CHAPTER 16

Molecular Basis of Transmissible Gastroenteritis Virus Epidemiology

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I. HISTORY OF TRANSMISSIBLE GASTROENTERITIS AND CLOSELY RELATED CORONAVIRUSES

A disease with the characteristics of transmissible gastroenteritis (TGE) was first reported in 1935 (Smith, 1956). The viral etiology of TGE was demonstrated 11 years later by Doyle and Hutchings (1946) in the United States. During the next 20 years TGE was reported in all other continents (Table I). Apparently, the disease occurred first in those countries that had imported North American stock and then was also introduced by European stock (Woode, 1969). There is evidence that TGE was not new to the pig, but became important to the pig industry concurrently with its intensification (Woode, 1969).

Until two decades ago, TGE outbreaks in the United States and Europe occurred mainly as acute epizooties on breeding farms, particularly during the winter months. The disease incidence appeared to have a cyclic course. After an outbreak, the disease and the virus disappeared and the herd immunity gradually waned in the next 2 to 3 years. A new outbreak was the consequence of the reintroduction of transmissible gastroenteritis virus (TGEV). A change in this epizootic pattern has occurred in recent decades together with the further intensification of swine breeding. TGEV has become more enzootic, partic-

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The Coronaviridae, edited by Stuart G. Siddell, Plenum Press, New York, 1995.

TABLE I. Initial Reports of Transmissible Gastroenteritis in Various Countries

Year	Country or Continent	References
1946	United States	Doyle and Hutchings (1946); R. Wesley (personal communication)
1956	England	Pritchard (1987)
1956	Japan	Sasahara *et al.* (1958)
1956	France	Jestin *et al.* (1987a,b)
1957	Denmark	Woode (1969)
1958	China	Woode (1969)
1959	Germany	Woode (1969)
1960	Spain	Concellón Martínez (1960)
1961	Russia	Woode (1969)
1961	Poland	Woode (1969)
1962	Romania	Woode (1969)
1962	The Netherlands	Woode (1969)
1966	Australia	Woode (1969); not confirmed
1966	Africa	Woode (1969); not confirmed
1966	Czechoslovakia	Woode (1969)
1967	Canada	Woode (1969)
1967	Belgium	Woode (1969)

ularly in large breeding farms in which, after a primary outbreak, the virus persists in infected weaned nursery pigs. This enzootic form of TGE cannot be recognized clinically and it has, therefore, become difficult to determine how widespread TGEV is in a country, unless serological surveys were performed (Pensaert *et al.*, 1993; Hill, 1989).

At present, TGE has dominantly been reported in developed areas, which could be caused by a higher incidence in these countries, but could also reflect that the disease is not an obligatory noticeable disease in developing countries (FAO, 1984). In Europe, the prevalence of TGE is decreasing. However, there are still pockets with a high incidence of TGE. For instance, in England only 3% of the national herd was seropositive for TGEV up to 1984 (Pritchard, 1987), but in East Anglia TGEV has been diagnosed virtually every year since 1956 (Pritchard, 1987); the last serious epizootic occurred from 1980 to 1982 (Pritchard and Cartwright, 1982; D. Paton, Central Veterinary Laboratory, UK, personal communication). Evidence of enzootic TGEV infection was found in 50% of pigs studied between 1981 and 1983 in East Anglia. The region, therefore, is a reservoir containing the virus. In general, TGEV outbreaks have diminished, but the virus is still present in Central Europe. In Belgium the number of seropositive fattening pigs has decreased from 15% (Callebaut *et al.*, 1989) to 7.6%, according to a seroepizootiological study carried out between 1989 and 1990. In this study 5% of the farms appeared to harbor seropositive animals (Pensaert *et al.*, 1992). In South European countries such as Spain, the incidence of TGE is low. Two geographical zones, Central and East Spain, can be differentiated. In Central Spain (Castilla and León), sera collected in 1985 and 1988 were seronegative for TGEV (Rubio *et al.*, 1987; Lanza *et al.*, 1993a) and the virus was

only occasionally diagnosed, generally associated with swine imports (Laviada *et al.*, 1988; Lanza *et al.*, 1990). Along the Mediterranean Coast, epizootic outbreaks of TGE-like disease have been observed every 3 years since 1980 (Plana *et al.*, 1982; Anon, 1989). TGEV-specific antibodies have been found in sows of two different intensive pig-breeding areas: Murcia and Catalonia. A survey made in 1987 in the East Coast showed that 1.3% of the breeding pigs were seropositive and there was a prevalence of infection in 5% of the farms (Cubero *et al.*, 1990, 1993b).

In the United States, TGE is more of a problem. In 1987 and 1988, TGE was reported in about half of the swine herds (Hill, 1989). It was also a major cause of viral enteritis, since 26% of the cases with neonatal diarrhea submitted to diagnostic laboratories were caused by TGEV, a figure equal to the enteritis caused by *Escherichia coli* (Hoefling, 1989). In the United States, TGEV has created greater economic vulnerability resulting from growth retardation and increased susceptibility to other infectious diseases (Hoefling, 1989). But recently the incidence of TGEV has also decreased in the United States. The percentage of farms with at least one sample testing positive for TGEV was 35.8% [National Animal Health Monitoring System (NAHMS), 1992], lower than the 50% found in a survey of Midwestern swine in the early 1980s (Egan *et al.*, 1982). In the United States, 24.3% of the farms still vaccinate the sows or gilts against TGE (NAHMS, 1992).

Recently, a variant of TGEV, the porcine respiratory coronavirus (PRCV), has been described (Pensaert *et al.*, 1986; Callebaut *et al.*, 1988). The discovery of this virus was based on a survey carried out in 1984 in Belgium, which showed an increase of animals with antibodies to TGEV (up to 68%), with no increase in the incidence of TGE in the preceeding winter, and in the absence of vaccination (Pensaert *et al.*, 1987). In 1986 and 1987, the virus spread to 100% of the swine farms in Belgium, 67% of which are still infected. PRCV-free farms became infected during the autumn (Pensaert *et al.*, 1992). This pattern, showing higher incidence of PRCV in the cold seasons, has repeated itself in later years both in Belgium and in France (Laude *et al.*, 1993). The virus has been observed in many European countries (The Netherlands, Denmark, England, Spain, and France), where it has now spread to almost the whole swine population (Brown and Cartwright, 1986; Pensaert *et al.*, 1987; Jestin *et al.*, 1987a,b; Laviada *et al.*, 1988; Have, 1991; Sánchez *et al.*, 1990). PRCV was detected in Spain for the first time in September 1986 (Martin-Alonso *et al.*, 1992). Thirty-one percent of sera collected in Castilla-León (Central Spain) and 64% of the farms in this area were seropositive for PRCV (Lanza *et al.*, 1993a,b). In England, a survey in 1987 of over 300 elite herds with a high health status revealed that 80.1% of the herds contained seropositive pigs (D. Paton, personal communication). In the United States, serological tests detected PRCV for the first time in Indiana in 1989 (Wesley *et al.*, 1990). Since then, other PRCV isolates have been reported in the United States and Canada (Halbur *et al.*, 1993; Jabrane and Elazhary, 1993). PRCV strongly cross-reacts with its ancestor TGEV (Callebaut *et al.*, 1988; Rasschaert *et al.*, 1990; Sánchez *et al.*, 1990, 1992; Wesley *et al.*, 1991a). PRCV may evoke a protective response to TGEV (Hooyberghs *et al.*, 1988; Paton and Brown, 1990; De Diego *et al.*, 1992; Laude *et al.*, 1993; Wesley

and Woods, 1993). PRCV has also been diagnosed in Asia and Eastern European countries (M. Pensaert and J. Musilova, personal communications).

A second enteric coronavirus, the porcine epidemic diarrhea virus (PEDV), serologically unrelated to TGEV, was isolated for the first time in Belgium in 1977 (Pensaert and Debouck, 1978) and then in England (Chasey and Cartwright, 1978) and Spain (Jiménez *et al.*, 1986a). PEDV is now present in all European countries. In 1983, two surveys performed in England, in the spring and in the autumn, showed 19.2 and 8.3% of the animals positive, respectively, which reflects the seasonal incidence of PED. In 1987, 9.2% of the sows tested were seropositive for PED in England. Currently, about 1–2% of the fecal samples submitted to the Central Veterinary Laboratory from all over England are PED positive. PEDV has not been found in the United States (R. Wesley, National Animal Disease Center, Iowa, personal communication). It is intriguing that during 35 years of research into TGE, PEDV had not been recognized before, suggesting that the infection is a recent event or that in the past its diagnosis was less efficient because TGEV-specific reagents were used to detect coronaviruses-causing enteritis.

II. SUSCEPTIBILITY TO TGEV INFECTION

A. Tropism and Host Cells

TGEV is basically an enteropathogen, although natural TGEV isolates can also replicate in the respiratory tract (Underdahl *et al.*, 1974; Kemeny *et al.*, 1975). The virus has occasionally been isolated from tonsils, trachea, and lungs. Signs of respiratory disorders were not observed despite the detection of pulmonary lesions (Kemeny *et al.*, 1975; Underdahl *et al.*, 1974, 1975). During infections with virulent isolates, the highest virus concentrations are found in the enteric tract. The jejunum and, to a lesser extent, the ileum and duodenal epithelium are the areas with most pathology. TGEV isolates that have been passaged in cell cultures gradually lose their tropism for the enteric tract, while they gain tropism for respiratory tissues (Harada *et al.*, 1969; Furuuchi *et al.*, 1978, 1979) and for alveolar macrophages, where TGEV replication is partially restricted (Laude *et al.*, 1984). Highly attenuated strains of TGEV, which replicate in the upper respiratory tract, including tonsils and lungs, do not replicate in the intestine of newborn pigs (Furuuchi *et al.*, 1979). TGEV replicates in the apical tubovascular system of villous absorptive cells in newborn pigs; this system is absent in pigs older than 3 weeks (Wagner *et al.*, 1973). The virus also grows in the mammary tissue of lactating sows (Saif and Bohl, 1983); infected sows shed virus in their milk (Kemeny and Woods, 1977). Intrafetal inoculation results in the production of villous atrophy (Redman *et al.*, 1978). The susceptibility of cells from different organs to TGEV should be carefully defined for each situation, since it is a function of the virus dose, the status of the swine used (gnotobiotic, colostrum deprived, or conventional), temperature, and age of the animal, among other factors (Furuuchi *et al.*, 1976; L. Saif, personal communication; M. L. Ballesteros and L. Enjuanes, unpublished results).

In infected animals, the virus can be recovered from macrophages and other cells of the reticuloendothelial system (Underdahl *et al.*, 1974). Macrophages from the intestinal mucosa (Chu *et al.*, 1982) and probably Küppfer cells are also infected, while porcine blood monocytes are not infected by TGEV (Laude *et al.*, 1984). The virus has also been isolated from mesenteric lymph nodes and Peyer's patches up to 9 days after infection. However, the infectious virus persisted only for 3–4 days in T-cell lines derived from these organs (M. J. Bullido and L. Enjuanes, unpublished results).

