogenesis of coronaviruses. Lab. Anim. Sci. 43 (1), 15–28.
Correa, I., Jimenez, G., Sune, C., Bullido, M.J., Enjuanes, L., 1988. Antigenic structure of the E2 glycoprotein from transmissible gastroenteritis coronavirus. Virus Res. 10 (1), 77–93.
Coste, A., Sirard, J.C., Johansen, K., Cohen, J., Kraehenbuhl, J.P., 2000. Nasal immunization of mice with virus-like particles protects offspring against rotavirus diarrhea. J. Virol. 74 (19), 8966–8971.
de Groot, R.J., Ziehbuhr, J., Poon, L.L., Woo, P.C., Talbot, P., Rottier, P.J.M., Holmes, K.V., Baric, R., Perlman, S., Enjuanes, L., Gorbalenya, A.E., 2010. Taxonomic structure of the Coronaviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., King, A. (Eds.), Virus Taxonomy. International Committee on Taxonomy of Viruses. Academic Press, New York.
de Haan, C.A., Haijema, B.J., Boss, D., Heuts, F.W., Rottier, P.J., 2005. Coronaviruses as vectors: stability of foreign gene expression. J. Virol. 79 (20), 12742–12751.
Dennehy, P.H., 2008. Rotavirus vaccines: an overview. Clin. Microbiol. Rev. 21 (1), 198–208.
Dormitzer, P.R., Both, G.W., Greenberg, H.B., 1994. Presentation of neutralizing epitopes by engineered rotavirus VP7's expressed by recombinant vaccinia viruses. Virology 204 (1), 391–402.
Durbin, J.E., Hackenmiller, R., Simon, M.C., Levy, D.E., 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell 84 (3), 443–450.
Enjuanes, L., Spaan, W., Snijder, E., Cavanagh, D., 2000. Nidovirales. In: Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., McGeoch, D.J., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.), Virus Taxonomy. Classification and Nomenclature of Viruses. Academic Press, New York.
Enjuanes, L., Sola, I., Almazan, F., Izeta, A., Gonzalez, J.M., Alonso, S., 2001. Coronavirus derived expression systems. Progress and problems. Adv. Exp. Med. Biol. 494, 309–321.
Escors, D., Ortego, J., Enjuanes, L., 2001. The membrane M protein of the transmissible gastroenteritis coronavirus binds to the internal core through the carboxy-terminus. Adv. Exp. Med. Biol. 494, 589–593.
Fischer, F., Stegen, C.F., Koetzner, C.A., Masters, P.S., 1998. Construction of a mouse hepatitis virus recombinant expressing a foreign gene. Adv. Exp. Med. Biol. 440, 291–295.
Godeke, G.J., de Haan, C.A., Rossen, J.W., Vennema, H., Rottier, P.J., 2000. Assembly of spikes into coronavirus particles is mediated by the carboxy-terminal domain of the spike protein. J. Virol. 74 (3), 1566–1571.
Haijema, B.J., Volders, H., Rottier, P.J., 2003. Switching species tropism: an effective way to manipulate the feline coronavirus genome. J. Virol. 77 (8), 4528–4538.
Jimenez, G., Correa, I., Melgosa, M.P., Bullido, M.J., Enjuanes, L., 1986. Critical epitopes in transmissible gastroenteritis virus neutralization. J. Virol. 60 (1), 131–139.
Johnson, M.A., Misra, R.M., Lardelli, M., Messina, M., Ephraums, C., Reeves, P.R., Bolcevic, Z., Noel, J.S., Hum, C.P., Van Mai, H., Dyall-Smith, M.L., Holmes, I.H., 1989. Synthesis in *Escherichia coli* of the major glycoprotein of human rotavirus: analysis of the antigenic regions. Gene 84 (1), 73–81.
Kapikian, A.Z., Hoshino, Y., Chanock, R.M., 2001. Rotaviruses, In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th edition. Lippincott-Raven, Philadelphia, pp. 1787–1833.
Kapke, P.A., Brian, D.A., 1986. Sequence analysis of the porcine transmissible gastroenteritis coronavirus nucleocapsid protein gene. Virology 151 (1), 41–49.
Kuo, L., Godeke, G.J., Raamsman, M.J., Masters, P.S., Rottier, P.J., 2000. Retargeting of coronavirus by substitution of the spike glycoprotein ectodomain: crossing the host cell species barrier. J. Virol. 74 (3), 1393–1406.
L'Haridon, R., Scherrer, R., 1976. In vitro culture of a rotavirus associated with neonatal calf diarrhea. Ann. Rech. Vét. 7 (4), 373–381.
Lai, M.M.C., 1996. Recombination in large RNA viruses: coronaviruses. Semin. Virol. 7, 381–388.
Lai, M.M., Cavanagh, D., 1997. The molecular biology of coronaviruses. Adv. Virus Res. 48, 1–100.
Lewicki, D.N., Gallagher, T.M., 2002. Quaternary structure of coronavirus spikes in complex with carcinoembryonic antigen-related cell adhesion molecule cellular receptors. J. Biol. Chem. 277 (22), 19727–19734.
Madhi, S.A., Cunliffe, N.A., Steele, D., Witte, D., Kirsten, M., Louw, C., Ngwira, B., Victor, J.C., Gillard, P.H., Cheuvart, B.B., Han, H.H., Neuzil, K.M., 2010. Effect of human rotavirus vaccine on severe diarrhea in African infants. N. Engl. J. Med. 362 (4), 289–298.
McCrae, M.A., McCorquodale, J.G., 1987. Expression of a major bovine rotavirus neutralisation antigen (VP7c) in Escherichia coli. Gene 55 (1), 9–18.
