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Lactogenic Immunity to Transmissible Gastroenteritis (TGE) of Swine Induced by the Attenuated Nouzilly Strain of TGE virus: Passive Protection of Piglets and Detection of Serum and Milk Antibody Classes by ELISA

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ABSTRACT

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Piglets of eight sows vaccinated by different routes with the attenuated TGE mutant coronavirus, Nouzilly (N) strain, and piglets from two field seropositive sows were challenged with a virulent TGE strain. On the day of challenge and 10 days after challenge, milk and serum samples from sows were analysed for their level of neutralizing antibodies, total immunoglobulin classes and TGE antibody classes by an ELISA. No direct relationship was seen between the level of protection of the litters and the titres of the different antibody classes on the day of challenge. However, an inverse correlation was seen 10 days after challenge between protection and the level of TGE antibodies.

INTRODUCTION

Transmissible gastroenteritis (TGE) causes acute and highly contagious viral enteritis in pigs, and is considered to be a major cause of death in newborn piglets. After natural infection or experimental oral infection of pregnant sows with a virulent strain of TGE virus, lactogenic immunity is highly protective for piglets, and neutralizing antibodies in milk are mainly associated with the IgA fraction (Bohl, 1981). In contrast, in sows immunized with cell culture-

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attenuated TGE strains, lactogenic immunity is poorly protective and neutralizing antibodies in milk are associated with the IgG fraction (Saif and Bohl, 1979). In a previous investigation (Aynaud et al., 1985), an attenuated TGE mutant virus, Nouzilly strain (N strain), resistant to acidity and digestive gastric enzymes, was used for oral immunization of seronegative pregnant sows. The post-vaccinal lactogenic immune response gave good protection to suckling piglets against virus challenge. However, the passive protection rate was not related to the level of neutralizing activity in milk and serum of sows after challenge exposure (Shiraï et al., 1988). In the present investigation, our objective was to analyse the immune response of pregnant sows vaccinated with the N strain of TGE, using an ELISA technique. The levels of IgG, IgM, and IgA were evaluated in serum and milk of sows on the day of, and 10 days after virulent challenge of the piglets.

MATERIALS AND METHODS

Cell culture

RP.D. is a pig kidney cell line which has been described elsewhere (Laude et al., 1981). The swine testis (ST) cell line (McClurkin and Norman, 1966) was kindly supplied by Dr. E.H. Bohl (Wooster, Ohio, U.S.A.). Both cell lines were cultured in minimal essential medium (MEM) containing 100 IU/ml penicillin and 100 μg/ml streptomycin sulphate, and were supplemented with 10% fetal calf serum. The virus, serum and milk whey used for the antibody titration were diluted in serum-free MEM containing twice the normal antibiotic concentration.

Viruses

Purdue-115 is a high-passage TGE strain, cultured in RP.D. cells. The virus suspension was harvested at the first sign of a cytopathic effect (about 15 h post-infection) and stored at $-70°C$. This strain was used for the virus neutralization (VN) antibody test. The Nouzilly strain (N) is a high-passage strain obtained in our laboratory by serial cycles (188) of survivor selection in gastric juice of adult pigs (Aynaud et al., 1985; Nguyen et al., 1987). This attenuated strain was used for vaccination of pregnant sows against TGE. Virus suspensions were prepared in the same way as the Purdue-115 strain. The virulent challenge Gep-II strain of TGE was isolated by P. Vannier (personal gift) from a field outbreak in France. A virulent virus stock (about 10^6 LD_{50}/ml) was prepared from the contents of the small intestine of colostrum-deprived newborn piglets 24 h post-inoculation with the Gep-II strain. This strain was free of rotavirus antigens, and was filtered (0.22 μm) before being used for the oral virulent challenge of the newborn piglets.

Virus neutralizing antibody titration

TGE virus neutralizing antibodies were titrated by a micro-method in ST cells (Aynaud et al., 1985). Briefly, doubling dilutions of the serum and milk were made in 96-well microplates, and 100 tissue culture-infectious $doses_{50}$ ($TCID_{50}$) of the Purdue-115 strain were added to each well. The mixtures of samples and virus were incubated for 60 min at 37°C in 5% CO_2, 95% air. One-hundred μl of fresh ST cell suspension (10^5/ml) in 15% fetal calf serum supplemented MEM was then added to each well. TGE antibody-positive serum was used as a reference. Results were read after 3 days of incubation, the titre of the sample was calculated as the arithmetic mean of triplicates of the lowest dilution which protected the cells from the cytolytic effect of the virus.