Even though TGEV usually causes an acute enteric disease, it also leads to persistent infections, in which the virus is not detected in the enteric tract (M. Pensaert, personal communication). Instead, the virus has been found in the respiratory tract of recovered pigs for more than 100 days after infection (Underdahl *et al.*, 1974, 1975). TGEV mutants can persistently infect adult swine with continuous virus shedding in the gut (R. Wesley and R. Roods, personal communication).

After oronasal inoculation, strain BEL85 of PRCV replicates in the nasal mucosa, tonsils, trachea, bronchi, bronchioles, and alveoli and alveolar macrophages, while in the intestine a few cells stained positively with immunofluorescence. Higher virus titers ($>10^8$ $TCID_{50/g}$ of tissue) have been detected in the lungs (O'Toole *et al.*, 1989; Cox *et al.*, 1990a; Laude *et al.*, 1993). Other tissues, including plasma (viremia was observed), mesenteric lymph nodes, and colon were consistently positive, while the virus was sporadically isolated from other lymph nodes, spleen, liver, and thymus. Even when the virus was inoculated directly to the lumen of the small intestine, a limited degree of virus replication, in a few enterocytes located in the transition from the crypts to the villi in the jejunum, was observed (Pensaert *et al.*, 1987). The cells were identified as villous enterocytes by electron microscopy (Popischil *et al.*, 1990) and immunocytochemistry (O'Toole *et al.*, 1989). PRCV is not found in rectal swab samples (Vancott *et al.*, 1993) unless it is administered to the pig artificially via a stomach tube (Wesley and Woods, 1993). A minimum of 10^3 $TCID_{50}$ of PRCV were needed to start the infection in the intestinal tract (Cox *et al.*, 1990b). This result indicates that the gut is not the target organ for PRCV and that the ability of these coronaviruses to infect a tissue is not an all-or-nothing phenomenon.

It has been debated whether PRCV causes respiratory disease or not. Signs of respiratory disorders caused by PRCV have not been observed by Pensaert's group, either after field infections or after experimental aerosol inoculation, using procedures that produce clinical signs using respiratory viruses such as influenza viruses and Aujeszky disease virus (Pensaert *et al.*, 1987). Also, experimental inoculation of pigs with the British PRCV has not produced disease, other than very occasional and mild rhinitis, although histopathological lesions are present (O'Toole *et al.*, 1989). In England, isolation of PRCV has been associated with respiratory problems, frequently with concurrent swine influenza (Lanza *et al.*, 1992). In contrast, two groups (Duret *et al.*, 1988; Van Nieuwstadt and Pol, 1989) have reported that PRCV can cause pneumonia. The pathology caused by various PRCV strains isolated in the United States also range from inapparent to severe bronchointerstitial pneumonia, which has been observed more recently in gnotobiotic pigs. Lesions in conventional pigs were

less severe, however. The pigs developed mild disease and fever (Halbur *et al.*, 1992). The Canadian isolate PRCV IQ90 produced morbidity and mortality rates reaching 100% and 60%, respectively (Jabrane and Elazhary, 1993). Conflicting results have been obtained with different PRCV isolates which differ in their genetic structure (see Section III.A.). This may explain the different results.

B. Virus Receptors

Two cell surface proteins appeared to be relevant to the entry of TGEV into susceptible cells: aminopeptidase N (APN) and a recently described 200-kDa protein. APN, an N-terminal exopeptidase with preference for neutral amino acids, has been clearly shown to act as a major receptor for TGEV and PRCV in cultured cells (Delmas *et al.*, 1992, 1993). APN is identical to CD13, a surface protein abundantly expressed in the brush border membranes of intestinal epithelial cells and fibroblast, on the apical surfaces of lung and renal cells, as well as in granulocytes, monocytes, and their bone marrow progenitors (Norén *et al.*, 1986; Look *et al.*, 1989). APN plays a role in the digestion of peptides in the gut (Kenny *et al.*, 1987). The distribution of APN strongly suggests that it is also major receptor for TGEV and PRCV *in vivo*. Two key experiments provide evidence for the involvement of APN as a receptor for TGEV and PRCV. First, cells refractory to these viruses became susceptible after being transfected with the cDNA encoding porcine APN. Second, monoclonal antibodies (MAbs) specific for this protein efficiently block the multiplication of these viruses in cell culture. APN also is the major receptor for human coronavirus 229E (Yeager *et al.*, 1992) and for cytomegalovirus (Söderberg *et al.*, 1993).

Additional factors are probably involved in the susceptibility of intestinal epithelial cells both for TGEV and PRCV. Since intestinal cells are not infected (or only infected with extremely low efficiency) by PRCV, the existence of an additional factor involved in virus entry or in later steps of replication has been proposed (Sánchez *et al.*, 1992; Delmas *et al.*, 1993; Laude *et al.*, 1993). A similar situation exists in murine coronaviruses (Yokomori *et al.*, 1993). On the viral side, there is evidence that two regions of the genome might be involved: the 5′-terminus of the S gene is deleted or altered in viruses that have lost enteric tropism (Sánchez *et al.*, 1992; Delmas *et al.*, 1993; Wesley *et al.*, 1991a; Britton *et al.*, 1991); alternatively, the open reading frame (ORF) 3a that codes for a 71–72 amino acid nonstructural polypeptide (Godet *et al.*, 1992), which has been converted to a pseudo-gene in many respiratory virus (Rasschaert *et al.*, 1990; Wesley *et al.*, 1989; Laude *et al.*, 1993), might also be a determinant of tropism and virulence for coronaviruses related to TGEV. It was suggested that the high susceptibility of newborn piglets to TGEV infection and the tropism of the virus for villous enterocytes may be related to a 200-kDa protein. Experimental evidence for the existence of a second receptor binding site for TGEV has recently been provided (Weingartl and Derbyshire, 1993a,b). A saturable, specific binding of TGEV to the plasma membrane of the villous enterocytes in neonate swine has been shown. This binding is inhibited by MAbs that recog-

nize a 200-kDa protein but not APN (Weingartl and Derbyshire, 1993a,b; 1994). This protein was present in tiny or undetectable amounts in cryptal enterocytes of newborn swine or villous enterocytes from weaned pigs, less susceptible to TGEV. At early times post-birth (less than 3-day old piglets) the villi are covered with APN containing epithelial cells, while the 200-kDa protein is only present on the tip of the villi and in a few epithelial cells of the villi.

C. Effect of Age on Infection

A clear relationship between TGEV pathogenicity and the age of the infected animals has been established. It has been shown that the dose of virus needed to infect an adult swine is 10^4-fold greater than that required to infect a neonate (Witte and Walther, 1976). Epizootic outbreaks affect all age groups and spread through the herd in 2 or 3 days. Only pigs up to 3 weeks of age often vomit and develop watery diarrhea that leads to dehydration, rapid weight loss, and death in 2 to 5 days. Mortality in these young pigs often approaches 100%. In weanlings (3 to 8 weeks old), mortality is usually less than 10 to 20%, but impaired feed adsorption causes growth retardation. Adult pigs become inappetent and develop diarrhea that usually lasts only 2 to 4 days. Although morbidity may approach 100%, mortality in this age group is usually less than 5% (Hill, 1989).

A sharp difference in the susceptibility of newborn swine to TGEV, depending on their access to colostrum and milk, even when the sows were seronegative for TGEV, has been observed (Furuuchi *et al.*, 1976; M. L. Ballesteros, C. M. Sánchez, J. Plana, and L. Enjuanes, unpublished results). These data suggest a specific effect of the colostrum on intestinal epithelial cell differentiation toward a TGEV-resistant state. This colostrum factor may influence the expression of the 200-kDa or APN TGEV receptors. Alternatively, factors present in the colostrum may interfere with binding of the virus to the receptors. Conventional colostrum-deprived newborn swine were fully susceptible (100% mortality) to high cell passage TGEV (PUR46-MAD strain), while 86% of their littermates that had access to colostrum and milk for 7 hr showed no disease symptoms (M. L. Ballesteros, C. M. Sánchez, J. Plana, and L. Enjuanes, unpublished results). Similar results have been obtained with gnotobiotic pigs and newborn animals that had access to colostrum, using attenuated strains of TGEV (M. Welter, personal communication). Thus, susceptibility to TGEV is a function of several host factors: age of animals, access to colostrum, and environmental conditions, in addition to the dose and virulence of the virus strain.

D. Transmission of the Virus

The main reservoir for TGEV and related viruses is probably the pig. Both fattening swine and lactating sows are responsible for transmission of TGEV to young animals. Contamination originates from feces, the milk, and aerosols generated in the respiratory tract (Kemeny *et al.*, 1975; Kemeny and Woods,

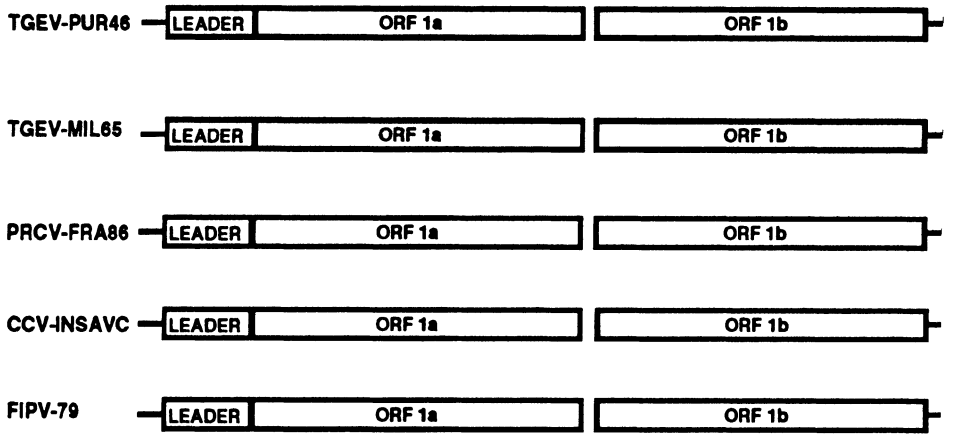


FIGURE 1.

1977; Kemeny, 1978). TGEV can also replicate in dogs, cats, foxes, starlings, domestic flies, and possibly in skunks, opossums, and muskrats (Saif and Wesley, 1992). No signs of infection have been detected in mice, rats, gerbils, or guinea pigs (Garwes, 1982).

TGEV replicates in the mammary gland of lactating sows, and infectious virus can be recovered from their milk and possibly be transmitted to piglets (Saif and Bohl, 1983). Intrauterine transfer of PRCV and TGEV has not been observed (Paton and Brown, 1990; Saif and Bohl, 1983). Transmission by the aerogenic route in TGEV epizootiology is under debate. Some authors suggest that it is relevant since infectious virus has been isolated 100 days after infection in the breath of infected pigs (Underdahl *et al.*, 1975; Torres-Medina, 1975). Other authors suggest that the aerogenic route is not important for TGEV, since the efficiency of spreading by this route was much lower than that for PRCV (Laude *et al.*, 1993). By contrast, PRCV spreads via air, based on the following findings: (1) aerosolized virus initiates the infection; (2) the virus disseminates very rapidly; (3) the virus transferred between farms with no apparent links; and (4) the virus spreads in countries with high hygienic standards, free of TGEV (Pensaert *et al.*, 1986; Jestin *et al.*, 1987a,b; Henningsen *et al.*, 1988; Have, 1991). PRCV is secreted orally nasally. There is no indication that the fecal–oral transmission plays a role in the epizootiology of the natural infection. The transmission of PEDV in infected farms in the center of Spain could not be easily explained, other than by the aerogenic route (Jiménez *et al.*, 1986a).

