Characteristics of mouse monoclonal antibodies to transmissible gastroenteritis virus of swine

by

Xiaoling Zhu

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TABLE OF CONTENTS

INTRODUCTION

Transmissible gastroenteritis (TGE) is an economically important, highly contagious enteric disease in swine characterized by severe diarrhea, vomiting and dehydration. The disease is most severe, frequently fatal, in young animals and less severe in animals over 3 weeks old (Saif and Bohl, 1986). The disease occurs in two forms: epizootic and enzootic. The epizootic form of the disease results in diarrhea and vomiting with a mortality as high as 100% among pigs less than two weeks of age and occurs seasonally in winter months. The enzootic form of the disease occurs in weaned pigs and is characterized by diarrhea with low mortality and low feed efficiency. The enzootic form of the disease occurs all year around and is increasingly becoming a major problem in nurseries. Conservative estimates indicate that TGE costs the U.S. swine industry 25 to 75 million dollars annually (Miller et al., 1982). The causative agent of TGE is a coronavirus refered to as TGE virus (TGEV). Only one serotype of TGEV is recognized (Kemeny, 1976). However, recent evidence indicates that antigenic variation may exist among TGEV (Laude et al., 1986; Pensaert et al., 1981).

The immunity to TGEV infection mainly depends on the presence of neutralizing antibodies within the intestinal tract. Following recovery of the animals from infection or vaccination with inactivated or attentuated virus, neutralizing antibodies can be detected in serum. Presence of neutralizing antibodies in secretions depends on the route of exposure or vaccination. Oral route is considered best for induction of high titers of IgA antibodies. Transfer of neutralizing antibodies, predominantly IgA, in the milk of an immunized dam to her offsprings confers protection against the disease in piglets (Bohl et al., 1972; Saif et al., 1972; Stone et al., 1977).

There are no satisfactory methods for treatment, prevention, and control of TGE. Laboratory diagnosis of the disease is usually based on the detection of viral antigens in epithelial cells of the small intestine by an immunofluorescence technique, microscopic detection of virus in intestinal contents by negative contrast transmission electron microscopy, isolation and identification of the virus, or detection of significant antibody response by a virus neutralization test. The routine diagnostic techniques do not differentiate isolates of TGEV from epizootic and enzootic forms of the disease, or differentiate vaccine strains from field strains. Identification and differentiation of various strains of

TGEV is of importance in epidemiologic studies. There is need for improved diagnostic tests for the identification and differentiation of TGEV.

TGEV belongs to the genus Coronavirus of the family Coronaviridae. The virus contains three major proteins revealed by polyacrylamide gel electrophoresis. Glycoprotein gp200(E2) with a molecular weight of 200 kilodaltons is a structural protein of surface projections, peplomers, and is able to induce neutralizing antibodies against TGEV. A protein associated with the viral genome, N protein, has a molecular weight of 50 kilodaltons. Transmembrane glycoprotein gp30(E1) has a molecular weight of 30 kilodaltons.

Thirty-three hybridomas secreting monoclonal antibodies (Mabs) directed against Miller strain of TGEV were developed at the Veterinary Medical Research Institute (Morales, 1984). These Mabs were divided into three groups based on their ability to react with TGEV and canine coronavirus (CCV) in indirect immunofluorescence assay (IFA) and virus neutralization tests. Group A, composed of six Mabs, reacted with TGEV and CCV by IFA but failed to neutralize either of these two viruses. Group B, consisting of eleven Mabs, showed virus neutralization activity for homologous (TGEV) and heterologous (CCV) immunogens. Group C, consisting of sixteen Mabs, detected

specific antigens on TGEV, and had no virus neutralization activity. Among these thirty-three hybridomas, eleven were stable. The objectives of this study were to characterize these eleven Mabs as to their immunoglobulin isotypes, ability to neutralize TGEV, and viral polypeptide specificity and to determine if these Mabs can be used to differentiate TGEV isolates.

LITERATURE REVIEW

Disease and etiology

Studies on TGEV have been carried out since 1946 when the viral etiology of TGE was described by Doyle and Hutchings. Our knowledge of morphological, physiochemical, and biological characteristics of TGEV has increased steadily being contributed by many investigators.

TGE is a highly contagious enteric disease of swine characterized by severe diarrhea, vomiting and dehydration. The pigs of all ages are susceptible to TGEV infection, but effects on newborn piglets are most severe. The mortality rate in newborn pigs frequently approaches 100%. The disease exists in two forms, an epizootic and an enzootic form (Saif and Bohl, 1986). Epizootic TGE occurs in a herd where most if not all of the animals are susceptible. When TGEV is introduced into such a herd, the disease usually spreads rapidly to swine of all ages, especially during winter. Most animals in such a herd develop clinical signs of disease. Lactating sows develop fever, anorexia and have reduced milk production. Suckling pigs become very sick, and develop diarrhea, vomiting, dehydration, and eventually die. Mortality in the pigs under 2-3 weeks of age may be as high as 100%. The epizootic form of the disease may

terminate in a few weeks or may persist in the enzootic form in the herd. The enzootic form of the disease is usually observed among weaned pigs and is a persistent problem in nurseries. However, enzootic TGE is also observed in nursing pigs of 2 weeks of age. Enzootic disease results in high morbidity, generally diarrhea, with low mortality. The enzootic form of the disease causes weight loss and low feed efficiency, and is increasingly becoming a major problem in nurseries.

TGE is an economically important disease. Conservative estimates indicate that TGE costs the U. S. swine industry 25 to 75 million dollars annually (Miller et al., 1982). In areas where there are high concentrations of swine, TGE is considered to be one of the major causes of illness and death in piglets (King, 1981; Miller et al., 1982). Swine producers are especially concerned about this disease for several reasons: i) mortality is high in newborn pigs; ii) there is no effective treatment for TGE; iii) control measures for the disease are frequently unsuccessful, and the available vaccines are of limited efficacy; and iv) many epidemiologic aspects of TGE, including vectors and reservoirs for the virus are poorly understood.

Pigs that have recovered from TGE are usually immune to subsequent challenge, presumably due to local immunity within the intestinal mucosa. Both the age of the animal at

initial infection and severity of the challenge may greatly influence the efficacy and duration of this active immunity (Saif and Bohl, 1986). Circulating antibodies (actively or passively acquired) provide little protection against a subsequent TGEV infection (Harada et al. 1969; Haelterman, 1965; Kodama et al., 1980). Protective antibodies are believed to be secretory IgA (Bay et al., 1953; Bohl et al., 1972; Porter and Allen, 1972; Sprino and Ristic, 1982). Ingestion of live virus which infects the intestinal tract and stimulates submucosal immunocompetent cells, has been considered to be essential for subsequent secretion of IgA (Bohl and Saif, 1975). Lactogenic immunity is the immunity provided to nursing piglets through milk of dams, and is of primary importance in providing newborn piglets with immediate protection against TGEV infection. This is accomplished naturally when immune sows allow their pigs to suckle about every 2 hours. TGEV antibodies in colostrum and milk of sows are primarily associated with IgA or IgG (Bohl et al. 1972; Saif et al., 1972). Stone et al. (1977) performed an experiment to ascertain efficacy of isolated colostral IgA, IgG and IgM to protect neonatal pigs against TGEV. They showed that all three classes of Igs in colostrum can protect the piglets from TGE during the first 10 days of life. They fed groups of hysterectomy-derived colostrum-deprived neonatal pigs with each Ig before and

after exposure with virulent TGEV. IgA antibodies to TGEV in milk provide the most effective protection, but IgG antibodies in milk were also protective if high titers could be maintained (Bohl and Saif, 1975). Probable reasons for greater efficacy of IgA anti-TGEV antibodies include: i) the concentration of IgA antibodies in milk is higher than IgG (Porter and Allen, 1972); ii) IgA antibodies are resistant to digestion by proteolytic enzymes (Underdown and Dorrington, 1974); and iii) IgA molecules are able to selectively bind to gut enterocytes (Nagura et al., 1978). IgA antibodies in milk are produced by the lactating sows as a consequence of an intestinal infection, while IgG antibodies are produced as a result of parental or systemic antigenic stimulation. Saif and Bohl (1979) proposed a "gut-mammary" immunogenic axis to explain the occurrence of IgA anti-TGEV antibodies in the milk following an intestinal infection. After antigenic sensitization in the gut, IgA immunocytes migrate to the mammary gland where they localize and secrete IgA antibodies into colostrum and milk.
The mechanism of virus neutralization is not completely understood. Nguyen et al. (1986) demonstrated that secretory IgA from milk and IgG from serum did not inhibit attachment of TGEV to ST cells and pig kidney cells, but they prevented the viruses from penetrating into cells. Moreover, secretory IgA enhanced virus attachment. It was

also found that pre-attached virus was still neutralizable and that IgG and secretory IgA had similar TGEV-neutralizing capacities.