Animals

Eight TGE seronegative (neutralizing antibody activity) Large White and Mei Shan pregnant sows were bred in our institute for the experiment. Two field seropositive pregnant sows were also used (no. 232 and 913).

Vaccination procedures

Eight sows received 10^7 PFU of the N strain, 42 to 49 days before parturition. Vaccine doses were given per os in 300 ml of MacIlvaine buffer (0.025 *M*, pH 4) to six fasting sows, and intramuscularly in 15 ml of PBS to two sows. Booster administration was carried out with the same dose of N strain virus, 7 to 15 days before farrowing, by different routes of administration (oral, intramuscular and conjunctival) as shown in Table 1. The animals which had been experimentally inoculated with TGE virus were housed in isolated units.

Challenge procedure

One ml of 1000 LD_{50} of Gep-II strain was orally-administered to all the piglets in a litter at 4 to 9 days old. Clinical signs and the mortality rate of the piglets were noted every day up until 15 days after challenge.

Collection and preparation of samples

Blood and milk samples were collected from each sow on the day and 10 days after challenge. Milk samples were obtained manually after milk let-down was induced by intravenous injection of oxytocin. Whey was prepared by centrifugation at $10\,000 \times g$ for 30 min. An aliquot was collected from the middle portion between the cream layer and the deposited casein and debris. Serum and milk whey were stored at −20°C until use.

Total quantity of immunoglobulin classes

Total amounts of IgG, IgM and IgA in the serum and milk were measured by a competitive, immunocaptive, ELISA technique as described previously (Bernard and Lantier, 1986). Briefly, the samples were diluted in PBS con-

taining 0.05% Tween 20 and 1% gelatin and mixed with chromatographically-purified peroxidase-conjugated (grade I, Boehringer, W. Germany) porcine IgG, IgM or IgA (Nakane and Pierce, 1966) on 96-well microplates (Dynatech microtiter). The mixture was transferred to microplates (Nunc Immunoplates 4-42404) coated with rabbit anti-porcine immunoglobulin and incubated for 2 h at 37°C. Dilution of a known quantity of purified immunoglobulin of the various classes, treated as described above were used to produce reference curves between 5 ng and 5 μg/well. The optical density at 415 nm was quantified after 1 h of contact with 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonate (ABTS) (Boehringer, W. Germany) and H_2O_2.

TGE antibody classes

The titres of TGE virus specific IgG, IgM and IgA antibodies in serum and milk were evaluated by an ELISA technique. A mixture of monoclonal antibodies (Delmas et al., 1986; Laude et al., 1986) specific for structural proteins of TGE virus was used for coating plates as described previously (Bernard et al., 1986). After washing, the plates were saturated overnight with 1% gelatin in PBS. Virus material from supernatants of ST cells infected with Purdue-115 strain of TGE virus (10^7 PFU) was incubated in PBS, containing 0.05% Tween 20 and 1% gelatin, in the precoated plates for 2 h at 37°C. Control plates were set up with supernatant from uninfected ST cell cultures. The diluted samples, containing unknown antibodies, were incubated in the same buffer in the microplates (100 μl/well) for 2 h at 37°C. The samples were then incubated with conjugated immune complexes (pig IgG or IgM or IgA-peroxidase labelled/specific anti-pig IgG, IgM, IgA) (Bernard and Lantier, 1985) for 2 h at 37°C. The enzymatic reaction was performed with ABTS under the same conditions as described above. Results were expressed as the difference in optical density of samples between infected and uninfected ST cell supernatant.

Statistical analysis

The relationships between the titres of the different antibody classes, and neutralizing antibodies in milk and serum collected on the day or 10 days after challenge exposure were estimated by the non-parametric Spearman rank-test.

RESULTS

Neutralizing antibody activities and protection

All of the piglets challenged with our virulent strain of TGE virus have shown clinical signs of diarrhea. This parameter was therefore not considered to be selective and we chose the mortality rate of the piglets, up to 15 days after challenge, to estimate the passive protection rate.