III. ANTIGENIC AND GENETIC VARIATION

A. Genome Organization and Virus Structure

TGEV contains a single-stranded positive-sense RNA genome of around 30 kb, which is infectious (Brian *et al.*, 1980), and generates eight mRNAs, includ-

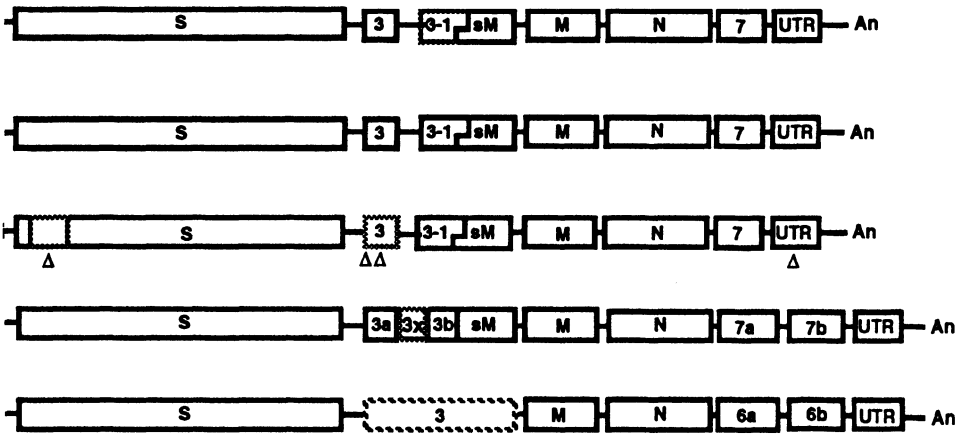


FIGURE 1. Comparison of the genomes of TGEV and related coronaviruses. ORFs are indicated. CCV ORF 3x is most likely not expressed. ORF 3 of FIPV is not well defined, although a 5.2-kb mRNA corresponding to this ORF has been described (de Groot *et al.*, 1987; Vennema *et al.*, 1992). Solid rectangles indicate identified or predicted ORFs; dotted rectangles indicate pseudogenes for which no mRNA has been detected; Δ, indicates presence of deletions; UTR, untranslated regions; An, poly A. The sequences used have been previously reported: TGEV-PUR46 (Rasschaert *et al.*, 1987), TGEV-MIL65 (Wesley *et al.*, 1989; Wesley, 1990); PRCV-FRA86 (Rasschaert *et al.*, 1990); CCV (Horsburgh *et al.*, 1992); FIPV (de Groot *et al.*, 1987; Jacobs *et al.*, 1987; Vennema *et al.*, 1992). The drawing is not to scale.

ing the genomic size mRNA (mRNA 1) and a nested set of seven subgenomic mRNAs. The mRNAs have a common leader of about 90 nucleotides (nt)-long (Page *et al.*, 1990; Sethna *et al.*, 1989) and poly A on the 3′ end (Jacobs *et al.*, 1986). These RNAs are named mRNAs 2 or S, 3, 3-1, 4 or sM, 5 or M, 6 or N, and 7, after the ORFs that are encoded by them (Fig. 1). The nomenclature used is according to the Coronavirus Study Group (Cavanagh *et al.*, 1990, 1994). These genes code for four structural proteins (S, sM, M, and N) (Jacobs *et al.*, 1986; Rasschaert *et al.*, 1987; Godet *et al.*, 1992). mRNA 7 codes for a potential fifth membrane-associated structural protein of 9 kDa (Tung *et al.*, 1992). A comparison of TGEV genome organization with that of closely related coronaviruses is shown (Fig. 1). TGEV, PRCV, canine coronavirus (CCV), and feline infectious peritonitis virus (FIPV) have a similar genome organization with some differences. FIPV and CCV have two ORFs at the 3′ end of the genome, instead of the single ORF7 in TGEV.

The most abundant structural proteins in TGEV are S, M, and N. S protein is the main inducer of neutralizing antibodies (Laude *et al.*, 1986; Jiménez *et al.*, 1986b). Four antigenic sites (A, B, C, and D) have been recognized on the spike protein; site A can be subdivided in three subsites (Aa, Ab, and Ac) (Correa *et al.*, 1988, 1990). These sites have been mapped on the S protein (Fig. 2) in the order C, B, D, and A, starting at the N-terminal end. Site C is located between amino acid 49 to 52; site B is between residues 97 to 144; site D between residues 382 to 389; and site A, around residues 538 (Aa), 543 (Ac), 586 (Aa-Ab), and 591 (Ab) (Correa *et al.*, 1990; Enjuanes *et al.*, 1990; Gebauer *et al.* 1991). Three of these

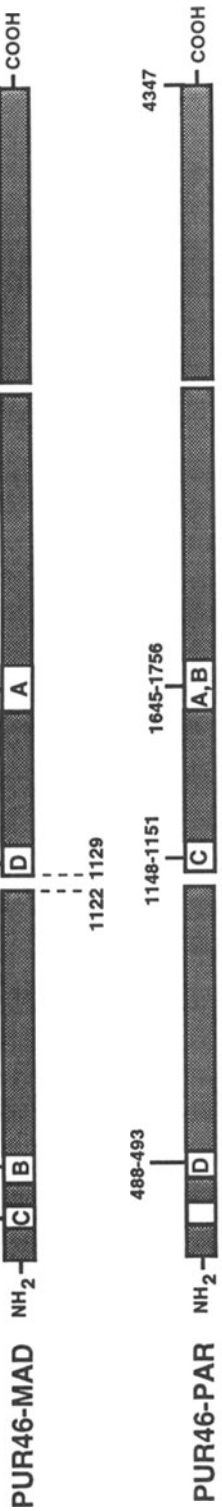


FIGURE 2. Correlation between the antigenic and the physical structure of TGEV S-glycoprotein. The nomenclature used for the antigenic sites by the groups at Paris (Delmas *et al.*, 1990a,b) and Madrid (Correa *et al.* 1988; Gebauer *et al.*, 1991) is indicated. The four antigen sites (A, B, C, and D) were defined by competitive radioimmunoassays using MAbs and the antigenic subsites by characterization of TGEV MAb-resistant mutants (Correa *et al.*, 1988). Numbers above the bars indicate changed residues responsible for altered amino acids in escape mutants selected with MAbs. Nucleotide numbers have been given according to the numbers for the PUR46 virus, adding six residues to compensate the deletion present between residues 1122 and 1129.

sites (B, D, and A) overlap with sites defined by Delmas *et al.* (1990a,b), who named them D, C, and A-B, respectively (Fig. 2). By competitive binding studies using MAbs from different laboratories, more than ten antigenic sites could be differentiated, five of which were involved in the neutralization of the virus, although site A was the major inducer of neutralizing antibodies in all laboratories (D. Garwes, H. Laude, R. Wesley, R. Woods, and L. Enjuanes, unpublished data). The second important sites involved in *in vitro* neutralization are sites D and B. Only some of the MAbs specific for these sites are neutralizing (Laude *et al.*, 1986; Jiménez *et al.*, 1986b; Correa *et al.*, 1988; Posthumus *et al.*, 1990a,b; Van Nieuwstadt *et al.*, 1988). Using neutralizing MAbs, Hohdatsu *et al.* (1987) have defined at least six different epitopes involved in the neutralization of TGEV isolates, which these authors classified into four groups. Interestingly, some critical epitopes for neutralization by MAbs appeared at high (176th) passage levels but were not present at a low (17th) passage of the Toyama strain of TGEV.

B. Evolution of TGEV Structural Proteins S, M, N, and sM

The binding of MAbs to TGEV, PRCV, and related coronaviruses demonstrated antigenic diversity in the three major viral proteins (Fig. 3). Among TGEV isolates, there is more diversity in the S protein than in the M or N protein. This might be related to the important biological activities of the coronavirus S protein. It is involved in the fusion of infected cells (Frana *et al.*, 1985; Fazakerley *et al.*, 1992); it binds to receptors on the cell surface (Holmes *et al.*, 1981, 1989; Delmas *et al.*, 1992, 1993); it is involved in the pathogenesis of murine hepatitis virus (MHV) (Fleming *et al.*, 1986); and it is the major inducer of complement-independent neutralizing antibodies (Jiménez *et al.*, 1986b; Delmas *et al.*, 1986). Of the four antigenic sites defined (C, B, D, and A) (Correa *et al.*, 1988, 1990; Delmas *et al.*, 1986; Gebauer *et al.*, 1991), sites C and B were particularly variable. Extending the comparison to PRCV, FIPV, feline enteric coronavirus (FECV) and CCV, these isolates also showed a wide variation in the sites C and B. Site D has an intermediate level of conservation, while site A was most conserved, particularly subsite Ac, which is present in porcine, feline, and canine coronaviruses (Fig. 3). From an antigenic point of view, the main difference observed between the attenuated PRCVs and the virulent TGEVs is the absence of the B site on the virulent isolates: MIL65, SHI56, and MAD88 (Fig. 3) (Sánchez *et al.*, 1990), indicating that this site is different in the virulent strains.

