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A viral subunit immunogen for porcine transmissible gastroenteritis

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An immunogenic component was isolated from both the Illinois (propagated in young swine) and the Miller (cell culture-adapted) strains of porcine transmissible gastroenteritis (TGE) virus. The viral subunit was released from the virion by sonication and was separated from intact virus and other viral components by isopycnic centrifugation. It had a buoyant density in sucrose of 1.02 g/ml. Further purification consisted of gel filtration through Sephadex G200, in which process the immunogen, with a molecular weight of approximately 25 000, was the last component to be eluted. A group of ten young, weaned swine, inoculated intramuscularly with two or three 1-mg doses of the viral subunit were protected against challenge with virulent TGE virus, probably by the induction of secretory IgA. The immunogen also induced a humoral immune response of variable magnitude (titers ranging from 8 to 5 625) in the animals. These antibodies are not believed to be directly related to protection against infection. They can, however, be easily identified by serologic procedures and may serve as a convenient indicator of responsiveness to the TGE viral immunogen.

porcine transmissible gastroenteritis; viral subunit; vaccine; TGE

Introduction

Transmissible gastroenteritis (TGE) virus is a pathogen that causes enteric disease in swine of all ages. The disease is especially severe in newborn pigs, and during epizootics the mortality rate among animals < 2 wk of age approaches 100%. In addition, an enzootic form of TGE exists as a persistent problem particularly among young weaned swine in nurseries. Since there exist no efficacious treatments, progression of the disease is limited primarily by the immune responses. This has led to efforts in many laboratories to develop vaccines and/or strategies for their administration. Thus far, these efforts in the United States or other countries have remained without success [3,8,14].

In most instances, intramuscular (IM) inoculation with intact TGE viruses has

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resulted in the production of circulating antibodies, sometimes in high titers, but less success has been achieved in inducing protective antibodies which are believed to be secretory IgA [1,5,6,16,20,24]. Ingestion of live virus that infects the intestinal tract and stimulates submucosal immunocompetent cells has been considered to be essential triggering for the secretion of IgA antibody [18]. Virulent virus is relatively efficient in inducing resistance to subsequent infection, but resistance to challenge after oral vaccination with attenuated strains of TGE virus, which cause limited damage to epithelial cells of the intestinal villi, has proved highly variable [3,4,11]. In addition, a problem associated with the use of live viruses as vaccines, in general, is that they may, under some conditions, revert to virulent and clinical disease may be the result. For this reason attention in recent years has been directed toward the use of viral subunits as immunogens. This paper describes a subunit component, isolated from a virulent strain of TGE virus, that has been shown by laboratory experiments to be both an effective and a safe immunogen for induction of protection of swine against challenge with the pathogen.

Materials and methods

Experimental animals

Pigs were of Duroc and Yorkshire breeding and were obtained from TGE-free herds. Baby pigs for propagation of TGE virus were removed from the sow after they had ingested colostrum and were infected with virus 24 to 48 h after birth. They were fed SPF-LAC porcine milk replacer (Borden Chemical, Pet Products, Norfolk, VA, U.S.A.) in disposable paper bowls and were housed in individual disposable boxes in isolation units maintained at 45°C and constant humidity.

For evaluation of immunogenicity of the viral subunit the animals weighed approximately 40 lbs at the start of the experiment. They were housed in groups of 6 to 8 in isolation units and were fed non-medicated grower rations.

Virus

Neonatal pigs were inoculated per os with 100 median neonatal pig infective doses (PID_{50}) of TGE virus (Illinois isolate). Animals were killed when clinical signs of TGE had developed, signifying that the pathogen had replicated, and the jejunum was collected for harvesting of virus. The presence of TGE was confirmed by fluorescent antibody (FA) staining for viral antigens. The intestine was homogenized in Eagle's minimum essential medium (MEM) containing 5% lactalbumin hydrolysate (LAH) and 2% porcine serum to make a 20% gut slurry. Macromolecular cellular debris was removed by centrifugation at 5 000 $\times g$ for 30 min in a 4°C refrigerated centrifuge. The supernatant fluid was collected for isolation of TGE virus and stored at -70°C.