Because of the severity of the disease and lack of suitable treatment, major emphasis has been placed on protection of neonatal swine from TGEV infection. Federally licensed attenuated modified-live virus vaccines are available through four biological companies (Matisheck et al., 1982; Graham, 1980; Henning and Thomas, 1981; Welter, 1980). Vaccines are also available through several intrastate biological companies. Efficacy of many of these vaccines was compared with that of autogenous virulent virus vaccines. In all instances, the autogenous vaccines or virulent TGEV are most efficacious (Moxley and Olson, 1986). Almost all commercially available vaccines produce partial immunity and have been reported to contribute to perpetuation of TGEV in problem herds (Larson et al., 1980). Viral subunits, small plaque variant of TGEV, and heterologous viruses have also been tested experimentally with varying degree of success (Gough et al., 1983; Woods and Pedersen, 1979; Woods and Wesley, 1986; Woods, 1984).

TGE virus can be propagated in either live pigs or cell culture systems. A variety of cell lines can be used for isolation and propagation of TGEV (Saif and Bohl, 1986). Swine testes (ST) cells are believed to be most sensitive

and reliable (Saif and Bohl, 1986) among various cell culture systems including porcine kidney cells, swine testes cells, secondary pig thyroid cells, and primary porcine salivary gland cells. In pigs, the main targets of the virus are the absorptive epithelial cells covering the small intestinal villi (Saif and Bohl, 1986). Virus replicates in the jejunum and duodenum to the highest titer, to a lesser extent in the ileum, and not at all in the stomach or colon (Hooper and Haelterman, 1966). There are also indications that TGEV may be harbored in the respiratory tract. Virus detection and transmission of the disease using lung extracts or pharyngeal swabs from naturally or experimentally TGEV-infected animals have been reported (Underdahl et al., 1974; Kemeny et al., 1976). Laude et al. (1984) reported replication of TGEV in swine alveolar macrophages and development of a distinct cytopathic effect on these cells. The virus infection of macrophages also led to a marked synthesis of type I interferon. It was proposed that alveolar macrophages might act as an extra-intestinal target for TGEV in vivo.

TGEV is ether and chloroform labile, trypsin resistant, and relatively stable in pig bile (Harada et al., 1968). Differences in stability under acidic conditions (pH2.0) were observed between TGEV strains (Aynaud et al., 1985). Field strains are stable at pH 3, but cell culture adapted

strains may be either stable or labile (Hess and Bachmann, 1976). An acid resistant mutant of TGEV has been developed (Aynaud et al., 1986). The complete virions of TGEV have a density in sucrose of about 1.19-1.20 g/ml, a molecular weight of $6\text{-}9\text{x}10^6$ and sedimentation coefficient of 495 S.

Structure of coronavirus

Based on its characteristic morphology, TGEV is classified in the genus coronavirus of the family coronaviridae (Tajima, 1970; Vetterlein and Liebermann, 1970) (Fig. 1). Coronavirus virions are round, pleomorphic, medium-sized particles measuring 60-220 nm in diameter and covered with a distinctive fringe of widely spaced, club-shaped surface projections (McIntosh, 1974). The projections are about 20 nm in length. These particles have been observed to bud from the membranes of the Golgi apparatus or endoplasmic reticulum and to accumulate in cytoplasmic vesicles (Becker et al., 1967). The coronaviridae is a family of at least 11 viruses which infect vertebrates (Siddell et al., 1983). Members of the group are responsible for diseases of clinical and economic importance, in particular, respiratory and gastrointestinal disorders. Coronaviruses were originally recognized as a separate group on the basis of the distinctive morphology in

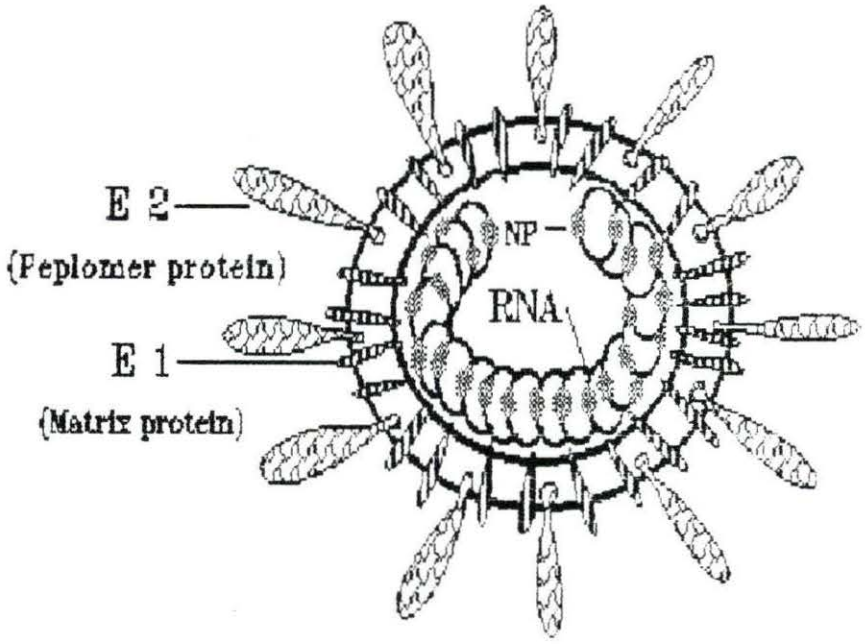

Fig. 1. Schematic drawing of transmissible gastroenteritis virus showing location of viral RNA and protein

negatively stained preparations (Tyrrell et al., 1968), but the group can now be defined by biological and molecular criteria.

The coronavirus genome is a linear molecule of single stranded RNA which is polyadenylated and infectious (Siddell et al., 1983; Brian et al., 1980). The RNA genome has a molecular weight of $5x10^6$ to $7x10^6$ daltons, corresponding to about 15,000 to 20,000 nucleotides. T1-resistant oligonucleotide fingerprinting of genome RNA and intracellular viral messenger RNA confirms the positive polarity of the genome and indicates that it does not have extensive sequence reiteration (Brian et al., 1980).

Many molecules of a basic phosphoprotein encapsulate the genomic RNA to form a long flexible nucleocapsid with a helical symmetry (Caul et al., 1979; Macnaughton, 1978; Stohlman and Lai, 1979; Sturman and Holmes, 1977). This phosphoprotein is designated as N protein and has a molecular weight of 50-60 kilodaltons. Depending on the plane of sectioning, these helical nucleocapsids appear as doughnuts or tubular strands 9 to 11 nm in diameter in thin sections of virions. The nucleocapsid lies within a lipoprotein envelope. The envelope consists of a lipid bilayer with two viral glycoproteins, E1 and E2 (Garwes and Pocock, 1975; Sturman et al., 1977; Sturman, 1980). The ratio of structural proteins in the virion is 8N:16E1:1E2