Comparison of S protein sequences of TGEVs and PRCVs (Fig. 4) has shown that the S protein from three TGEV strains, MIL65, BRI70-FS772, and TOY56, has 1449 amino acids, two more residues than three clones of the high passage PUR46 strain and two vaccine strains (Sánchez *et al.*, 1990; Register and Wesley, 1994; C. M. Sánchez and L. Enjuanes, unpublished results). The two amino acid deletion is not present in the European PRCVs. An identity of 97% between the S-proteins of MIL65 and the PUR46 strains was observed at both amino acid and nucleotide level. Seventy-two percent of the amino acid changes were located within the N-terminal half of the S protein, which comprises the exposed globular-shaped portion of the peplomer. Only nine residue differences occur

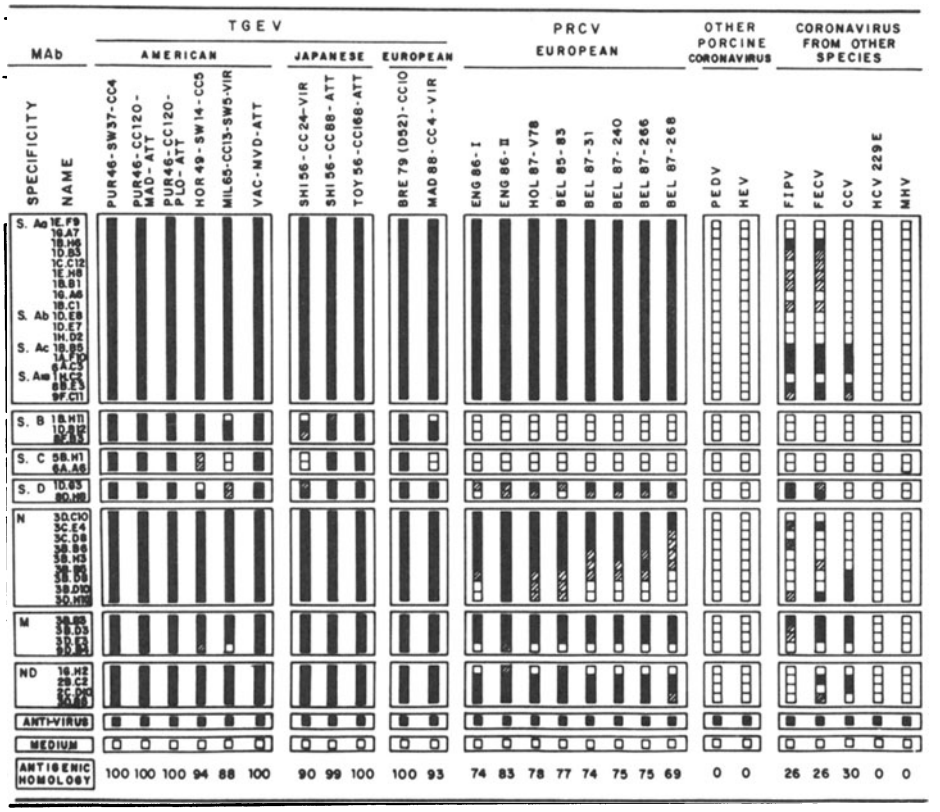


FIGURE 3. Binding of MAbs to coronaviruses. The value of the MAb binding to the PUR46-CC120-MAD strain, determined by RIA, was taken as the reference value (100). The characteristics of the viruses used and the specificity of the MAbs have been reported (Sánchez *et al.*, 1990). The antigenic homology of each virus isolate relative to the reference virus PUR46-CC120-MAD was expressed as a percentage (Sánchez *et al.*, 1990). Symbols: □, 0 to 30; ▨, 31 to 50; ■, 51 to 100. The antivirus sera were TGEV specific in the case of TGEV, PRCV, FIPV, FECV, and CCV and specific for the homologous virus in the case of PEDV, HEV, HCV 229E, and MHV. ND, not determined.

within the C-terminal half of the peplomer, which encompasses the stalk structure and the membrane anchoring domain. The N-terminal amino acid variation in the S-protein has also been detected in MHV and avian infectious bronchitis virus (IBV) (Luytjes *et al.*, 1987; Kusters *et al.*, 1989). In this region (S1) of IBV virus, there are two areas of high amino acid variability (Niesters *et al.* 1986; Cavanagh *et al.*, 1988). No clustering of amino acid changes indicative of highly variable domains were apparent in the S protein of TGEV. The amino acid changes in the S protein of MIL65 and PUR46 strains of TGEV are apparently substitutions that do not affect epitopes involved in *in vitro* TGEV neutralization, since neutralizing MAbs representing five different noncompeting sites were unable to distinguish between these TGEV strains (Wesley, 1990). All these observations on the S protein of TGEV indicate that the peplomer protein is highly conserved among TGEVs.

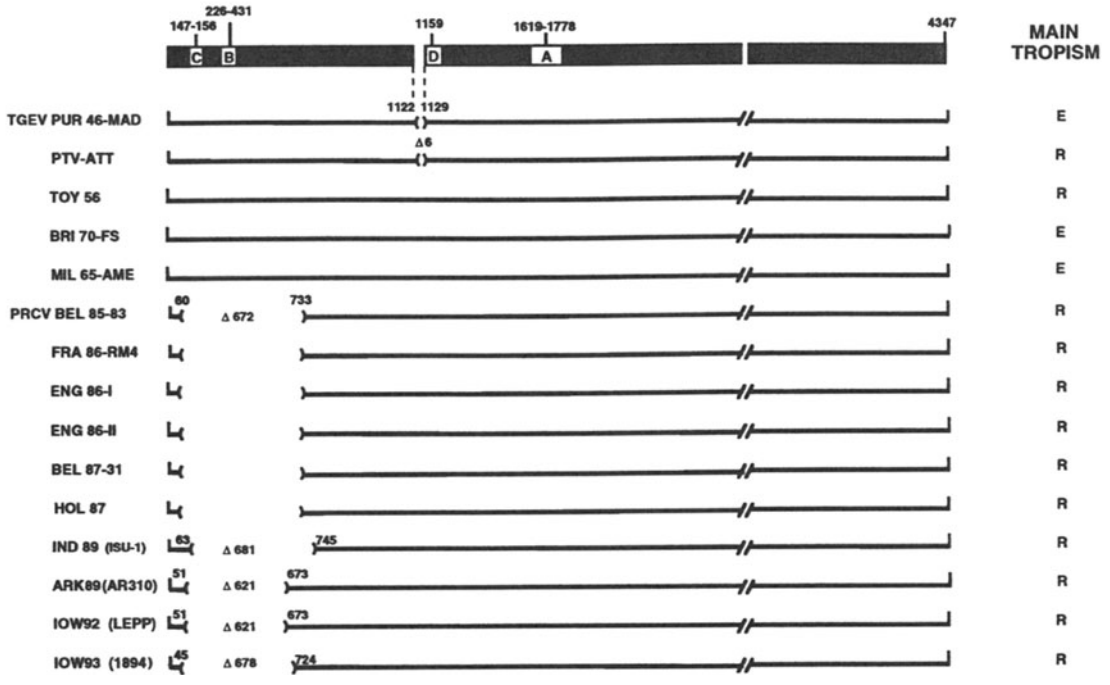


FIGURE 4. Simplified comparison of TGEV and PRCV S genes from European and American isolates. The bar indicates the S gene of PUR46-MAD. Antigenic sites have been identified by letters. Numbers above the bar indicate the location of nucleotides related to the antigenic sites on S protein. The lines represent S genes from other strains. The position of the deletions are indicated by brackets. Numbers above brackets indicate residues flanking the deletion. E and R indicate the predominant tropism of the isolate: enteric or respiratory, respectively. Sequence data were taken from different authors: PUR46-MAD, PTV-ATT (a Purdue type virus, attenuated, formerly named NEB72), TOY56, BEL85-83; ENG86-I, ENG86-II, BEL87-31, and HOL87 (Sánchez *et al.*, 1992); BRI70-FS (Britton and Page, 1990), MIL65-AME (Wesley, 1990); FRA86-RM (Rasschaert *et al.*, 1990); IND89 (Wesley *et al.*, 1991a,b), ARK89, IOW92, IOW93 (Halbur *et al.*, 1993; P. Paul, personal communication).

The genetic relationship among enteric and respiratory isolates of the TGEV cluster has been determined, based on the RNA sequences of the S protein (Sánchez *et al.*, 1992). An evolutionary tree relating these isolates indicated that a main virus lineage evolved from a recent progenitor virus that was circulating around 1941 (Fig. 5). From this, secondary lineages originated, PUR46, TOY56, MIL65, BRI70, and the PRCVs, in this order. Least-squares estimation of the origin of TGEV-related coronaviruses showed a significant constancy in the fixation mutation rate with time, that is, the existence of a well-defined molecular clock. A mutation fixation rate of $7 \pm 2 \times 10^{-4}$ nucleotide substitutions per site and per year was calculated for TGEV-related viruses. This rate falls into the range reported for other RNA viruses. Sequencing data of the S gene of PRCV virus indicate that there is a high similarity, as compared with TGEV, in the areas encoding antigenic sites A and D. A deletion of 672 nucleotides in the 5′ region, which in TGEV codes for sites C and B, leaves PRCV without these sites (Callebaut *et al.*, 1988; Rasschaert *et al.*, 1990; Sánchez *et al.*, 1990, 1992; Wesley *et al.*, 1990; Britton *et al.*, 1991). PRCV was detected about 40 years later than TGEV in Europe, where it spread very fast. All the European PRCVs have an identical deletion of 224 amino acids in the same position within the amino terminal half of the spike protein, suggesting that they were all derived from the same precursor (Sánchez *et al.*, 1992). In contrast, American PRCVs, which were detected for the first time in 1989 (Wesley *et al.*, 1990), have deletions of different sizes (207 to 227 amino acids) located in slightly different positions (Fig. 4) (Vaughn *et al.*, 1994; Halbur *et al.*, 1992, 1993; P. Paul, personal communication), suggesting that they originated independently.

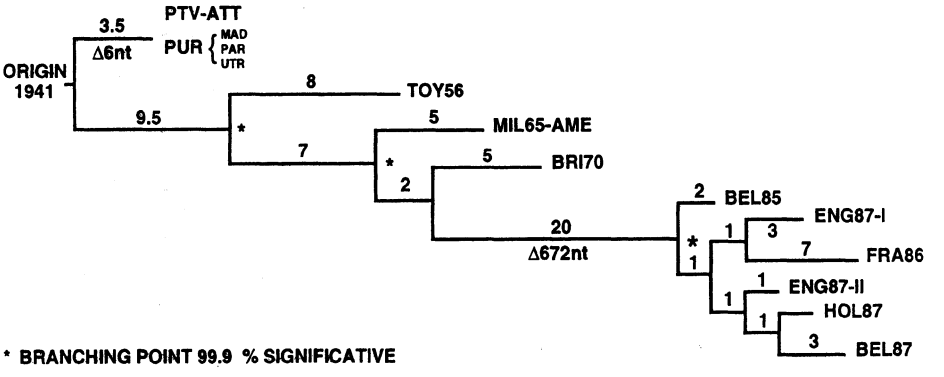


FIGURE 5. Evolutionary tree of TGEV related coronaviruses. Neighbor-joining and least-squares methods of tree reconstruction procedures were applied to the first 1956 nt of 13 virus isolates (Sánchez *et al.*, 1992). Numbers in the diagram indicate residue substitutions between branching points. Δ, Indicates the introduction of a deletion between branching points; *, indicates that all the descendents of this fork have, with a probability of 99.9%, a recent common ancestor.

A comparison of the primary structure of the peplomer proteins of TGEV and FIPV and of the 3′ end of their genome indicates that they are closely related, both in their structural and nonstructural proteins (de Groot *et al.*, 1987; Jacobs *et al.*, 1987). Sequence analysis of the S protein genes revealed one domain, from amino acids 1 to 274, in which the nucleotide sequences were 39% similar, whereas in the second domain, from residues 275 to 1447, the identity was 93%. Comparison of the 3′ ends of the FIPV and TGEV genomes revealed that the highest sequence similarity (98.5%) is in the 3′-noncoding sequences. Large insertions and deletions in the FIPV and TGEV genomes were detected that could be the result of RNA recombination events (de Groot *et al.*, 1988; Vennema *et al.*, 1992). The amino acid sequence of the CCV S protein has 91.1%, 81.6%, and 24% identity with FIPV, TGEV, and MHV, indicating a closer relationship with FIPV. This conclusion is reversed, however, if the M and N proteins are compared (Horsburgh *et al.*, 1992). The CCV S protein has 1452 amino acids, slightly larger that the 1447 or 1449 of TGEVs.