The cell culture-adapted Miller number 3 isolate of TGE virus was propagated in monolayers of swine testes (ST) cells as roller cultures, with Eagle's MEM containing 0.5% LAH and 100 meq/ml kanamycin as the nutrient medium. Cultures in 750 cm^2

bottles were 6 days of age when inoculated with 1 000 plaque-forming units of virus and were incubated at 37°C in a humid 1% CO_2 atmosphere for 3 days before virus was harvested. The entire contents of each roller bottle (cell layer and culture medium) were frozen in a dry ice–alcohol bath and thawed at 37°C for three times to release virus from cells. Cellular debris was removed by centrifugation at 5 000 × *g* for 20 to 30 min, and the supernatant fluid containing virus was stored at −70°C.

Isolation of viral subunit

The same procedure was used for the preparation of immunogen from the in vivo propagated Illinois isolate and from the in vitro replicated Miller number 3 isolate of TGE virus. Precipitation of TGE virus occurred following the addition of 7% polyethylene glycol 6 000 (PEG) and 2.3% sodium chloride. The mixture was held at 40°C for 1.5 to 2.5 h for precipitation to be completed and then was centrifuged at 9500 × *g* for 30 min. The pellet was dispersed in Eagle's MEM to a volume 1/20th of the original and was layered over a discontinuous sucrose gradient (10, 25, 40% sucrose in TRIS-EDTA buffered saline at pH 7.4). Rate-zonal centrifugation was carried out at 100 000 × *g* for 1.5 h. Since the buoyant density of the dehydrated TGE virus associated with PEG is 1.122 g/ml, the virus sedimented to the 25–40% sucrose interface. Material at this position was collected, sonicated with a Biosonic IV sonic oscillator (Bronwill, VMR Scientific, San Francisco, CA, U.S.A.) for 10 s at low power, and dialyzed against TRIS-EDTA buffered saline (pH 7.4) to remove sucrose. Then it was fractionated by isopycnic centrifugation through a continuous sucrose density gradient (10 to 60% sucrose in TRIS-EDTA-buffered saline, pH 7.4) for 18 to 20 h at 260 000 × *g*. The virus freed from PEG by sonication had the buoyant density characteristic of coronaviruses, 1.18 g/ml, and the immunogen had a buoyant density of 1.02 g/ml. The band with a buoyant density of 1.02 g/ml was fractionated by upward flow gel filtration through Sephadex G200 (Pharmacia Fine Chemicals, Piscataway, NY, U.S.A.) at a flow rate of 6 ml/h and fractions were collected with an Ultrarac model 7000 fraction collector (LKB Instruments, Inc., Rockville, MD, U.S.A.). Two components were evident by absorption at a wave length of 254 μm. The immunogen was the viral subunit with a molecular weight of approximately 25 000 and thus was the last fraction to be eluted.

Molecular weight determination

The approximate molecular weight of the subunit immunogen was determined by electrophoresis through polyacrylamide gel under denaturing conditions, so that the optimal medium for gel filtration could be selected. The technique used for this determination was that of Bishop and Roy [2]. Gels consisted of 7.5% (w/v) acrylamide with 2.5% cross-linkage and contained 1-M urea and 0.1% (w/v) sodium dodecyl sulfate. They were prepared in 0.1-M phosphate buffer (pH 7.0). Phosphate buffer containing urea and the detergent was also used in the electrophoretic chamber. Bromophenol blue (0.005%) was incorporated in the samples as a tracking dye. Electrophoresis of the immunogen and a solution containing a series of standard

proteins of known molecular weights (SDS-PAGE Standards-Low Molecular Weight, Bio-Rad Laboratories, Richmond, CA, U.S.A.) was performed at 2 mA/tube for 1 h and then at 5 mA until the bromophenol blue dye reached the bottom of the tube. Protein was fixed and stained for 2 h in Coomassie brilliant blue R-250 (Eastman Kodak, Rochester, NY, U.S.A.) prepared in 7% acetic acid and then gels were destained in a mixture of ethanol, acetic acid and distilled water (3 : 1 : 10). On the basis of its mobility relative to the standards, the molecular weight of the immunogen was calculated to be approximately 25 000.