(Sturman, 1980). The E1 glycoprotein with a molecular weight of 20 to 30 kilodaltons, is a transmembrane protein that differs from the glycoproteins of other virus groups in several important ways (Holmes et al., 1981; Siddell et al., 1983; Sturman and Holmes, 1983). It is more deeply embedded in the envelope, only a small glycosylated amino-terminal region is exposed on the outer surface of the lipid bilayer (Rottier et al., 1984; Sturman, 1980). The amino acid sequence of E1, deduced from the nucleotide sequence of the cloned E1 gene, suggests that the glycoprotein penetrates the lipid bilayer via two hydrophobic domains and a larger domain lies beneath the bilayer (Armstrong et al., 1984; Cavanagh, 1984). Analysis of the functions of coronavirus glycoproteins by differential inhibition of synthesis with tunicamycin indicated that E1 glycoprotein is accumulated in the Golgi apparatus instead of the plasma membrane (Holmes et al., 1981). This observation explains why coronaviruses bud from the Golgi apparatus instead of from the plasma membrane. The glycosylated region of E1 protein is exterior to the virion envelope and the nonglycosylated region is within the envelope, as pronase treatment removes a 5K glycosylated portion of E1 (Sturman and Holmes, 1977). Antibodies to E1 can neutralize viral infectivity only in the presence of complement (Collins et al., 1982). The second coronavirus glycoprotein, E2, has a molecular weight

of 180 to 200 kilodaltons (Siddell et al., 1983), and is a major component of peplomers (Garwes and Pocock, 1975; Sturman and Holmes, 1983). Antibodies to E2 can neutralize viral infectivity (Garwes et al., 1978/1979). The presence of E2 on the plasma membrane can render coronavirus infected cells susceptible to cell-mediated cytotoxicity. In many cases, E1 and E2 glycoproteins with different degrees of glycosylation are incorporated into virions (Siddell et al., 1983; Laude et al., 1986). In addition to these characteristic proteins, others which do not appear to fit into any consistent pattern have been described. A 14 kilodalton protein, and glycoproteins of about 60 to 70 kilodaltons described for mouse hepatitis virus (MHV), bovine coronavirus (BCV), and porcine hemagglutination encephalitis virus (HEV) (Siddell et al., 1983). Wesley and Woods (1986) identified a 17 kilodalton polypeptide in TGE virus-infected cells. This 17KD polypeptide is not a surface feature of the virion. This polypeptide is antigenic and reacted significantly in the immunoprecipitation test with serum from pigs at 60 days after infection with purified TGEV.

Relationship between TGEV and other mammalian coronaviruses

Antigenic relationships among the coronaviruses have been studied by molecular and immunological methods. Based on serologic results, mammalian coronaviruses are divided into three serogroups: i) human coronavirus 229E, canine coronavirus (CCV), feline infectious peritonitis virus (FIPV), and TGEV; ii) human coronavirus OC43, rat coronavirus, bovine coronavirus, HEV, and MHV; and iii) porcine epidemic diarrhea virus (Pedersen et al., 1978; Pensaert and DeBouck 1978; Pensaert et al., 1981; Wege et al., 1982; Siddell et al., 1983).

A close antigenic relationship has been demonstrated between TGEV and CCV by immunofluorescence (Pedersen et al., 1978) and cross virus-neutralization tests (Reynolds et al., 1980), by the similarity of the molecular weight of their polypeptides on sodium dodecyl sulfate gels (Garwes and Reynolds, 1981), by electroblotting (Horzinek et al., 1982) and by enzyme-linked immunosorbent assay (Horzinek et al., 1982). Woods and Wesley (1986) compared the immune response in sows given attenuated TGEV and tissue culture-adapted CCV. It was suggested that TGEV and CCV shared at least one common neutralizing determinant that may be involved in protection.

TGEV and FIPV shared the common determinants on the three major virion proteins (Horzinek et al., 1982). TGEV was neutralized by feline anti-FIPV serum, and the reaction was potentiated by complement. This heterologous neutralization involved antibody reacting with E2 and E1 glycoproteins. A radioimmunoprecipitation assay involving disrupted TGEV and feline anti-FIPV antibody showed that three major viral proteins of TGEV were recognized by feline serum. Enzyme immunoassays also showed recognition of FIPV antigens by anti-TGEV serum. Neutralizing antibody against TGEV have been produced after sows were vaccinated with FIPV, although the titer was lower than that obtained from the animals vaccinated with TGEV (Woods and Pedersen, 1979; Woods, 1984).

TGEV is not antigenically related to two other porcine coronaviruses, HEV and porcine epidemic diarrhea virus (PEDV or CV777) (Chasey and Cartwright, 1978; Pensaert and DeBouck, 1978; Pensaert et al., 1981). Presence of only one serotype of TGEV is commonly accepted (Kemeny et al., 1976), however, TGEV variants have been described. Woods (1978) developed and tested a small plaque (sp) variant of TGEV. This strain was derived from a persistently infected swine leukocyte cell line originally infected with virulent TGEV. The sp strain was avirulent for 3-day-old susceptible pigs and pregnant gilts. The sp virus elicited protective

antibody when inoculated into pregnant gilts via oral and intranasal routes, intramammary, or via both of these routes. Aynaud etal. (1985) obtained two TGEV mutants (188-SG and 152-SG) from a low passage virus strain (D-52) by 188 and 152 cycles of stomach juice treatment and multiplication in cell culture. Compared to the high-passage Purdue-115 and the original D-52 strains, these mutants were more stable at pH 2.0, more resistant to pepsin and trypsin, and characterized by a small plaque phenotype. In vivo, the two mutants were avirulent to 4-day-old piglets and sows after oral inoculation. Laude et al. (1986) indicated the occurrence of distinct antigenic differences among TGEV strains by using monoclonal antibodies.

Utilization of monoclonal antibodies in coronavirus studies

Kohler and Milstein (1975) first produced Mabs through hybridoma technology. They fused mouse plasmacytoma cells with normal splenic lymphocytes from a mouse that had been immunized with ovine erythrocytes. The fused plasmacytoma-lymphocyte cells (hybridomas) were grown in a medium that only permitted growth of hybridoma cells. It was found that several hybridomas secreted antibodies that specifically recognized an antigenic determinant (epitope) on ovine erythrocytes. The tremendous significance of these findings

led to the award of a Nobel Prize to Milstein and Kohler in 1984.

Monoclonal antibodies are homogeneous populations of identical antibody molecules. The entire process of producing Mabs involves immunizing the animal, performing the fusion between myeloma cells and splenic cells, screening medium supernatants for antibody activity, cloning antibody-producing hybrids, and growing hybrids for antibody production. The myeloma cell lines originally used to make hybrids produced their own heavy and light chains. The resulting hybrids secreted immunoglobulin molecules that were mixtures of products from spleen cells and myeloma cells' heavy and light chains. Variants of those cell lines have been selected primarily for three characteristics: i) a vigorous rate of growth in vitro; ii) lack of a purine salvage pathway enzyme, hypoxanthine phosphoribosyl transferase; and iii) loss of ability to secrete immunoglobulins of their own. To induce cell fusion, a virus, specifically a paramyxovirus like Sendai virus, was originally used as a fusing agent. Now, polyethylene glycol is commonly used in hybridoma technology.

The basis for selecting cells that have undergone fusion is the classic hypoxanthine-aminopterin-thymidine (HAT) selection system of Littlefield (1964). The main biosynthetic pathways for purines and pyrimidines can be

blocked by the folic acid antagonist aminopterin. However, the cell can still synthesize DNA via the so-called salvage pathways, in which preformed nucleotides are recycled. These pathways depend on the enzymes thymidine kinase (TK) and hypoxanthine guanine phosphoribosyl transferase (HGPRT). Thus, if the cells are provided with thymidine and hypoxanthine, DNA synthsis can still occur. If one or other enzyme is absent, DNA synthesis ceases. When cells are fused with other cells that supply the genes for the missing enzyme, the hybrids are able to grow in HAT medium, while spleen cells will die in tissue culture simply because they are short-lived cells and myeloma cells will die due to the lack of TK or HGPRT.

Hybridoma cells can be maintained indefinitely in tissue culture or by passage as tumor in syngeneic mice. The fused cells grow as a suspension in the peritoneal cavity of the mice, and the ascitic fluid contains concentrations as high as 10 mg/ml of secreted antibody.

High specificity and availability of mouse Mabs have proven invaluable for expanding biological research in coronavirus. The use of Mabs has facilitated studies on defining biological activities associated with the viral proteins, recognizing antigenic relationship among coronaviruses, and identifying epitope maps on a particular viral strutural protein.