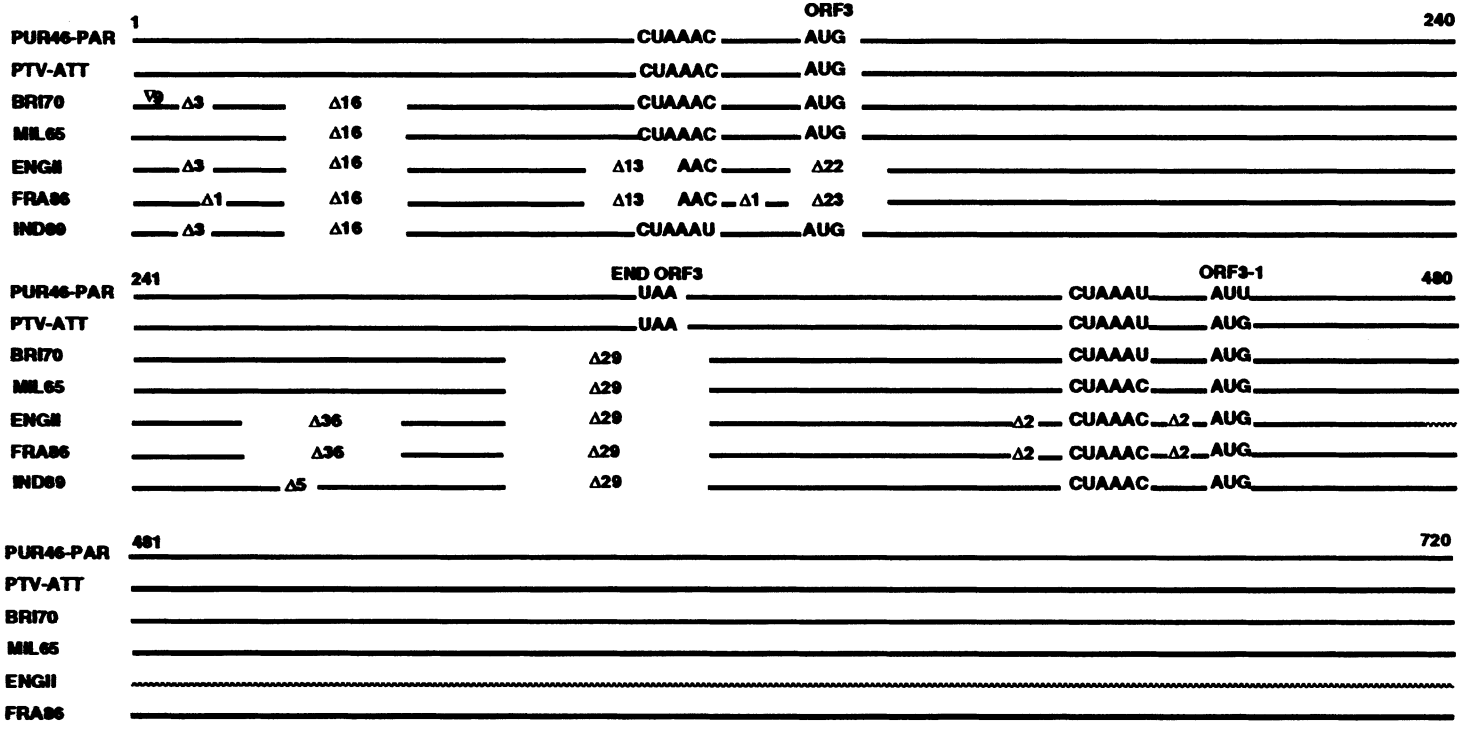
The identity between the M proteins of two TGEV strains (ENG70 and PUR46) is 98% at the nucleotide and amino acid level (Britton *et al.*, 1988a). There are 11 or 12 amino acid substitutions between the British and the PUR46 strain, as determined by Kapke *et al.* (1987) or by Laude *et al.* (1987), respectively.

In the TGEV N protein, three antigenic domains (A, B, and C) have been defined using MAbs (Martín-Alonso *et al.*, 1992). By studying the binding of these MAbs to virus isolates, it was shown that the three domains were highly conserved in TGEV isolates, but that B domain differs between TGEVs and PRCVs (Sánchez *et al.*, 1990). The amino acid sequences of the N protein genes from the British (ENG70) and the PUR46 strains of TGEV are 97.9% identical, and most of the changes are relatively conservative (Kapke and Brian, 1986; Britton *et al.*, 1988b). The data available on the three structural proteins of TGEV isolates indicate that there is also high conservation among isolates collected from remote areas and analyzed after different passage numbers in cell culture.

The sM protein of TGEV has been recently described (Godet *et al.*, 1992). A comparison of the nucleotide sequences of sM genes from virulent (BRI70, MIL65) or attenuated (PUR46, PTV-ATT) TGEVs and PRCVs (ENGII, FRA86) showed a high degree of conservation (Fig. 6) (C. M. Sánchez, M. L. Ballesteros, and L. Enjuanes, unpublished results).

C. The Nonstructural Proteins

The genes coding for three nonstructural proteins of TGEV (nsp 3, 3-1, and 7) of seven strains of TGEV, including two European PRCVs (ENGII and FRA86) and one American isolate (IND89), have been aligned (Fig. 6). Gene 3 is one of the areas of major genetic variability among TGEVs. There are major differences between the noncoding areas of these genes (Fig. 6) (Wesley *et al.*, 1989, 1990, 1991a,b; Britton *et al.*, 1989, 1991; Rasschaert *et al.*, 1990; Laude *et al.*,


ORF3
1
240
PUR46-PAR
PTV-ATT
BRI70
MIL65
ENGII
FRA86
IND69
CUAAAC
AUG
Vp2
Δ3
Δ16
Δ13
AAC
Δ22
Δ1
Δ23
CUAAAU
241
END ORF3
480
ORF3-1
UAA
Δ29
Δ36
Δ5
Δ2
CUAAAU
AUU
AUG
CUAAAC
481
720

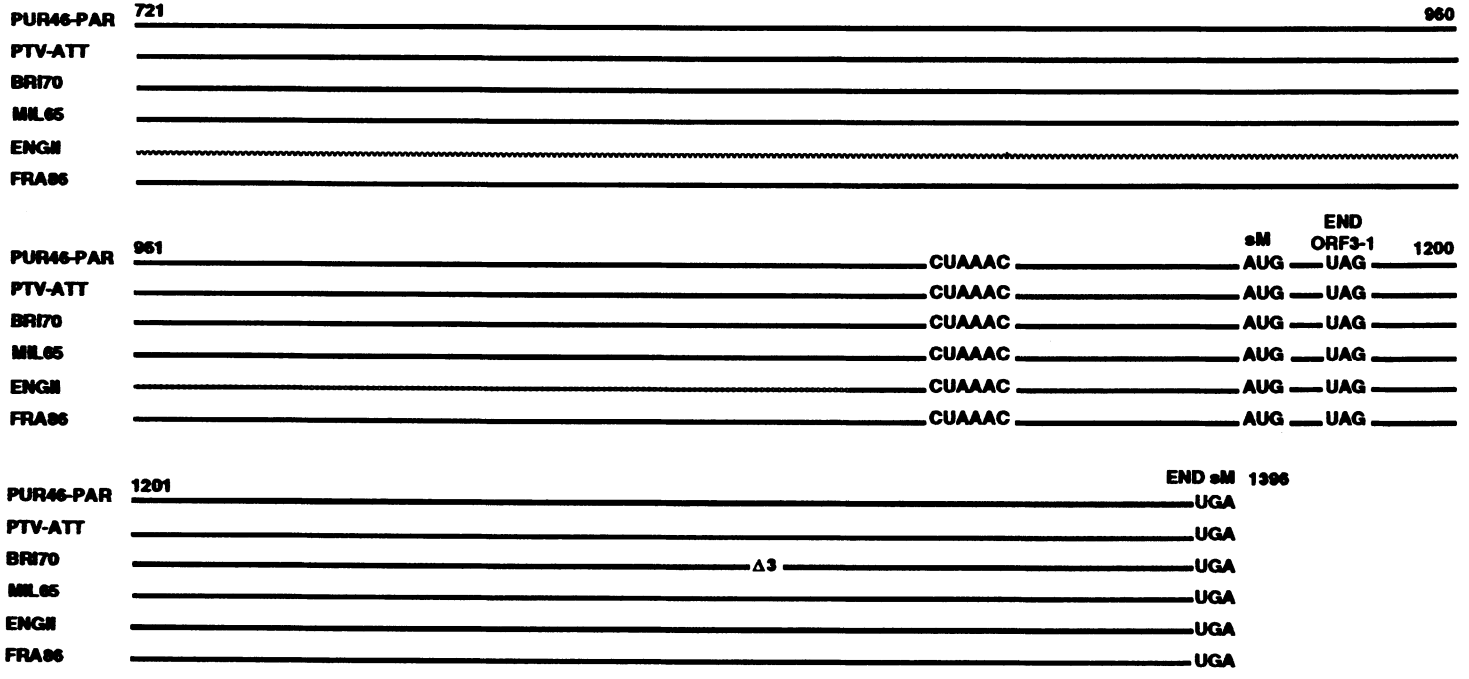


FIGURE 6. Simplified alignment of ORFs 3, 3-1, and sM of enteric and respiratory coronavirus of the TGEV group. Sequences of the indicated ORF were aligned using the Pileup program of Genetics Computer Group (Wisconsin University). Sequence starts after S gene termination codon. The approximate position and sequence of start and stop codons and of the consensus sequence are shown. The number of deleted nucleotides is shown after Δ. Solid and dotted bars represent determined or undetermined sequences. The sequences were obtained from: PUR46-PAR (Rasschaert *et al.*, 1987); PTV-ATT (Purdue type virus, attenuated, formerly named NEB72) (Sánchez *et al.*, 1992); BRI70 (Britton *et al.*, 1991); MIL65 (Wesley *et al.*, 1989); ENGII (Britton *et al.*, 1990b, 1991); FRA86 (Rasschaert *et al.*, 1990); IND89 (Wesley *et al.*, 1991a).

1993; C. M. Sánchez and L. Enjuanes, unpublished results). Between the 3′ end of the S gene and the start of gene 3, there are deletions in six different positions, in the seven viral isolates. In contrast, in ORFs 3-1 and 4 (translated and nontranslated areas), deletions were only observed in two and one position, respectively. The presence of a deletion (ENGII, FRA86) or a mutation (IND89) has altered the ORF 3 consensus sequence CUAAAC in PRCV isolates, preventing its expression. In contrast, ORF 3-1, which is expressed in PRCVs, is not functional in TGEVs isolates: PUR46, PTV-ATT, and BRI70 due to alterations in the consensus sequence. Finally, TGEV MIL65 strain expresses both 3 and 3-1 ORFs. In ORFs 3 and 3-1 amino acid identity was higher than 95%. The lack of expression of ORF3 in PRCVs and ORF3-1 in some TGEVs indicates that they are not essential for *in vitro* or *in vivo* replication. Although, alterations in these ORFs might be involved in the pathogenicity of these isolates.