McGonigal, T.P., Turon, M.C., Komar-Hartnett, K., Kister, S.E., Smith, R.E., 1992. Expression of the gene coding for the major outer capsid protein of SA-11 rotavirus in a baculovirus system. Virus Res. 23 (1–2), 135–150.
Navas, S., Seo, S.H., Chua, M.M., Sarma, J.D., Lavi, E., Hingley, S.T., Weiss, S.R., 2001. Murine coronavirus spike protein determines the ability of the virus to replicate in the liver and cause hepatitis. J. Virol. 75 (5), 2452–2457.
Offit, P.A., Clark, H.F., 1985. Maternal antibody-mediated protection against gastroenteritis due to rotavirus in newborn mice is dependent on both serotype and titer of antibody. J. Infect. Dis. 152 (6), 1152–1158.
Ortego, J., Escors, D., Laude, H., Enjuanes, L., 2002. Generation of a replication-competent, propagation-deficient virus vector based on the transmissible gastroenteritis coronavirus genome. J. Virol. 76 (22), 11518–11529.
Ortego, J., Sola, I., Almazan, F., Ceriani, J.E., Riquelme, C., Balasch, M., Plana, J., Enjuanes, L., 2003. Transmissible gastroenteritis coronavirus gene 7 is not essential but influences in vivo virus replication and virulence. Virology 308 (1), 13–22.
Parashar, U.D., Glass, R.I., 2009. Rotavirus vaccines—early success, remaining questions. N. Engl. J. Med. 360 (11), 1063–1065.
Parashar, U.D., Gibson, C.J., Bresse, J.S., Glass, R.I., 2006. Rotavirus and severe childhood diarrhea. Emerg. Infect. Dis. 12 (2), 304–306.
Penzes, Z., Gonzalez, J.M., Calvo, E., Izeta, A., Smerdou, C., Mendez, A., Sanchez, C.M., Sola, I., Almazan, F., Enjuanes, L., 2001. Complete genome sequence of transmissible gastroenteritis coronavirus PUR46-MAD clone and evolution of the purdue virus cluster. Virus Genes 23 (1), 105–118.
Rasko, J.E.J., 1997. Granulocyte-macrophage colony-stimulating factor and its receptor. In: Garland, P., Quesenberry, P., Hilton, D. (Eds.), Colony-stimulating factors: molecular and cellular biology. Marcell Dekker, New York, pp. 163–202.
Risco, C., Anton, I.M., Sune, C., Pedregosa, A.M., Martin-Alonso, J.M., Parra, F., Carrascosa, J.L., Enjuanes, L., 1995. Membrane protein molecules of transmissible gastroenteritis coronavirus also expose the carboxy-terminal region on the external surface of the virion. J. Virol. 69 (9), 5269–5277.
Rossen, J.W., Strous, G.J., Horzinek, M.C., Rottier, P.J., 1997. Mouse hepatitis virus strain A59 is released from opposite sides of different epithelial cell types. J. Gen. Virol. 78 (Pt 1), 61–69.
Salas-Vidal, E., Plebanski, M., Castro, S., Perales, G., Mata, E., Lopez, S., Arias, C.F., 1990. Synthesis of the surface glycoprotein of rotavirus SA11 in the aroA strain of Salmonella typhimurium SL3261. Res. Microbiol. 141 (7–8), 883–886.
Sanchez, C.M., Jimenez, G., Laviada, M.D., Correa, I., Sune, C., Bullido, M., Gebauer, F., Smerdou, C., Callebaut, P., Escribano, J.M., Enjuanes, L., 1990. Antigenic homology among coronaviruses related to transmissible gastroenteritis virus. Virology 174 (2), 410–417.
Sanchez, C.M., Izeta, A., Sanchez-Morgado, J.M., Alonso, S., Sola, I., Balasch, M., Plana-Duran, J., Enjuanes, L., 1999. Targeted recombination demonstrates that the spike gene of transmissible gastroenteritis coronavirus is a determinant of its enteric tropism and virulence. J. Virol. 73 (9), 7607–7618.
Sola, I., Alonso, S., Zuniga, S., Balasch, M., Plana-Duran, J., Enjuanes, L., 2003. Engineering the transmissible gastroenteritis virus genome as an expression vector inducing lactogenic immunity. J. Virol. 77 (7), 4357–4569.
Sune, C., Jimenez, G., Correa, I., Bullido, M.J., Gebauer, F., Smerdou, C., Enjuanes, L., 1990. Mechanisms of transmissible gastroenteritis coronavirus neutralization. Virology 177 (2), 559–569.
Thiel, V., Herold, J., Schelle, B., Siddell, S.G., 2001. Infectious RNA transcribed in vitro from a cDNA copy of the human coronavirus genome cloned in vaccinia virus. J. Gen. Virol. 82 (Pt 6), 1273–1281.
Torrecilhas, A.C., Faquim-Mauro, E., Da Silva, A.V., Abrahamsohn, I.A., 1999. Interference of natural mouse hepatitis virus infection with cytokine production and susceptibility to Trypanosoma cruzi. Immunology 96 (3), 381–388.
Velazquez, F.R., Matson, D.O., Calva, J.J., Guerrero, L., Morrow, A.L., Carter-Campbell, S., Glass, R.I., Estes, M.K., Pickering, L.K., Ruiz-Palacios, G.M., 1996. Rotavirus infections in infants as protection against subsequent infections. N. Engl. J. Med. 335 (14), 1022–1028.
Yount, B., Curtis, K.M., Fritz, E.A., Hensley, L.E., Jahrling, P.B., Prentice, E., Denison, M.R., Geisbert, T.W., Baric, R.S., 2003. Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus. Proc. Natl. Acad. Sci. U. S. A. 100 (22), 12995–13000.