Collins et al. (1982) have developed Mabs with specificities to three viral polypeptides of murine hepatitis virus-4 (strain JHM). Anti-E2 alone had direct neutralizing activity for MHV-4 virus, while in the presence of complement both anti-E2 and anti-E1 Mabs neutralized virus. Only anti-E2 had the ability to inhibit the spread of infection. They proposed that the viral glycoprotein E2 likely contained both attachment and fusion activities of MHV-4. Wege et al. (1984) characterized major antigenic domains associated with functions related to virulence by using a panel of monoclonal antibodies specific for surface peplomer glycoprotein (E2) of MHV JHM strain. Their results indicated that the site responsible for cell fusion was associated with an epitope group carried by gp170 and gp98 which constructed peplomer protein. Vautherot and Laporte (1983) observed antigenic variation between different bovine enteric coronavirus (BECV) isolates. It was also recognized that cross-reactivity between BECV, human respiratory coronavirus (OC43), HEV, human enteric coronavirus and bovine respiratory coronavirus (BRCV) existed. Laude et al. (1986) isolated thirty-two hybridoma cell lines producing monoclonal antibodies against three major structural proteins as well as E2 protein precursor of TGEV. Their study showed that all major neutralization-mediating determinants were found to be carried by the peplomers (E2

protein), but not their precursor. Comparison of nine TGEV strains confirmed their close antigenic relationship, but revealed the occurrence of distinct antigenic differences detected by anti-E2 monoclonal antibodies. Delmas et al. (1986) further explored the antigenic structure of the E2 protein and established the topography of the epitopes by means of competition radioimmunoassay. They found that two major sites (A and B) on E2 glycoprotein were highly conserved among TGEV strains. Fiscus et al. (1985) demonstrated that a competitive ELISA involving the use of enzyme-conjugated monoclonal antibodies to the E1 glycoprotein of FIPV was a simple and rapid method for the detection of antibodies in feline sera. This method was expected to replace a more cumbersome immunofluorescence assay which is most commonly used to aid diagnosis by determining the reactivity of sera to FIP.

MATERIALS AND METHODS

Cell culture

The swine testes (ST) cell line was used to propagate and isolate TGEV (McClurkin and Norman, 1966). ST cells were grown in Eagle's minimal essential medium (MEM) (GIBCO, Grand Island, NY) supplemented with fetal bovine serum (FBS) (10%) (J.R. Scientific, Woodland, CA), lactalbumin enzymatic hydrolysate (LAH) (5 g/liter) (Sigma, St. Louis, MO), sodium bicarbonate (2.9 g/liter) and 1% of antibiotic-antimycotic solution (Penicillin 10,000 units/ml, Streptomycin 10,000 ug/ml and fungizone 25 ug/ml) (GIBCO). ST cells were propagated in 75 cm^2 tissue culture flasks (Costar, Cambridge MA) at a 3-4 day interval between subculturing. ST cells were grown at 37 C in a humid 5% CO_2 atmosphere.

A SP2/O myeloma line was used to subclone hybridomas and prepare conditioned medium (CM) for the growth of hybridomas. Myeloma cells were kindly provided by Dr. Van Deusen (National Veterinary Service Laboratory, U.S.D.A., Ames, IA). The SP2/O cells were grown in regular DMEM medium (GIBCO) that consisted of a high glucose concentration (4500 mg/ml) supplemented with 10% fetal bovine serum (J.R. scientific), gentamicin (50 ug/ml) and L-glutamine (29.9 mg/100 mls). The cells were propagated in 25 cm^2 tissue culture flasks

(Corning, New York) in a humidified incubator with an atmosphere of 5-7% CO_2 in air and a temperature of 37 C. The cells were fed every 2-3 days by placing 1 ml of cell suspension into 9 mls of fresh regular DMEM medium in 25 cm^2 tissue culture flasks. The SP2/O cells were treated with 8-azaguanine every month to eliminate the presence of mutants and maintain susceptibility to aminopterin.
DMEM-8-azaguanine consisted of regular DMEM with 8-azaguanine (2 ug/ml). The conditioned medium was prepared by harvesting medium in which SP2/O cells had been growing for 2 to 3 days. The conditioned medium was harvested by centrifugation at 200 xg for 10 minutes to remove cells and filtered through 0.22 um filter to assure sterility.

Subcloning hybridomas

The hybridomas were subcloned by an end point dilution technique. The primary hybridomas were developed previously at the Veterinary Medical Research Institute (Morales, 1984) and stored in liquid nitrogen. The hybridomas were thawed and propagated. Before cloning, the cells were counted and diluted to a final concentration of 300 cells/ml. The SP2/O cell suspension of $5x10^6$ cells/ml in HAT medium (which consisted of equal volume of DMEM with 20% FBS and CM in addition of HAT was used as cell diluent when hybridomas

were further diluted to 3 cells/ml. The cells were dispensed into each well of a 96-well tissue culture plate (0.2 ml/well), and the plate was incubated at 37 C in a humidified atmosphere and 5-7% CO_2. Beginning on the fourth day after seeding, each well was examined daily for a single growing colony. The supernates from these wells were screened for the production of antibodies by an indirect immunofluorescence assay. The colonies which secreted anti-TGEV Mabs were fed with HAT medium and propagated further by transferring to a larger flask. The cloned hybridomas were frozen in liquid nitrogen and used for producing ascitic fluids enriched in Mabs to be characterized.

Ascitic fluid production

The hybridoma cells secreting desired Mabs were centrifuged and resuspended in a regular DMEM medium without any serum at approximately $2x10^6$ cells/ml. A 0.5 ml cell suspension was injected into the peritoneal cavity of the BALB/c mouse which was primed with pristane at least two weeks before injection. The mouse was observed for abdominal swelling three times weekly. When swelling became pronounced, the ascitic fluids were aspirated with a disposable myelography needle (Becton-Dickinson, Itasca, IL)

and a 5ml syringe. The ascitic fluids were harvested every other day until the mouse died. Each ascitic fluid harvest was clarified by centrifugation in Vacutainer tubes with silicone plug at 200 xg for 10 minutes to maximize yield. The myelography needles were reused after washing them with a protein detergent and sterilizing them with 70% ethanol. Ascitic fluids containing monoclonal antibodies were used for characterization of these monoclonal antibodies. Ascitic fluids were also prepared with SP2/O cells.

Virus propagation

Miller, Illinois, and high passaged Purdue strains of TGEV were used as standard virus strains. Miller strain was obtained from the American Type Culture Collection (Rockville, MD) and was the virus strain used for developing hybridomas and screening production of Mabs. Purdue strain was obtained from Dr. R. D. Woods (National Animal Disease Center, Ames, IA). Illinois strain was obtained from Dr. M. Ristic, University of Illinois, Urbana, IL. Virus stocks were prepared by infecting 5-day-old monolayers of ST cells in 150 cm^2 tissue culture flasks (Corning, New York) at a multiplicity of infection (MOI) of approximately 0.1 plaque forming units (PFU)/cell. After 90 minutes adsorption at 37 C, 20 ml of MEM containing 2% fetal bovine serum (FBS) was

added. At 16 to 24 hours post infection, when about half of the cells had rounded up, detached from the glass and formed syncytia, the cell cultures were frozen and thawed 3 times to harvest virus. The cell debris was removed by low speed centrifugation and the supernates were titrated for TGEV. The virus suspension was dispensed into small aliquots and stored at -70 C.

Virus isolation

TGEV was isolated from small intestines of pigs with TGE. These TGE cases were from Iowa herds submitted to the Veterinary Diagnostic Laboratory of Iowa State University during the period of 1985 to 1986. The intestines were cut into small pieces and ground in a sterile mortar with a small amount of sterile sand. After the tissues were well homogenized, 5 ml of MEM-LAH containing 2% FBS and antibiotics (Penicillin 50,000 units/ml, streptomycin 50,000 ug/ml and fungizone 125 ug/ml) were added to 1 gm of tissue. The sample solution was clarified by centrifugation at 12,000 rpm (Sorval RC-5B, DuPont Instrument) for 30 mins. The supernates were collected and stored at -70 C as the source of TGEV field isolates. When the complete monolayers of ST cells incubated with MEM-LAH containing DEAE-Dextran (50 ug/ml) at 37 C for 30 minutes. After that, cell monolayers

were rinsed with MEM with 2% FBS briefly, and 1 ml of TGEV suspected tissue suspension was added to each flask. The inoculum was allowed to be absorbed for 90-120 mins, then 5 ml of MEM containing 2% FBS was added to each flask. The flask was incubated at 37 C for 48 hours and examined twice a day for the presence of CPE. If CPE occurred, the flask was frozen and thawed for further detection of TGEV by an immunofluorescence (FA) test. The flasks, which did not develop CPE at 48 hours post infection, were frozen at that time, and the tissue cultures were processed in the same way to detect TGEV.