CCV and FIPV have ORFs equivalent to the TGEV ORFs 3 and 3-1. In addition, CCV has an extra ORF (named 3x) that, to date, has not been detected in this group of viruses and could potentially encode a 71 amino acid protein overlapping with ORFs 3a and 3b of CCV. Due to the context of the ORF, it is very unlikely that it is expressed and probably represents an evolutionary redundant sequence that is no longer required by the virus (Horsburgh *et al.*, 1992). Amino acid identity in ORFs 3 and 3-1, between TGEV and CCV, is high: 83.5% and 92.7%, respectively.

The genomic organization at the 3′ end of TGEV differs from that of CCV and FIPV in that the latter viruses contain additional ORFs (Fig. 1) (de Groot *et al.*, 1988; Vennema *et al.*, 1992; Horsburgh *et al.*, 1992). At the 3′ end, TGEV has ORF 7, CCV has ORFs 7a and 7b, while FIPV has ORFs 6a and 6b. In addition, TGEV ORF 7 has a 69 nt deletion. These nucleotides are present in all FIPV 6a ORFs. The presence rather than absence of ORF 6b appears to be the common theme in FIPV and CCV, strongly suggesting that these nucleotides have been lost in TGEV (Vennema *et al.*, 1992). Paired comparison of the colinear parts of the amino acid sequences of the TGEV and CCV ORF 7, with FIPV ORF 6, have shown an identity of 96% and 77%, respectively (Vennema *et al.*, 1992).

The antigenic and genetic homology among the isolates of the TGEV cluster strongly suggests that they have diverged from a common ancestor. Differences in the antigenic and genomic maps of coronaviruses, including TGEV and FIPV, show that in their divergence certain transcription units have been lost, gained, or translocated (de Groot *et al.*, 1987, 1988; Sánchez *et al.*, 1990, 1992). There are three regions where deletions can occur at a higher frequency: within the S gene, between the S and M genes, and downstream of the N gene (Wesley *et al.*, 1990, Rasschaert *et al.*, 1990; Britton *et al.*, 1991; Sánchez *et al.*, 1992; Horsburgh *et al.*, 1992; Vennema *et al.*, 1992). The relevance of RNA recombination to the evolution of these viruses has been suggested by the occurrence of recombination during TGEV infections (Ballesteros *et al.*, 1994). The porcine, feline, and canine coronaviruses can infect the same cell type in the gut of their natural host species (Reynolds *et al.*, 1980; Stoddart and Scott, 1989; Woods *et al.*, 1981), which would facilitate recombination between them. More specifically, CCV (Woods and Wesley, 1986), FIPV (Woods *et al.*, 1981), and obviously TGEV can infect swine.

IV. CLASSIFICATION OF TGEV AND RELATED CORONAVIRUSES

Based on the antigenic cross-reaction among coronaviruses, four antigenic groups have been defined (Siddell *et al.*, 1982; Sturman and Holmes, 1983; Wege *et al.*, 1982; see also Chapter 1, this volume), two of which include mammalian viruses and the other two, avian viruses. In this grouping several isolates, not all of which are yet recognized as coronavirus species, remain unclassified: namely, hemagglutinating encephalomyelitis virus (HEV) (Mengeling *et al.*, 1972); human enteric necrotic coronavirus (HENCV) (Resta *et al.*, 1985); rabbit coronavirus (RbCV) (Small and Woods, 1987); mink coronavirus [which has been tentatively related to TGEV and PEDV (Have *et al.*, 1992), although these results have not been confirmed], and cheetah coronavirus (ChCV), which is antigenically related to the feline coronaviruses by immunofluorescence (Evermann *et al.*, 1989). The RbCV could be related to TGEV based on *in vivo* protection experiments (Small and Woods, 1987), but there is evidence that this virus may have a gp65 glycoprotein and a proteolytically cleaved peplomer (Descoteaux *et al.*, 1985), both of which are not characteristic for the TGEV group.

TGEV and related viruses can be classified on the basis of their protein composition and antigenic properties. The protein composition of TGEV and related coronaviruses (Table II) shows that these viruses have three families of abundant proteins with molecular weight 160–220 kDa (S protein), 47–56 kDa (N protein), and 22–36 kDa (M protein) (Laude *et al.*, 1986; Jiménez *et al.*, 1986a,b; Cavanagh *et al.*, 1990, 1994). In addition, TGEV has another structural protein, sM (Godet *et al.*, 1992). Some coronaviruses have yet another structural glycoprotein (HE, gp65), which forms dimers through disulfide bridges. These dimers form a second "crown" below the one formed by the S protein and have hemagglutinating activity. TGEV and the related coronaviruses PRCV, FIPV, FECV, and CCV (Sánchez *et al.* 1990) do not have gp65. Other coronaviruses, including rat coronavirus (RCV), human coronavirus (HCV) OC43, HENCV, bovine coronavirus (BCV), and diarrhea virus of infant mice (DVIM) have gp65 as an abundant component. MHV and HEV contain variable amounts of the hemagglutinin esterase protein, depending on the particular isolate or the host cell.

Another characteristic useful for classification of coronaviruses is the cleavage of the spike protein into two halves. The peplomer protein of TGEV and related isolates (PRCV, FIPV, FECV, CCV, and HCV 229E) is not cleaved, in contrast to the antigenically unrelated coronaviruses: turkey coronavirus (TCV), HCV OC43, BCV, MHV, IBV, and, tentatively, HEV. These later viruses have a protease cleavage site on the peplomer protein that is absent on the uncleaved S proteins (Table II and references therein).

The binding of 42 MAbs, which recognized at least 25 epitopes, to enteric isolates of TGEV, collected during a period of 41 years in three distinct geographical areas (America, Europe, and Asia), PRCV isolates, and other coronaviruses, showed a strong antigenic homology among the corresponding viral proteins (S, M, and N) of TGEV, PRCV, FIPV, FECV, and CCV (Fig. 3). In contrast, no cross-reactivity with PEDV, HCV 229E, HCV OC43, HECV, BCV, or MHV

TABLE II. Coronavirus Proteins

Protein	Virus															
	TGEV	PRCV	PEDV	CCV	FECV	FIPV	RbCV	TCV	HCV229E	HCVOC43	HENCV	HEV	BCV	DVIM	MHV	IBV
S	160–220	<220	200	204	180–200	180–200		180	180	190	190	180[c]		180	180	128
S1	—	—		—	—	—	(82)[c]		107[d,e]	110		(100)[c]	120	—	90	90
S2	—	—		—	—	—	(81)[c]	(95)[c]	92[d,e]	90			100	—	90	84
			70[b]				76[b]	75[b]								
							71[b]	72[b]								
HE	—	—	—	—	—	—	(65)[c]	(66)[c]	±[d,e]	65	60	±[d]	65	69	±[d]	—
N	47–56	47–56	58	50	45–50	45–50	54	52	50	55	50	56	52	58	50	50–54
									39[e]							
M	23–33	23–33	27–32	22–32	25–30	25–30	34	27	21–25	26	23	26	26	25	23	23–36
References[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

[a]References: 1. Garwes and Pocock, 1975; Jiménez *et al.*, 1986a,b. An additional protein of 21 kDa has been reported by other authors (Horzinek *et al.*, 1982). 2. Callebaut *et al.*, 1988. 3. Egberink *et al.*, 1988; Knuchel *et al.*, 1992; Utiger *et al.*, 1993. 4. Garwes and Reynolds, 1981; Horzinek *et al.*, 1982. 5. Boyle *et al.*, 1984; Fiscus and Teramoto, 1987b. 6. Evermann *et al.*, 1981; Boyle *et al.*, 1984; Fiscus and Teramoto, 1987a,b. 7. Descoteaux *et al.*, 1985. 8. Dea and Tijssen, 1988. 9. Kemp *et al.*, 1984; Schmidt and Kenny, 1982; Spaan *et al.*, 1988. 10. Hogue and Brian, 1986. 11. Resta *et al.*, 1985. 12. Callebaut and Pensaert, 1980; Pocock and Garwes, 1977. 13. King and Brian, 1982; Hogue *et al.*, 1989; Parker *et al.*, 1989. 14. Sugiyama *et al.*, 1986. 15. Sturman and Holmes, 1977; Sturman, 1977. 16. Cavanagh, 1981.

[b]Possibly degradation products of higher-molecular-weight proteins.

[c]Bracketed numbers were tentatively assigned to a protein family.

[d]Cleavage occurs only at low rate or varies depending on the virus strain and its host cell (Spaan, 1990; Sturman *et al.*, 1985; Sugiyama *et al.*, 1986).

[e]Variable. Not confirmed.

was detected (Sánchez *et al.*, 1990). Interestingly, all TGEV, PRCV, FIPV, FECV, and CCV shared the antigenic subsite Ac, defined by three MAbs that neutralized all these isolates (Sánchez *et al.*, 1990). The presence of the antigenic subsite Ac in a coronavirus could be taken as the basis to define an antigenic cluster, which groups TGEV, PRCV, FIPV, FECV, and CCV with all members having: (1) common epitopes in the three structural proteins; (2) no conventional cleavage site in the peplomer protein; and (3) no gp65 glycoprotein.

HCV 229E was classified into the TGEV serological group (Pedersen *et al.*, 1978) based on the binding of polyvalent antibodies, as determined by immunofluorescence. In contrast, no reactivity was detected by radioimmunoassay (RIA) or ELISA (Sánchez *et al.*, 1990), with a panel of 42 MAbs (Correa *et al.*, 1988) or with a second collection of independently derived MAbs (Laude *et al.*, 1986; P. Talbot, personal communication). Also, the binding of TGEV- or HCV 229E-specific polyvalent antisera adsorbed with noninfected cells to these viruses in a RIA was absent or slight (Sánchez *et al.*, 1990). Reynolds *et al.* (1980) did not detect neutralizing activity for CCV or TGEV in the antisera specific to the 229E strain of human coronavirus. This suggests that the cross-reactivity observed by Pedersen *et al.* (1978) could be due to nonstructural antigenic determinants (Garwes, 1982). No cross-hybridization between the HCV 229E RNA and a cDNA probe complementary to the N and M genes of TGEV has been reported, while a strong hybridization was observed with TGEV, FIPV, and CCV (Shockley *et al.*, 1987).