Immunization and challenge of swine

The TGE viral subunit immunogen was emulsified with Freund's complete or incomplete adjuvant, or suspended in 10% aluminum hydroxide, as indicated in the text. Each animal received 1 mg of immunogen as a 1-ml dose IM into a rear leg. Antibodies to TGE were assayed by a plaque reduction-virus neutralization procedure in cell culture [25]. Antibody titers were calculated according to the Reed and Muench [17] formula.

Results

Effectiveness of vaccination

Two groups of pigs were immunized. Group I consisted of 6 TGE-seronegative pigs. They received a primary injection of antigen in complete Freund's adjuvant, followed by a second injection in incomplete adjuvant, 30 days later. Four of the animals were challenged on the sixtieth day after the primary immunization.

Sera were collected to monitor the humoral immune response. Four of the pigs in this group were challenged per os with 1×10^7 PID_{50} of virulent Illinois isolant of TGE virus on the sixtieth day. The remaining two pigs were given a third dose of antigen on day 135 and were challenged 30 days later. As a control to group I, 6 pigs received injections of adjuvant only. Group II consisted of 4 pigs, each of which received 1 mg of immunogen in aluminum hydroxide. A second inoculation was given 30 days later and the animals were challenged per os on day 45. Four control animals received saline–aluminum hydroxide as inoculum.

All swine were observed closely for clinical signs of TGE. Attempts were made to isolate the virus from feces and rectal swabs, using ST cell cultures for propagation of virus. Specimens from rectal swabs and feces, on microscope slides, also were stained with TGE-virus specific fluorescent antibody (TGE-FA) in an effort to detect the presence of the virus. When animals became ill, they were killed and specimens were collected from the jejunum to be stained with TGE-FA and for virus isolation in order to confirm diagnosis of the disease.

The results are summarized in Tables 1 and 2. All swine that received the immunogen developed circulating antibodies that neutralized TGE virus. However, seroconversion with antigen obtained from the cell culture-adapted Miller isolant of

TABLE I

Seroconversion of young swine (group I) following vaccination with TGE viral subunit immunogen in Freund's adjuvant[a]

Pig number	TGE virus[b]	Serum antibody titer[c] – day after initial inoculation					14 Days post-challenge	Day of challenge	Clinical TGE
		0	30	45	69	165			
678	Miller No. 3	<5	<5	ND[d]	38	ND	72	60	NCS[e]
679	Miller No. 3	<5	<5	ND	25	ND	45	60	NCS
682	Illinois	<5	38	ND	248	ND	ND	60	NCS
684	Illinois	<5	22	ND	80	ND	250	60	NCS
W-18	Illinois	<5	177	120	ND	3310	7040	165	NCS
W-19	Illinois	<5	1244	1356	ND	3540	7990	165	NCS
680	None[f]	<5	<5	ND	<5	ND	160	60	Yes
683	None	<5	<5	ND	<5	ND	40	60	Yes
686	None	<5	<5	ND	<5	ND	32	60	Yes
687	None	<5	<5	ND	<5	ND	ND	60	Yes (died)
W-16	None	<5	<5	<5	ND	<5	10	165	Yes
W-17	None	<5	<5	<5	ND	<5	128	165	Yes

[a] Pigs received immunogen on days 0 and 30; pigs number 678, 679, 682 and 684 were challenged 30 days later. Pigs number W-18 and W-19 were given a third dose of immunogen on the 135th day before also being challenged 30 days later. Control animals were inoculated with adjuvant only.

[b] TGE viral isolant serving as source of subunit immunogen.

[c] Reciprocal of the highest dilution of serum to inhibit plaque formation in cell cultures.

[d] ND = Not done.

[e] NCS = No clinical signs.

[f] Control animals.

TABLE 2

Seroconversion of young swine (group II) following vaccination with TGE viral subunit immunogen in an aluminum hydroxide as adjuvant[a]

Pig number	TGE virus[b]	Serum antibody titer[c]				Day of challenge	Clinical
		Day post-immunization			14 days post-challenge		
		0	30	45			
661	Illinois	<5	10	8	125	45	NCS[d]
664	Illinois	<5	289	5625	2525	45	NCS
665	Illinois	<5	625	1261	1316	45	NCS
666	Illinois	<5	279	1106	1903	45	NCS
660	None[e]	<5	<5	<5	32	45	Yes
662	None	<5	<5	<5	46	45	Yes
663	None	<5	<5	<5	80	45	Yes
667	None	<5	<5	<5	61	45	Yes

[a] Pigs in group II received two doses of vaccine, prepared from Illinois TGE virus, 30 days apart and were challenged 15 days after the secondary inoculation; control animals received aluminum hydroxide adjuvant only.