Indirect immunofluorescence assay

An indirect immunofluorescence assay (IFA) was used to screen cell culture supernates for the production of antibodies and to detect TGEV isolates. The ST cells were grown in wells of eight-chamber Lab-Tek tissue culture slides (Miles Scientific, Naperville, IL). When complete cell monolayers were formed, ST cells were infected with TGEV Miller strain at a MOI of approximately 0.1 PFU/cell or 0.1 ml of tissue suspension. At 16-24 hours post infection when CPE developed in a small portion of the ST cell monolayer, or at 48 hours post infection, the cells were fixed with acetone:methanol (4:1) for 10 minutes. After washing with

phosphate buffered saline (PBS), the slides were stained with anti-TGEV antibody or stored at 4 C for later use. For staining, 100 ul of cell culture supernates, dilutions of ascites or serum were added to each well, and the slides were incubated at 37 C in a humid chamber for 30 mins. The slides were washed with PBS 3 times, and 100 ul of optimum dilution of fluoresceinated goat antimouse IgG (heavy and light chain) (Cooper Biomedical, Malvern, PA) was added to each well. The optimum dilution of the conjugate was determined in a preliminary experiment and was the highest dilution that gave positive fluorescence using anti-TGEV Mabs without nonspecific background. The slide was incubated at 37 C for another 30 mins, then washed with PBS 3 times and air dried. After removing the plastic divider, the slide was mounted in 50% glycerol in PBS with a cover slip, and examined under a fluorescence microscope. The uninfected ST cell monolayers, which were processed the same way, were used as negative controls. The antibody titer of each ascitic fluid in IFA was determined by preparing a series of 10-fold dilutions. The last dilution which gave positive fluorescence on TGEV-infected ST cells, without nonspecific background on mock-infected ST cells, was considered the FA titer of that particular Mab.

Competitive immunofluorescence staining

Mab MA4 was conjugated with fluorescein isothiocyanate using a procedure similar to that described by Mengeling et al. (1963). The ascitic fluid containing Mab MA4 was diluted with an equal volume of saline (.85% NaCl), and then precipitated with ammonium sulphate at a final concentration of 50% for 60 minutes at 22 C. The precipitates were collected by centrifugation at 8,000 rpm (10,000 xg) (Sorvall RC-5B, DuPont Instrument) for 20 minutes. The pellets were resuspended in saline to the same volume as the original ascitic fluid. The precipitation was performed two more times. After the third precipitation, the pellets were resuspended in saline to one half the original volume of the ascitic fluid. The resuspended globulin solution was dialyzed against saline overnight at 4 C with changes. The protein concentration was determined by protein assay based on Bradford method (Bradford, 1976). Bovine serum albumin (BSA) was used as standard protein. The globulin solution was standardized to obtain 10 mg protein/ml of solution using carbonate-bicarbonate buffer (pH 9.0) and saline as diluent. Fresh fluorescein isothiocynate (FITC) (0.5 mg FITC per mg of protein) was combined with globulin solution (pH 9.0) in an ice bath and the conjugation was completed by stirring overnight at 4 C. The free fluorescein was separated from the fluorescein-protein complex with a Bio-Gel P-6 column.

The column was prepared as follows: 21 g Bio-Gel P-6 was hydrated in 300 ml of PBS overnight at 22 C with constant shaking. After 24 hours, the slurry was packed into a 400 mm x 20 mm column containing a coarse fritted glass disc in its base. The conjugated protein was loaded on to the column and the eluate containing the first peak of yellow colored conjugate collected. The unbound dye migrated slowly and was retained in the column. The conjugate was absorbed with rabbit liver powder (RLP) (30 mg RLP per total mg protein). After overnight adsorption, the mixture was clarified by centrifugation first at 8,000 rpm (Sorvall RC-5B, DuPont) for 20 minutes and then at 30,000 rpm for 60 minutes. The supernate was dispensed in small aliquots, frozen and used as a final conjugate.

The competitive immunofluorescence assay was performed as follows: MA4 conjugate was mixed with a series of dilutions of homologous or heterologous Mabs. The mixture was used to stain TGEV-infected ST cell monolayers to determine if immunofluorescence was blocked. If the competing Mab shared the same epitope with MA4 Mab, the immunofluorescence would be blocked by the competing Mab; if MA4 recognized a different epitope than that recognized by the competing Mab, the immunofluorescence would not be blocked. Blocking of immunofluorescence would indicate that either the competing Mab shares the same epitope as MA4 or is

topographically closely located and blocks the immunofluorescence due to steric hindrance. No blocking would indicate that the competing Mab is directed to a different epitope.

Virus neutralization test

The virus neutralizing capability of the Mabs was quantitated by the plaque reduction method (Saif and Bohl, 1979). The ascitic fluids containing Mabs were heat-inactivated at 56 C for 30 minutes before testing. Serial twofold dilutions of the ascitic fluids were mixed with an equal volume of Miller strain of TGEV. A dilution and volume of virus suspension was chosen, which would yield 200 PFU (as determined by the plaque assay). The ascites-virus mixture was held at 37 C for 60 minutes, then the unneutralized virus was titrated by the plaque assay method. The neutralization titer of each Mab was expressed as the reciprocal of the last dilution which resulted in 80% reduction in the number of plaques compared to the virus-medium mixture. Porcine anti-TGEV hyperimmune serum and preimmune serum were used as positive and negative controls respectively to ensure specificity. The virus neutralization activity of each Mab was also tested in the presence of complement by adding 20 hemolytic units of guinea pig complement (GIBCO) to the ascites-virus mixture.

Radioimmunoprecipitation assay

A radioimmunoprecipitation assay (RIP) was used to identify viral proteins which Mabs were directed against. ST cell monolayers were infected with the Miller strain of TGEV at a high MOI of 100 PFU/cell. At 7.5 hours postinfection, the medium was replaced with methionine-free MEM. From 8 to 12 hours after infection, the TGEV-infected ST cells were incubated with ^{35}S-methionine (100 uCi/ml) containing MEM. Cell lysates of TGEV-infected or mock-infected cells were prepared in lysis buffer (50 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% Triton X-100, and 1 mM PMSF). One ml of cell lysates were prepared from one 75 cm^2 flask. The cell lysates were preabsorbed with SP2/0 ascites and protein-A sepharose 4B before performing the assay. The lysates (50 ul) were mixed with 10 ul of ascitic fluid and incubated overnight at 4 C. Immune complexes were collected by addition of protein-A sepharose 4B beads and incubated at room temperature for 60 mins. After protein-A beads were washed 3 times with lysis buffer and 3 times with deionized distilled water, then resuspended in 50 ul of sample buffer (0.125 M tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and placed in boiling water bath for 5 minutes. The samples were electrophoresed at 30 mA constant current through a discontinuous SDS-polyacrylamide gel (10%) crosslinked with bis-acrylamide and initiated by TEMED and ammonium persulfate

(Laemmli, 1970). The ionic strength of acrylamide gel and running buffer were 0.375 M tris-HCl (pH 8.8) and 0.025 M tris-HCl (pH 8.3) respectively. Immune reactions were detected on autoradiographs prepared by exposing the dried gels to Kodak XAR-5 film and intensifying screen at -70 C for 48 hours. Porcine anti-TGEV hyperimmune serum and preimmune serum as well as mock-infected ST cells were used as controls. High and low molecular weight standard proteins (Bio-Rad) were used as reference standars to determine size of viral proteins.

Immunoglobulin isotype determination

The immunoglobulin isotype of Mabs was determined by an agarose gel diffusion test using subclass specific antisera (Miles Lab, Elkart, IN). Hybridoma cell culture supernates were concentrated 10 times by precipitation with ammonium sulphate at 50% saturation and used in this test.

RESULTS

Eleven out of thirty-three hybridomas were shown to be stable and secreting Mabs against TGEV as tested by IFA. These eleven hybridomas were subcloned and propagated. The cell culture supernates and ascitic fluids of these Mabs were collected as the source of Mabs for characterization.