Sequence data, in contrast to antigenicity and cross-hybridization data, have shown significant sequence homology in three genes coding for the main structural proteins of HCV 229E, PEDV, and other TGEV-related viruses. On the basis of S gene sequences, there is a closer relationship of PEDV with the TGEV-related subset than with the MHV-related subset and IBV (Duarte and Laude, 1994), confirming the reported sequence homology between these viruses in their N and M genes (Raabe and Siddell, 1989; Schreiber *et al.*, 1989; Bridgen *et al.*, 1993; Duarte *et al.*, 1994). A multiple alignment of the S protein sequences of PEDV, TGEV, FIPV, CCV, and HCV 229E has been constructed (Duarte and Laude, 1994), and a significant sequence relationship between PEDV and HCV 229E was found, with level of identity of 60% (S2 region) and 37% (S1 region). The percentage identity of the PEDV N protein with these coronaviruses ranged from 12 to 19% with MHV, IBV, HCV OC43, and BCV and from 32 to 37% with FIPV, CCV, PRCV, TGEV, and HCV 229E (Bridgen *et al.*, 1993). A comparison of the amino sequences of the M proteins showed that the HCV 229E M protein has a higher sequence similarity to the homologous protein of TGEV (HCV/TGEV, 68%; HCV/MHV, 58%; HCV/BCV, 57%; and HCV/IBV, 52%) (Raabe and Siddell, 1989).

Some cross-reactivity, as detected by immunoblotting, has been reported among the N proteins of various coronaviruses previously considered antigenically unrelated: FIPV, PEDV, and HEV (Yaling *et al.*, 1988); TGEV, HCV 229E, MHV 3, and HEV (Yassen and Johnson-Lussenburg, 1978); and MHV-A59 and IBV-M41 (H. G. M. Niesters, unpublished observations). More work is needed to determine whether all coronaviruses are antigenically related at the N-protein level.

In spite of the sequence homology between PEDV and viruses related to

TGEV, no antigenic cross-reaction has been detected using both polyvalent or MAbs with this group of viruses (Pensaert and De Bouck, 1978; Garwes and Reynolds, 1981; Callebaut *et al.*, 1988; Enjuanes *et al.*, 1990; C. M. Sánchez and L. Enjuanes, unpublished results). Also, no antigenic relationship was detected between TGEV and HEV. These results are interesting, in the case of PEDV, as it causes a disease similar to the one produced by TGEV. In summary, using sequencing data there is a cluster of viruses formed by TGEV, PRCV, CCV, FIPV, FECV, PEDV, and HCV 229E. Using serological criteria, an antigenic cluster including TGEV, PRCV, CCV, FIPV, and FECV is differentiated.

V. DIAGNOSIS OF TGEV, PRCV, AND RELATED CORONAVIRUSES

A rapid diagnosis of TGE is important to discriminate it from enteritis caused by enteropathogenic *E. coli*, in order to determine if treatment with antibiotics is required. Several techniques have been developed for TGEV diagnosis: immunofluorescence, reversed passive hemagglutination, ELISA, RIA, and hybridization with DNA probes. TGEV antigens can be specifically detected by immunofluorescence. Coronaviruses of the TGEV cluster can be distinguished with type-, group- and interspecies-specific MAbs (Sánchez *et al.*, 1990). Reversed passive hemagglutination, based on the agglutination of erythrocytes coated with TGEV-specific antibodies, is more sensitive than immunofluorescent staining of primary cultures of porcine kidney cells inoculated with the specimens (Asagi *et al.*, 1986). It is also simple and rapid.

PRCV induces a serological response that originally could not be distinguished from that of TGEV-infected swine. This was a considerable drawback, since the movement of pigs between countries is frequently restricted until evidence that the stock is specifically free from TGEV infection has been obtained. This requires a diagnostic procedure capable of differentiating the two viruses. For this purpose, competitive RIA (Sánchez *et al.*, 1990) and ELISA (Callebaut *et al.*. 1989) with type- and group-specific MAbs have been developed. These assays are sensitive tests for the detection of epitope-specific antibodies, and provide no false-positive results. As a small percentage of false-negative results may occur with this test, a negative result must be confirmed, using several serum samples from the same farm. A cDNA clone containing 396 base pairs (bp) from the 5′ end of the TGEV S gene of MIL65 virus has been used to differentiate TGEV from PRCV. The probe also hybridizes to CCV but not to FIPV (Wesley *et al.*, 1991b).

To differentiate coronaviruses from other agents that cause similar disease and to determine the mechanism by which coronaviruses perpetuate enzootic or epizootic outbreaks, cloned cDNA probes, representing 2 kb from the TGEV genome, have been used in dot blot hybridization assays to detect viral RNA from cell culture and from fecal specimens. The cloned sequence encompasses the 3′-noncoding region, the nucleocapsid protein gene, and a large portion of the membrane protein gene. ^{32}P-labeled cDNA probes prepared from these clones detected as little as 23 pg of homologous RNA, but did not detect RNA

from the nonrelated virus, even when amounts of up to 10 ng per dot were used. In cell culture fluids, these probes detected TGEV, FIPV, and CCV, but not HCV 229E, HCV OC43, BCV, HEV, and MHV A59 (Shockley *et al.*, 1987).

To differentiate PEDV from TGEV and other porcine coronaviruses, an ELISA blocking test, based on crude virus preparations (Callebaut *et al.*, 1982), or an ELISA test that uses purified PEDV adapted to grow on Vero cells (Hofmann and Wyler, 1990) have been developed.

VI. IMMUNE PROTECTION

The main economic losses caused by TGEV result from high mortality rates in newborn piglets, under the age of 10 days. These piglets do not have a fully mature immune system, and the time is too short to elicit a protective immune response. Passive immunity from colostrum and postcolostral milk is crucial in providing protection to neonates against TGEV infection (Abou-Youssef and Ristic, 1975; Bohl and Saif, 1975). Key experiments (Stone *et al.*, 1977; Wesley *et al.*, 1988; De Diego *et al.*, 1992) showed that both the IgG and sIgA immunoglobulin fractions of colostrum and postcolostral milk from immune sows confer protection when fed to susceptible piglets. Thus, newborn animals can be protected by natural lactogenic immunity provided by sows or by artificial lactogenic immunity using serum or protective MAbs.

To induce mammary antibodies, the sows have to be immunized 2 weeks before delivery, preferentially in the intestinal tract (Saif and Wesley, 1992). Stimulation of the mucosal humoral immune system against TGEV also can be induced by priming of the bronchus-associated lymphoid tissue (Cox *et al.*, 1993; De Diego *et al.* 1992; Wesley and Woods, 1993). Immunization in the mammary glands gave variable results. Some authors claimed protection (Bohl *et al.*, 1975; Saif and Bohl, 1983; Woods, 1984), while others (Aynaud *et al.*, 1986) did not find the intramammary route a valid alternative to the oral route. The available data agree on the low efficacy of the intramuscular route (Saif and Wesley, 1992; Moxley and Olson, 1989; Moxley *et al.*, 1989).

Three types of viruses have been used to develop lactogenic immunity: (1) virulent TGEV; (2) attenuated TGEV; and (3) TGEV-related coronaviruses (PRCV, FIPV, and CCV). The oral administration of nonattenuated TGEV in sows has generally resulted in protective levels of immunity for the sow and passive (lactogenic) immunity for suckling pigs (Haelterman, 1965; Bohl *et al.*, 1972; Saif and Wesley, 1992; Moxley and Olson, 1989; De Diego *et al.*, 1992). Because vaccination with nonattenuated TGEV has the obvious risk of spreading pathogenic virus (Bohl, 1982), vaccine research has been focused on attenuated and variant strains of TGEV (Chen and Kahn, 1985; Aynaud *et al.*, 1985, 1988; Fitzgerald *et al.*, 1986; Nguyen *et al.*, 1987; De Diego *et al.*, 1992; Cox *et al.*, 1993, Wesley and Woods, 1993) or on the use of related coronaviruses (Woods, 1984; Hooyberghs *et al.*, 1988). Attenuated TGEV vaccines have only limited efficacy. The problems were inconsistent results in their experimental evaluation, occurrence of epizooties, and persistence of TGEV in vaccinated herds (Saif and Wesley, 1992; Moxley and Olson, 1989).

Comparative studies of virulent and attenuated strains of TGEV revealed that the former are more stable to pancreatic enzymes (Chen, 1985; Furuuchi *et al.*, 1975, 1976), acidity (Chen, 1985; Hess and Bachmann, 1976; Laude, 1981), and porcine intestinal fluids (Chen, 1985). These properties may protect virulent TGEV from inactivation during its passage through the upper part of the gastrointestinal tract and permit viral replication in the small intestine, where it stimulates enteric immunity. A variant of TGEV (derived from the attenuated Purdue strain), resistant to trypsin and α-chymotrypsin, induced lactogenic immunity (Chen and Kahn, 1985). Using a survivor selection process in gastric juice, a mutant of TGEV, selected from a low passage strain (D-52), acquired simultaneous resistance to acidity and pepsin or trypsin cleavage (Aynaud *et al.*, 1985; Nguyen *et al.*, 1987). This mutant induced protective immunity when administered by the oral route but not by the intramuscular or intramammary routes (Aynaud *et al.*, 1988). Molecular characterization of attenuated vaccine strains of TGEV has shown alterations in mRNAs 2 and 3 of these viruses, affecting the spike and the nonstructural protein 3 (Register and Wesley, 1994).

Protection by the TGEV-related coronavirus PRCV has been studied by several groups. Some of them have found no protection (Hooyberghs *et al.*, 1988; Van Nieuwstadt *et al.*, 1989; Paton and Brown, 1990), while others have described a significant level of protection (Bernard *et al.*, 1989; Cox *et al.*, 1993; De Diego *et al.*, 1992; Wesley and Woods, 1993). The poor protection observed by the first group might be due to the lack of or insufficient antigenic stimulation of the gut-associated lymphoid tissue during PRCV infection of the sows. PRCV is known to have a respiratory tropism. An intermediate degree of protection induced by PRCV has been described. Infection of pigs with this virus primes the systemic and mucosal humoral immune system against TGEV, so that subsequent challenge with TGEV results in a secondary antibody response and in a decreased duration of infectious TGEV excretion. Experimental vaccination of seronegative naive gilts with PRCV induced lactogenic immunity against TGEV. The overall survival rate ranged from 47 to 70%, but was variable from sow to sow (Bernard *et al.*, 1989; De Diego *et al.*, 1992; Wesley and Woods, 1993).

These results suggest that there is a link between respiratory infection with PRCV and secreted protective antibody in the mammary glands of postparturient gilts. The level of virus-neutralizing antibody in serum and colostrum that was induced by PRCV vaccination did not correlate with piglet survival (Wesley and Woods, 1993; Bernard *et al.*, 1989). This has also been recognized as a consistent feature of TGEV vaccination and challenge experiments (Saif and Wesley, 1992), which suggests that the immunodominant neutralizing epitopes for PRCV and TGEV are probably not the major contributors to passive protection. Pigs previously infected with PRCV develop a rapid secondary immune response upon infection with field strains of TGEV. The duration of the TGE outbreak is shortened and the loss of piglets is substantially reduced. This situation may open new ways for vaccination against TGEV. These results are consistent with the apparent correlation between dissemination of PRCV and the reduction in incidence of TGEV observed by Jestin *et al.* (1987a,b).