[b] TGE viral isolant serving as source of subunit immunogen.

[c] Reciprocal of the highest dilution of serum to inhibit plaque formation in cell cultures.

[d] NCS = no clinical signs.

[e] Control animals.

TGE virus was delayed until after a second inoculation of the vaccine, and only low titers of circulating antibody were observed, whereas specific antibodies were evident following a single inoculation with the component derived from the highly virulent Illinois isolant (Table 1). Very high titers of antibodies occurred after a third inoculation with immunogen prepared from the Illinois TGE virus. Both aluminum hydroxide (Table 2) and Freund's adjuvants (Table 1) were effective in serving to promote an immune response to the subunit immunogen. No tissue reaction was observed at the site of inoculation of swine receiving the immunogen with aluminum hydroxide but granulomas characteristic of the adjuvant were observed when the immunogen was given with Freund's adjuvant. Control pigs, housed with the animals which received immunogen, did not sero-convert prior to challenge with virulent TGE virus.

No clinical signs of TGE were observed after challenge in any of the pigs receiving either the Miller or the Illinois TGE viral subunit, nor was the virus isolated from any fecal specimens obtained from them. All unvaccinated control swine, however, developed clinical signs of TGE, with diarrhea being the most consistent indication of infection. One pig became severely ill and died on the sixth day post-challenge. Fecal samples obtained from the sick control animals contained virus which stained with TGE-FA. Also, the pathogen was found in epithelial cells of the jejunum of the pig that died, both by virus isolation in cell culture and by staining with TGE-FA. Severe villous atrophy was evident.

Safety testing of TGE viral subunit immunogen

Six 24-h-old pigs were given 1 mg of TGE viral subunit immunogen orally, as a 1-ml dose. The immunogen for 4 of the animals was prepared from the Illinois strain of TGE virus; for 2 of the animals its source was the Miller number 3 isolate. The pigs were held as a group in an isolation unit for 14 days during which time they were observed closely for clinical signs of TGE and attempts were made to isolate TGE virus in ST cell cultures from fecal specimens and rectal swabs. Also preparations from rectal swabs and feces on microscope slides were stained with TGE-FA conjugate to identify the pathogen. Two additional pigs which did not receive immunogen were housed in contact with them. None of the 24-h-old pigs which received the TGE viral subunit orally developed any signs of disease, nor did pigs in contact with them become ill. Virus was not isolated from fecal specimens. There was no evidence that the preparation contained virulent material.

Discussion

Many preparations of TGE virus have been used experimentally in efforts to develop a good vaccine for control of this disease [9,10,12,15,21,22]. Some products have been made available commercially. As a matter of convenience, the IM route has been generally chosen for administration of the antigen. In many cases success has been achieved in a humoral immune response but almost uniformly there has been a failure of this response to provide satisfactory protection [4]. Bohl and Saif [7] and Saif et al. [18] have proposed that the problem in the development of protection against infection is associated with the class of immunoglobulin, production of which is stimulated. Infection of the intestinal tract results predominantly in secretion of IgA but IM inoculation with the virus results in synthesis of IgG. The IgA which is produced locally neutralizes TGE virus in the lumen of the small intestine but little or no IgG is released into this site of infection. Furthermore, secretory IgA is relatively stable in the environment of the digestive tract; IgG is not.

At least one commercially available vaccine consists of an attenuated virus that is given both orally and IM to pigs in order to increase its efficacy. The degree of protection afforded has been disappointing, probably because the low level infection of the gut that occurs is inadequate to stimulate a protective immune response [4]. Also, the inconvenience of multi-doses and multi-routes is a disadvantage of this vaccine. However, an even more serious problem, as with any live vaccine, is that there exists some theoretical danger to highly susceptible animals. These animals may be infected either accidently at the time of immunization, with virus contaminating the environment, or later as a result of reversion of the attenuated virus to virulent pathogen during passage through the host species. None of the vaccines federally licensed in the United States is approved for use in newborn or suckling pigs [4].