Reactivities of Mabs

All of the 11 Mabs reacted specifically in an IFA test with ST cells infected with the Miller strain of TGEV (Fig. 2 and Fig. 3). No reactivity was observed with mock infected ST cell monolayers (Fig. 4). The immunofluorescence staining patterns varied with different Mabs. Immunofluorescence appeared evenly distributed in the cytoplasm with Mabs MD9, ME5, MG5, MC6, and MF2 (Fig. 2). A surface membrane immunofluorescence staining was also observed with these Mabs (Fig. 2). Immunofluorescence with MB2, MH11, MA5, MA4, MG7 and ME9 appeared as fine granulation essentially limited to the perinuclear area (Fig. 3). The FA titers of ascites in indirect immunofluorescence were expressed as the last dilution of ascitic fluids which stained TGEV-infected ST

Fig. 2. TGEV-infected ST cells were stained with Mab MG5, directed against E1 viral protein. Note diffuse distribution of immunofluorescence in the cytoplasm as well as surface membrane immunofluorescence staining of large balloon shaped cells (arrow). Mag x300

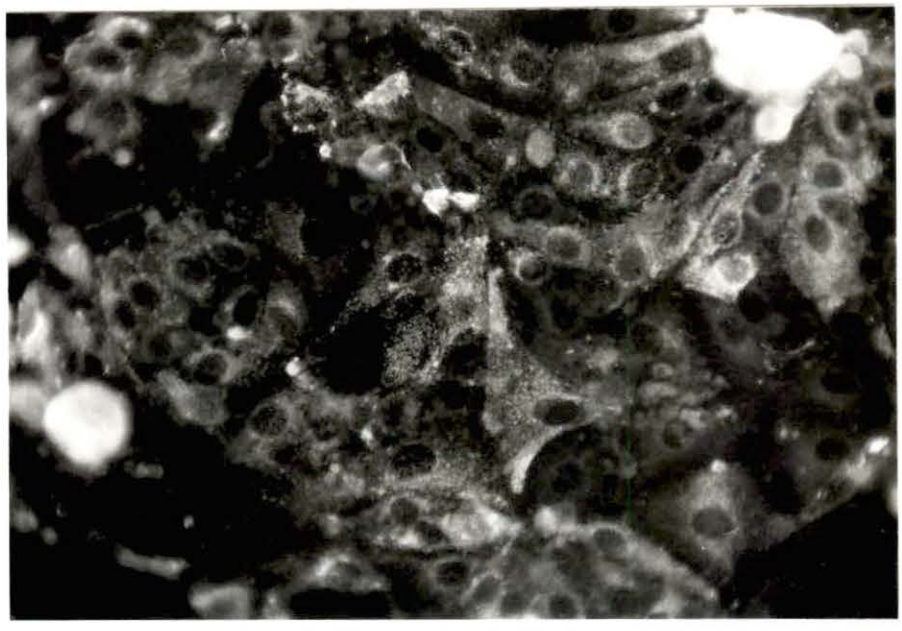

Fig. 3. TGEV-infected ST cells stained with Mab MA4, directed against E2 viral protein. Immunofluorescence appears as diffuse cytoplasmic granular fluorescence as well as fine granulation in the perinuclear area. Mag x580

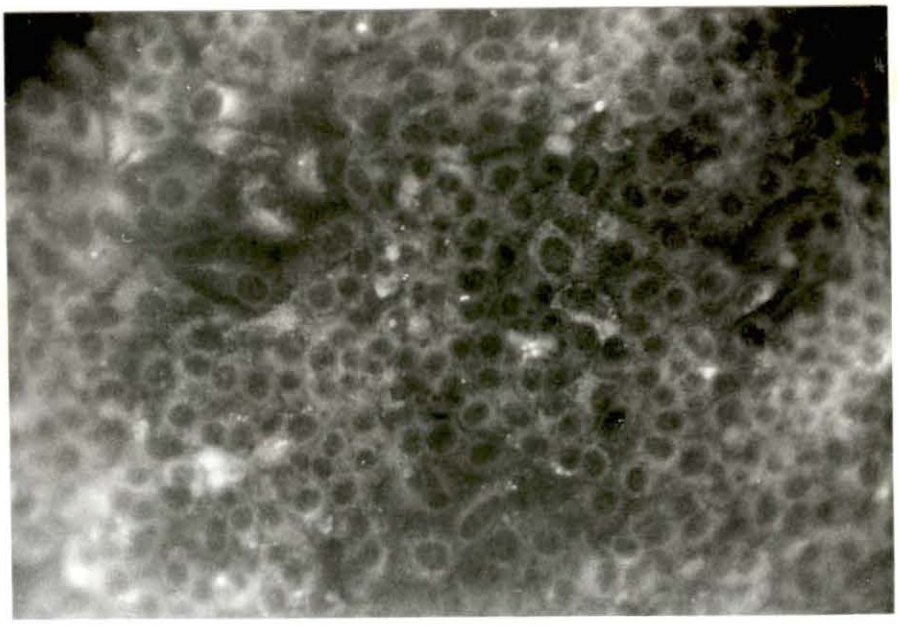

Fig. 4. Mock-infected ST cells stained with Mab MA4. No specific immunofluorescence was observed. Mag x580

cell monolayers brightly without producing any immunofluorescence on mock-infected ST cell monolayers. The IFA titer for Mabs varied from 10^3 to 10^7 and is shown in Table 1.

Virus neutralization activity

Virus neutralization capabilitiy of eleven Mabs was tested in the presence or absence of guinea pig complement. Results of neutralization tests showed that four out of eleven Mabs had high neutralization titers (range 16,000 to 64,000) when titrated against the Miller strain of TGEV. The presence of guinea pig complement did not change their neutralization titers. The other seven Mabs did not show significant neutralization titers (less than 40), even in the presence of guinea pig complement (Table 1).

Viral polypeptide specificity

Porcine anti-TGEV hyperimmune serum precipitated three major viral proteins from ^{35}S-methionine labelled lysates from TGEV-infected cells on PAGE. Additional proteins appeared repeatedly with a lower molecular weight than the E1 and E2 glycoproteins, and they are believed to be the precursors of E1 and E2 glycoproteins (Fig. 5). The results

Table 1. Virus neutralization titer and IFA titer of mouse anti-TGEV Mabs

Mabs	V. N. Titer[a] without C'	V. N. Titer[a] with C'	IFA Titer[b]
MD9	<40	<40	$1x10^3$
ME5	<40	<40	$1x10^3$
MG5	<40	<40	$1x10^4$
MC6	<40	<40	$1x10^4$
MF2	<40	<40	$1x10^4$
ME9	<40	<40	$1x10^3$
MG7	<40	<40	$1x10^4$
MB2	$16x10^3$	$16x10^3$	$1x10^4$
MH11	$32x10^3$	$32x10^3$	$1x10^6$
MA5	$32x10^3$	$32x10^3$	$1x10^5$
MA4	$32x10^3$	$32x10^3$	$1x10^7$

[a]Last dilution of ascites which neutralized 200 PFU of Miller strain of TGEV in 0.2 ml of MEM.

[b]Last dilution of ascites which gave immunofluorescence on TGEV-infected ST cells and no immunofluorescence on mock-infected ST cells.

of reactivity of Mabs with viral polypeptides is shown in Table 2 and Figs 5-7. Mabs reacted with E1 or E2 protein of TGEV. MA4 and MA5 reacted with E2 AND E2 precursor proteins (Fig. 5). None of the Mabs reacted with N protein. No reactivity was observed with mock-infected ST cells.

Epitope specificity

Epitope specificity of Mabs toward TGEV was tested by competitive immunofluorescence assay staining of TGEV-infected ST cells with fluorescein-conjugated MA4 mixed with series of dilutions of Mabs. Immunofluorescence staining of TGEV-infected cells by fluorescein-conjugated MA4 was blocked by Mabs MA4, MB2 and MA5, but not by MH11, ME9, and MG7 (Table 2). The Mabs, MA4, MB2 and MA5, appeared to share the same epitope on E2 protein and was dominant epitope as 3 of the 6 anti-E2 Mabs were directed to this epitope. This epitope was referred to E2.1. The epitope recognized by MH11 was a distinct neutralization epitope and was referred to as E2.2. Mabs ME9 and MG7 were nonneutralizing, were directed to E2 and the epitope recognized by these Mabs was referred to as E2.3.

Table 2. Some characteristics of mouse anti-TGEV Mabs

Mabs	Isotype	Viral Protein Specificity[a]	Epitope Specificity[b]
MD9	IgG3	E1	N.D.
ME5	IgG3	E1	N.D.
MG5	IgG1	E1	N.D.
MC6	IgG3	E1	N.D.
MF2	IgG3	E1	N.D.
ME9	N.D.	E2	E2.3
MG7	N.D.	E2	E2.3
MB2	IgG2a	E2	E2.1
MH11	IgG2a	E2	E2.2
MA5	IgG2a	E2	E2.1
MA4	IgG2a	E2	E2.1

[a]Tested by radioimmunoprecipitation.