Vaccination against TGE with heterologous coronaviruses such FIPV

(Woods and Pedersen, 1979; Woods, 1984) and CCV (Woods and Wesley, 1986) only provided partial protection against TGEV. The reverse experiments, that is, vaccination to protect against FIPV with heterologous live virus vaccines (TGEV, CCV, and HCV 229E), also did not provide satisfactory results (Barlough *et al.*, 1984, 1985; Toma *et al.*, 1979; Woods and Pedersen, 1979; Scott, 1987).

VII. NEW TRENDS IN VACCINE DEVELOPMENT

An effective vaccine against TGE should protect newborn piglets through lactogenic immunity. At least two approaches could be undertaken: (1) the production of noninfectious antigens that are targeted to the gut, or (2) the use of live vectors with enteric tropism. Both approaches require the definition of the B- and T-cell epitopes involved in protection and the search for molecules promoting IgA responses. In addition, the first approach requires the incorporation of molecules with affinity for enteric gut cells. B-cell epitopes involved in protection are, most frequently, those involved in the induction of neutralizing antibodies, although viral proteins that do not have this activity have been shown to induce protection against herpes simplex virus (Chan *et al.*, 1985), cytomegalovirus (Reddehase *et al.*, 1987), and other viral systems (Whitton *et al.*, 1989; Klavinskis *et al.*, 1989). At least five antigenic sites are involved in the induction of TGEV-neutralizing antibodies: sites A, B, and D, and the sites on the S protein defined by MAbs 5G1 and 5D5 from R. D. Woods' and R. Wesley's laboratory (personal communication). Of these five domains, site A is the major inducer of neutralizing antibodies. Sites A and B are complex, conformational, and glycosylation-dependent. Site D can be represented by synthetic peptides, although glycosylation has a minor effect on its conformation. Site C is continuous and glycosylation-independent. A peptide from site D, which includes residues 379 to 386 (SFFSYGEI) from the S protein of TGEV, induced neutralizing antibodies. This peptide induced antibodies with a higher titer in neutralization when coupled to a second S protein derived peptide, which includes residues 1160 to 1180 (Posthumus *et al.*, 1990a,b). The combination of these peptides could be the first candidate for a subunit vaccine. Unfortunately, the neutralizing epitopes selected in these studies were defined using swine testicle cells and intestinal porcine epithelial cells in culture (C. M. Sánchez and L. Enjuanes, unpublished results), and may differ from the epitopes that induce protection *in vivo*. This is a real possibility, since no correlation has been found between neutralization titer and protection by some authors (Bernard *et al.*, 1989; Saif and Wesley, 1992; Wesley and Woods, 1993).

The T-cell epitopes potentially involved in protection against TGEV are being defined by using virus-specific T-cell hybridomas (Bullido *et al.*1989) and polyclonal T cells. T-cell epitopes have been identified on the three major structural proteins of TGEV: S, M, and N. A dominant T-helper epitope defined in the N protein helps the synthesis of TGEV-neutralizing antibodies specific for the S protein *in vitro* (Antón *et al.*, 1995).

IgA isotype immunoglobulin is more stable in the gut than those of the IgG isotype (Porter and Allen, 1972). Several types of T-cell factors have been impli-

cated in the promotion of IgA responses: interleukin 5 (Harriman *et al.*, 1988; Lebman and Coffman, 1988; Strober and Harriman, 1989), transforming growth factor β (Coffman *et al.*, 1989; Sonoda *et al.*, 1989), and a factor that binds to the Fc portion of the IgA immunoglobulin on the surface of B cells (Mestecky and McGhee, 1987). Genes coding for these factors should be cloned and expressed in combination with viral antigens in order to study their role in the induction of secretory immune responses against TGEV.

Two types of molecules have shown to be effective as carriers of unrelated antigens in the stimulation of gut-associated lymphoid tissue after oral administration: cholera toxin B subunit and the pili of the enterotoxic K88 strain of *E. coli*. Most nonviable antigens are often inefficient in promoting antibody responses. They require large (milligram) quantities of immunogen and yield, if any, modest antibody responses (Czerkinsky *et al.*, 1987, 1989; Mestecky, 1987). A notable exception is cholera toxin (CT), which is a potent immunogen (Pierce, 1978). CT and *E. coli* labile toxin (LT) have been shown to exert potent adjuvant effects in mice on gut-immune responses to unrelated antigens presented orally (Elson and Ealding, 1984; Lycke and Holmgren, 1986; Nedrud *et al.*, 1987). There has been a debate on the requirements of the holotoxin (A and B subunits of CT or LT). The best adjuvant effect is induced by the complete toxin. This adjuvant effect appears to be closely linked to the ADP-ribosylating action of CT and LT, associated with enhanced cyclic AMP formation in the affected cells, and thus it may prove difficult to eliminate the enterotoxic activity without loss of adjuvant activity. However, as an antigen carrier system providing specific binding to epithelium, including the M cells of the intestinal Peyer's patches, both CT and its nontoxic binding subunit moiety have been shown to markedly enhance the mucosal immune response to various foreign antigens or epitopes covalently linked to these molecules (Holmgren *et al.*, 1993). An antigenic determinant representing multimers of site D from the peplomer protein of TGEV has been expressed as a fusion protein on the carboxy-terminus of the LT-B subunit. The recombinant product induced TGEV-neutralizing antibodies (Smerdou *et al.*, 1994). The LT-B-site D antigen was expressed using attenuated forms of *Salmonella typhimurium*, which have a tropism for the Peyer's patches (Curtiss *et al.*, 1986). Double mutants of this recombinant *Salmonella*, defective in the synthesis of cAMP and the cAMP receptor, persisted in the gut lymphoid organs for about 3 weeks and induced TGEV antibodies (Smerdou *et al.*, 1994). Its role in protection is being evaluated. Other attempts to develop genetically engineered vaccines using prokaryotic vectors have failed. Most of the S protein gene has been expressed at high levels in *E. coli*. Subcutaneous immunizations with the recombinant antigen did not induce neutralizing antibodies (Hu *et al.*, 1984, 1987) nor induce protection *in vivo*.

A 23-kDa subunit immunogen obtained from purified TGEV by sonification, isopycnic centrifugation, and gel filtration through Sephadex G200 was administered intramuscularly to gilts prior to farrowing. It protected piglets suckling on the vaccinated gilts (Gough *et al.*, 1983a,b). These results were unexpected, as intramuscular inoculation of inactivated TGEV did not provide protection (Saif and Wesley, 1992) and have not been confirmed.

Expression in eukaryotic vectors is required for those TGEV antigenic determinants that are dependent on glycosylation, such as sites A and B, of the S glycoprotein. Two types of live eukaryotic vectors have been considered to induce protection against TGEV: poxvirus and adenovirus. Porcine poxviruses and adenoviruses induced systemic and secretory IgA responses in pigs (Tuboly *et al.*, 1993). Attempts to immunize against TGEV with TGEV–vaccinia recombinants expressing most of the peplomer protein have resulted in the induction of TGEV-neutralizing antibodies (Hu *et al.*, 1987) but not in protection. Adenoviruses have the advantage of their tropism for gut- and bronchus-associated lymphoid tissue. Also, these viruses have a DNA genome that facilitates their use as vector (Graham and Prevec, 1992; Prevec *et al.*, 1989; Schneider *et al.*, 1989; Lubeck *et al.*, 1989; Bett *et al.*, 1993). Using human adenoviruses-based vectors, the whole spike protein and truncated fragments of this glycoprotein have been expressed. The Ad5–TGEV recombinant induced TGEV-neutralizing antibodies when administered to hamsters (Sánchez *et al.*, 1994). Ad5–TGEV recombinants infected swine. The tropism of these recombinants is being determined using a recombinant Ad5-luc vector (Mittal *et al.*, 1993), expressing luciferase activity. Both respiratory and enteric tissues were infected and the enzymatic activity expressed (J. M. Torres, C. M. Sánchez, F. Graham, and L. Enjuanes, unpublished results). Porcine adenoviruses are being developed as vectors to induce protection against TGEV. The genome of porcine adenovirus type 3 (Seshidar-Reddy *et al.*, 1993) and type 4 (Kleibocker *et al.*, 1993) have been cloned and partially sequenced and deletions on the E3 gene introduced to facilitate the cloning of heterologous genes, including the S gene of TGEV.

Expression of TGEV proteins under the control of the baculovirus polyhedrin promoter or in filamentous fungi (as *Aspergillus nidulans* or the Mucor system) may be an economic way of producing large amounts of glycosylated antigens (Van Brunt, 1986). The S, M, and N proteins of TGEV have been expressed using baculovirus (Britton *et al.*, 1990a; Pulford *et al.*, 1990; Tuboly *et al.*, 1993). The recombinant baculovirus developed by Tuboly *et al.* (1993) contained inserts ranging from 1.6 kb, encoding sites C, B, and D, to 3.3 kb, encoding all the major antigenic sites (C, B, D, and A). Piglets immunized with the recombinants developed a strong antibody response, but only recombinants expressing at least part of antigenic site A induced *in vitro* neutralizing antibodies (T. Tuboly and J. B. Derbyshire, personal communication).

There is an increasing interest in the use of RNA viruses that do not replicate through a DNA intermediate as vectors for the expression of heterologous genes. This is the case of alphaviruses (Schlesinger, 1993; Bredenbeeck *et al.*, 1993; Liljeström and Garoff, 1991). Coronaviruses have a genome of 30 kb, which is too large to be easily manipulated. This has prompted the isolation and characterization of defective subgenomic RNAs, which are easily generated in the murine coronavirus MHV. Identification of the minimum requirements to replicate these subgenomic RNAs (Makino *et al.*, 1988; Makino and Joo, 1993), the packaging signal (Makino *et al.*, 1990; van der Most and Bredenbeek, 1991; Fosmire *et al.*, 1992), and the possibility of engineering cDNAs coding for these RNAs (Koetzner *et al.*, 1992; van der Most *et al.*, 1992; Masters, 1992; Masters *et al.*, 1994) are helping the development of vectors based in coronaviruses.

TGEV subgenomic RNAs have been identified. A cDNA has been derived from a 10-kb defective RNA of TGEV. This cDNA has been cloned and sequenced (Mendez *et al.*, 1994). The subgenomic RNA is packaged into capsids and it can be used to develop a TGEV-based vector to induce secretory immunity in swine (C. Smerdou, A. Mendez, M. L. Ballesteros, C. M. Sánchez, and L. Enjuanes, unpublished results).

Anti-idiotypic antibodies are in theory an interesting source of antigen, especially those mimicking the complex antigenic site A, the major inducer of neutralizing antibodies in TGEV. The use of antibodies as antigen may have the advantage of their adsorption into the gut, particularly in the first days after birth. Induction of neutralizing antibodies to TGEV by porcine anti-idiotypic antibodies generated against a murine-neutralizing MAb has been shown in mice (Hariharan *et al.*, 1989). These antibodies neutralized TGEV *in vitro*, but no protection studies *in vivo* have been reported. Monoclonal anti-idiotypic antibodies of the γ and β (internal-image) type, which induced and Ab3 TGEV-neutralizing, are now being tested *in vivo* for protection (Suñé *et al.*, 1991).

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