A strain of TGE virus developed by Woods [26] has a tropism for crypt cells of the small intestine rather than for the villous epithelial cells. Consequently, atrophy of villi does not occur following infection and clinical signs of TGE are not observed. It

has been proposed that this isolate may be the basis for a vaccine. Since the crypt cells mature into absorptive villous epithelial cells, however, one must give very careful consideration to the advisability of introducing this new virus into swine herds.

For many viral systems, attention is being directed towards the development of subunits as immunizing agents [19,23]. These subunit immunogens can be produced either by disrupting and fractionating virions or by interrupting the sequence of viral replication at some point after which essential antigens are synthesized but before assembly of intact and virulent virions occurs. Subunits used as antigens offer the advantage of being completely safe because the nucleic acid essential for replication of the virus can be eliminated. Toxic factors can be removed. Also selection of components can be made in order to induce antibodies of more limited specificity and of the most suitable activities. Most experimental subunit vaccines consist of surface components, frequently glycoproteins, which are involved in attachment of the pathogen to the host cell prior to invasion [13]. Immune reactions directed against these antigens may prevent disease, protecting against primary infection, but may not produce potential pathologic consequences associated with some antigen–antibody interactions.

A subunit of TGE virus has been isolated in this laboratory and it induced a protective immune response in vaccinated pigs. This component was discovered serendipitously as a by-product of a protocol used for the purification of virus. At the final isopycnic centrifugation during the procedure that was being used, a band of protein with a buoyant density of only 1.02 g/ml always was observed in the sucrose gradient. In an attempt to identify the component, it was found to absorb neutralizing activity from hyperimmune serum (unpubl. observations). Therefore, the fraction was inoculated into rabbits and pigs with the hope that it would induce production of highly specific antibody for use as fluorescein-conjugate to be utilized in diagnostic procedures. Secondarily, it was found also to provide protection to the pigs against challenge with virulent TGE virus. Further evaluation of its efficacy as a vaccine followed and is described in this paper.

The efficacy of the IM-inoculated subunit vaccine, as contrasted with the failure of intact virus administered by the same route, to provide protection against TGE virus is not readily explained. Possibly the relative size of the two is a factor, having a significant effect upon metabolic reactions and types of cells and sites involved in processing of antigens. The smaller subunit might be more rapidly disseminated throughout the body, being processed at many sites, including the liver, and stimulating immunocytes at various locations, thus inducing all classes of antibodies. The larger virions are degraded primarily in regional lymph nodes and the spleen, and involvement of phagocytic cells may be of greater importance; IgG is the predominant class of antibody produced.

Based on the serologic analyses, circulating antibody was produced in response to the TGE viral subunit. The amount of this antibody that was detected was highly variable from animal to animal, ranging from a titer of 8 for pig number 661 to a titer of 5625 for pig number 664. Since the swine with the lowest titers were as resistant to TGE as were those with the highest titers this humoral antibody may be incidental to protection against clinical disease but may nonetheless serve as a convenient indica-

tion that the animal was responsive to the immunogen. It is believed that the protective antibody which was produced but could not be measured was secretory IgA released into the lumen of the intestine. Unpublished preliminary experiments with a limited number of pigs have indicated that fluids removed from the lumen of the intestine of immunized swine contain antibodies sensitive to 2-mercaptoethanol and of a molecular size larger than IgG. Critical identification of these immunoglobulins as secretory IgA must, however, await determination of the presence of α-chain and secretory piece and is the subject of further research.

TGE virus is relatively labile. Its physical appearance, in electron microscopy, can be altered through handling. The virus in its native form probably is a spherical entity but characteristically it appears pleomorphic. As a coronavirus, it is surrounded by closely spaced peplomers that appear as a halo. These peplomers are readily lost from the surface during procedures involved in preparation for microscopic examination. The subunit immunogen is believed to be a surface component of the virion and likely is associated with the peplomers that are readily lost. It probably is a glycoprotein associated with attachment of the virion to host cells as the initial step in infection [13]; immune reactions directed against this component provide protection by prevention of initiation of infection of the intestinal villi. The exact nature of the viral subunit immunogen is the subject of continued research.

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