[b]Tested by blocking of immunofluorescence staining of TGEV-infected cells with fluorescein- conjugated MA4 Mab.

Fig. 5. Autoradiogram of SDS-PAGE showed radioimmuneprecipitation of ^{35}S-methionine-labelled TGEV-infected (lane 2, 6, 8, 9, 11) and mock-infected (lane 1, 3, 4, 5, 7, 10) ST cell lysates by hyperimmune porcine anti-TGEV serum (lane 1 and 2), preimmune serum (lane 3 and 4), and Mab, ME5 (lane 5 and 6), MA5 (lane 7 and 8), MA4 (lane 9), and MF2 (lane 10 and 11). Note hyperimmune serum precipitated at least three proteins whereas Mabs precipitated E1 or E2 proteins

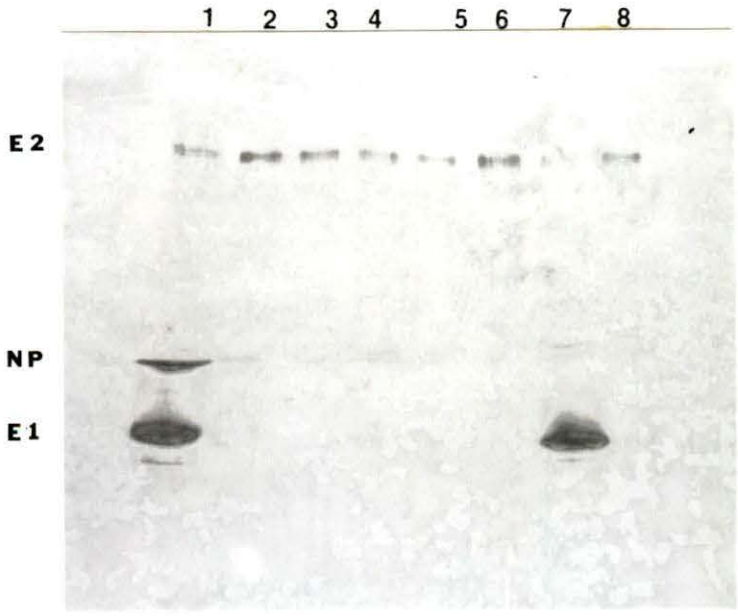

Fig. 6. Autoradiogram of SDS-PAGE showed immunoprecipitation of ^{35}S-methionine-labelled TGEV-infected ST cell lysates by hyperimmune serum (lane 10 and Mabs, ME9 (lane 2), MB2 (lane 3), MG7 (lane 4), MH11 (lane 5), MA5 (lane 6), MF2 (lane 7), and MA4 (lane 8).

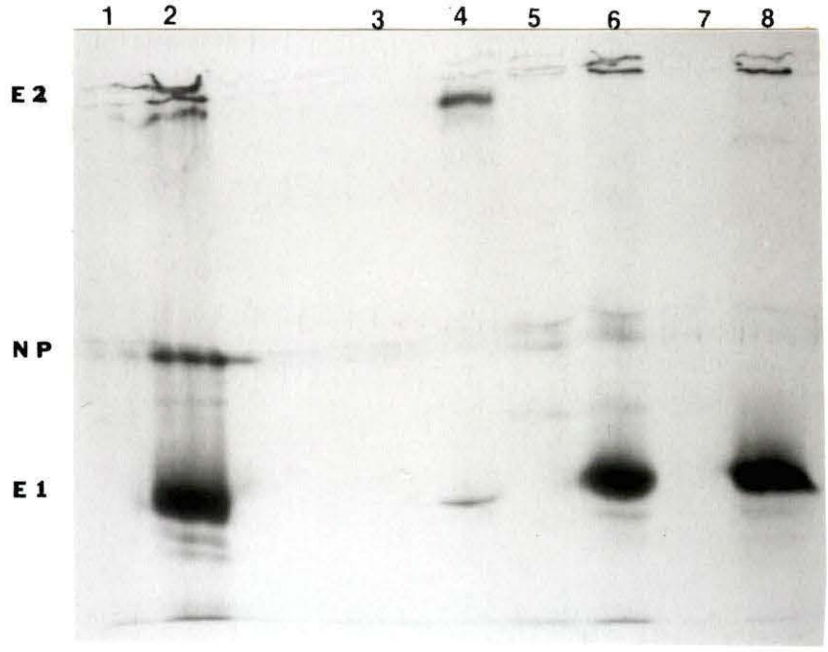

Fig. 7. Autoradiogram of SDS-PAGE showed immunoprecipitation of ^{35}S-methionine-labelled TGEV-infected (lanes 2, 4, 6, 8) and mock-infected (lane 1, 3, 5, 7) ST cell lysates by hyperimmune porcine anti-TGEV serum (lane 1 and 2), and Mabs, MH11 (lane 3 and 4), MG5 (lane 5 and 6), and MD9 (lane 7 and 8)

Immunoglobulin isotype

Immunoglobulin isotypes of nine Mabs were determined by an immunodiffusion test. Cell culture supernatants were used in this test. All of the Mabs tested belonged to an IgG isotype representing three subclasses of IgG: IgG1, IgG2a, and IgG3 (Table 2). The isotype of ME9 and MG7 could not be determined by this test. It is possible that concentration of Mabs produced by these hybridomas was low and was not detectable by immunodiffusion test.

Reactivity patterns of Mabs to different TGEV isolates

The reactivity of Mabs with different TGEV isolates was measured by IFA. Cell culture-adapted TGEV strains: Miller, Purdue, Illinois, were used as standard TGEV isolates. Eight field isolates were isolated from pigs with TGE and were confirmed to be TGEV by staining with porcine anti-TGEV hyperimmune serum in an indirect immunofluorescence test. The intestines from pigs with TGE were collected from different geographic areas in Iowa during 12 months. The field isolates were passaged on ST cells for not more than four passages to minimize original antigenic structural change. Four neutralizing Mabs as well as porcine anti-TGEV hyperimmune serum recognized all of the

isolates tested (Table 3), while reactivity of nonneutralizing Mabs to these isolates varied tremendously (Table 4). Some Mabs recognized certain isolates but not the others. Each isolate was shown to have a unique reactivity pattern with Mabs.

Table 3. Reactivity patterns of neutralizing anti-TGEV Mabs with TGEV isolates[a]

TGEV isolates	Mabs				Hyper-immune anti-TGEV serum	Pre-immune serum
	MB2	MH11	MA5	MA4		
Standard strains						
Miller	+++	+++	+++	+++	+++	-
Illinois	+++	+++	+++	+++	+++	-
Purdue	+++	+++	+++	+++	+++	-
Field isolates						
IA35	+++	+++	+++	+++	+++	-
IA57	+++	+++	+++	+++	+++	-
IA77	+++	+++	+++	+++	+++	-
IA37	+++	+++	+++	+++	+++	-
IA24	+++	+++	+++	+++	+++	-
IA65	+++	+++	+++	+++	+++	-
IA17	+++	+++	+++	+++	+++	-
IA09	+++	+++	+++	+++	+++	-

[a]-: no immunofluorescence; +++: >75% cells with immunofluorescence.

Table 4. Reactivity pattern of non-neutralizing anti-TGEV Mabs with TGEV isolates[a]

TGEV isolates	MD9	ME5	MG5	MC6	MF2	ME9	MG7
Standard strains							
Miller	+++	+++	+++	+++	+++	+++	+++
Illinois	-	-	-	-	-	++	-
Purdue	-	-	-	-	-	-	+++
Field isolates							
IA35	+	-	-	-	-	-	-
IA57	-	-	-	-	++	-	-
IA77	-	-	-	-	-	-	++
IA37	-	-	-	-	-	-	++
IA24	-	+	-	-	++	-	-
IA65	-	-	-	-	+++	++	-
IA17	-	-	-	-	-	-	-
IA09	-	-	-	-	+++	-	-

[a] -: no immunofluorescence;
+: 25% cells with immunofluorescence;
++: 26-50% cells with immunofluorescence;
+++: >50% cells with immunofluorescence.

DISCUSSION

Eleven Mabs to TGEV have been characterized to determine their polypeptide specificity toward virion proteins, virus neutralization activity, and immunoglobulin isotypes. The results showed that Mabs which had viral neutralization activity recognized only E2, and Mabs recognizing E1 had no virus neutralization activity. The results concur with the earlier findings that E2 is responsible for inducing virus neutralizing antibodies. Garwes et al. (1978/1979) compared partially purified preparations of virus surface projections, subviral particles derived by detergent treatment of the virus and inactivated virus particles for their ability to induce neutralizing antibodies in pregnant sows. Neutralizing antibodies were demonstrated in serum and colostrum from animals that received whole virus or preparations of surface projections. Whereas subviral particles failed to stimulate neutralizing antibody formation. Laude et al. (1986) reported that all major neutralization-mediating determinants of TGEV were carried by peplomers. Antigenic structural analysis of some other members of coronaviradae, such as mouse hepatitis virus and bovine enteric coronavirus, also showed that E2 was involved in inducing

neutralizing antibodies (Vautherot and Laporte, 1983; Collins et al., 1982). In contrast, anti-E1 Mabs did not neutralize virus infectivity in this study even in the presence of guinea pig complement. The results agree with those of Laude et al. (1986) but disagree with those of Woods et al. (1986). These differences in neutralization ability of anti-E1 Mabs are possibly due to different epitope specificity of the Mabs reported by Woods and Wesley (1986). Another possible explanation is that lyophilized guinea pig complement used in the present study is not ideal for testing neutralization potential of Mabs. Collins et al. (1982) reported that Mabs against E1 of MHV resulted in virus neutralization only when fresh guinea pig complement was added to the Ab-virus reaction mixtures, and this is believed to be a lytic effect of complement on the virus. Isotype of immunoglobulins can also affect complement mediated neutralization. Mouse IgG2a, IgG2b and IgG3, but not IgG1, have been shown to activate complement (Brown et al., 1985). Since majority of Mabs characterized in the present study belonged to either IgG2a or IgG3 subclasses except IgG1 (Table 2), lack of complement mediated virus neutralization can not be explained by the immunoglobulin isotypes.

The mechanism for TGE virus neutralization activity is not clear. Nguyen and his co-workers (1986) indicated that neutralizing secretory IgA and IgG did not inhibit attachment of TGEV to susceptible cells, but prevented the virus from entering cells. We found that all of the neutralizing Mabs were of the IgG2a isotype, and it is unclear if the immunoglobulin isotype of Mabs plays any role in viral neutralization activity.

Viral glycoprotein E2 and E1 also seem to be more antigenic in the mouse than N protein. Among 11 Mabs characterized, 6 of them were directed against E2, 5 against E1, and none of them were against N protein. Similar results were obtained by Laude and his co-workers (1986). They developed 32 hybridoma cell lines producing Mab against 3 major structural proteins of TGEV. Twenty-three Mabs recognized either E2 or its precursors or both. Four Mabs were directed against E1. Three were against N protein. Another possible explanation is that N protein is located within the capsid and may not have been available to the mouse immune system, whereas E2 and E1 are located on the surface of virions.

Preliminary data on epitope specificity of Mabs by competitive immunofluorescence assay helped to define at least three functional domains on E2 protein, one

recognized by MA5, MB2 and MA4, namely E2.1, a second one recognized by MH11, namely E2.2, and the last one recognized by nonneutralizing Mabs MG7 and ME9, namely E2.3. Mabs MA5 and MB2 blocked the staining of MA4 and may either share the same epitope or a topographically related epitope. Further studies need to be conducted by using more quantitative tests, such as competitive ELISA, to characterize epitopes on TGEV proteins. Data on reactivity patterns of Mabs to eleven isolates showed that epitopes on E2.1 and E2.2 are more conserved than the E2.3 epitope, since Mabs to E2.1 and E2.2 recognize all of the isolates and Mabs to E2.3 recognize only some isolates (Tables 3 and 4). Conservation of these epitopes on peplomer proteins indicates that these are important epitopes and possibly play an essential role in virus function. These epitopes may also be important in immunogenicity of TGEV. Delmas et al. (1986) found that most of the neutralization-mediating determinants clustered in the small area of the E2 protein, and this area was found to be highly conserved among TGEV strains. How the epitopes defined in the present study relate to those reported by Delmas et al. is not yet known.

A correlation between FA titer and viral neutralizing titer was observed. The Mabs with neutralizing activity showed higher FA titer than those with noneutralizing

activity. Immunofluorescence staining pattern of different Mabs also varied. With anti-E2 Mabs, immunofluorescence staining appeared perinuclear and granular, while with anti-E1 Mabs, the infected cells were brightly and intensely stained, and showed a surface membrane immunofluorescence. The ability of anti-E1 Mabs to bind to infected cell surface implies that this transmembrane polypeptide of TGEV is expressed at cell surface independent of virus maturation, since coronaviruses mature by budding into intracytoplasmic vesicles (Wege et al., 1982). Collins et al. (1982) also demonstrated by immunoelectron microscopy that E1 proteins of MHV were expressed at the surfaces of infected cells. They suggested that expression of virus specific surface components during eclipse may render the infected cell susceptible to specific antibody-dependent or cellular host defense mechanisms. Spread of MHV infection is facilitated by the ability of viral polypeptides to mediate cell fusion.

Occurrence of only one serotype of TGEV is commonly accepted (Kemeny et al. 1976). However, there is an indication that variation of TGEV may occur in nature. Autogenous TGEV vaccines have been shown to be effective whereas modified live or inactivated virus vaccines have failed. Pensaert et al. (1981) have recently reported a new

TGEV-related coronavirus. This new virus is antigenically indistinguishable from TGEV but causes only a respiratory and no enteric infection in pigs. Laude et al. (1986) also showed the occurrence of distinct antigenic differences among TGEV strains. Antigenic heterogeneity among other coronaviruses, such as MHV and feline infectious peritonitis virus, is common (Sturman and Holmes, 1983). The results in the present study indicate that antigenic heterogeneity exists among isolates but needs to be further examined.

The Mabs characterized in this study have several potential applications. Virus neutralizing Mab will be valuable in identifying specific epitopes responsible for inducing neutralizing antibodies. Once the immunogenic epitopes are characterized, they can be produced in large quantities by recombinant DNA techniques. These Mabs may also allow us to develop Mab resistant (mar) variants. Treatment with Mabs induces viruses to undergo small structural variations, which may result in change of biological function and pathogenecity of TGEV. A similar study with MHV has successfully been performed by Fleming et al. (1986). They selected antigenic variant viruses using anti-E2 Mabs. One of the Mabs selected demyelinating variants which had reduced neurovirulence, whereas the parent virus was highly neurovirulent. Based on this study

they proposed that a subregion of the E2 molecule is particularly important in neurovirulence of JHMV and in the pathogenesis of JHMV infection in mice. These and additional Mabs to TGEV will be helpful for detecting antigenic heterogeneity among TGEV isolates, for developing improved methods for diagnosis and possibly for immunotherapy of TGE.

SUMMARY

Eleven hybridomas were selected from a panel of hybridomas developed at the Veterinary Medical Research Institute for their continuous secretion of Mabs against Miller strain of TGEV. Mabs secreted by these hybridomas were partially characterized. Four of the Mabs had high neutralization titers for TGEV. The remaining seven did not neutralize TGEV even in the presence of complement. All four neutralizing and two of the nonneutralizing Mabs reacted with gp200 or E2 protein of TGEV in a radioimmunoprecipitation test. The remaining five Mabs reacted with the gp30 or E1 protein of TGEV. Epitope specificity was determined by blocking of immunofluorescence of a fluorescein-labelled Mab by homologous and heterologous Mabs. Neutralizing Mabs were found to be directed against two different epitopes. Reactivity of these Mabs was tested with three laboratory adapted standard strains of TGEV and eight wild type isolates of TGEV from Iowa. Neutralizing Mabs reacted with all of the eight wild type isolates and three standard strains of TGEV. In contrast, noneutralizing monoclonal antibodies which stained Miller strain in an indirect immunofluorescence test, varied in their reactivity with

various wild type isolates. These data confirm earlier observations that E2 is the major neutralizing protein of TGEV and the two different epitopes recognized by the neutralizing Mabs are conserved. In contrast, antigenic heterogeneity occurs among TGEV isolates on epitopes recognized by the noneutralizing Mabs on E1 and E2.

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