The passive protection rate of piglets and the neutralizing antibody activity levels in serum and milk of immune sows are presented in Table 1. Neutralizing antibody activities were always higher in serum than in milk. After challenge

TABLE 1

Relationships between the neutralizing antibody response in serum and milk of vaccinated and naturally infected sows and the passive protection rate of suckling piglets challenged with the virulent TGE virus strain

Sow		Piglet protection			TGE neutralizing antibody titre			
					Serum		Milk	
No.	Vac./boost.[a]	Age[b]	Sur./Tot.[c]	%	Ch[d]	Ch+10[e]	Ch[d]	Ch+10[e]
232	Nat[f]	9	4/4	100	512	512	32	64
168	OR/CJ	8	9/9	100	128	4096	16	256
224	IM/IM	4	13/13	100	512	2048	8	64
196	IM/IM	8	8/9	89	512	8196	8	128
1	OR/OR	6	10/13	77	512	4096	4	128
913	Nat	6	8/11	73	512	2048	64	1024
434	OR/IM	4	6/9	67	512	4096	32	512
216	OR/IM	8	5/8	62	4092	32768	512	4096
195	OR/OR	8	3/6	50	64	8192	4	512
231	OR/IM	6	4/9	44	4096	32768	512	16384
960	—[g]	9	0/4	0	<4	16	ND[h]	ND

[a]Route used for vaccination (vac.) and boosting (boost.): OR=oral, IM=intramuscular, CJ=conjunctival.
[b]Age of piglets on the day of challenge.
[c]Number of survivors 15 days after challenge exposure/total number of piglets challenged.
[d]Collected on the day of challenge exposure.
[e]Collected 10 days after challenge exposure.
[f]Unvaccinated seropositive sow (natural infection).
[g]Unvaccinated seronegative sow (control).
[h]Not done.

TABLE 2

Mean total immunoglobulin levels in the serum and milk of vaccinated sows on the day and 10 days after challenge of their piglets with virulent TGE virus strain

	Serum			Milk		
	IgG[a]	IgA	IgM	IgG[a]	IgA	IgM
Ch[b]	18.8 (3.5)	0.7 (0.36)	2.7 (1.4)	0.87 (0.44)	5.7 (2.8)	0.94 (0.34)
Ch+10[c]	20.8 (2.3)	0.66 (0.14)	3.1 (0.7)	1.22 (0.71)	9.5 (4.3)	0.96 (0.82)

[a]Total immunoglobulin of G, A and M classes: mean mg/ml (standard deviation).
[b]Collected on the day of challenge exposure of piglets.
[c]Collected 10 days after challenge exposure of piglets.

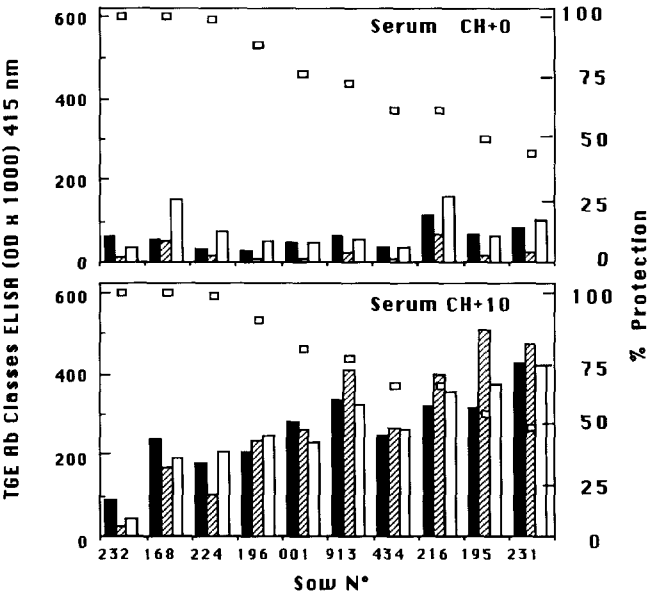

Fig. 1. TGE antibody class response in serum (optical density, ELISA technique) and passive protection rate of 10 litters, on the day or 10 days after virulent challenge exposure of the piglets. Closed bars, IgG antibodies; hatched bars, IgA antibodies; open bars, IgM antibodies; open rectangles, protection rate (% surviving piglets).

exposure of piglets, a strong increase in neutralizing antibody activity was observed in serum and milk of immune sows with the exception of sow no. 232. This observation suggested a secondary immune response caused by virulent TGE virus excreted by challenged piglets. Whatever the conditions of immunization, there was no correlation between the neutralizing activity level in serum or milk and the passive protection rate of piglets ($P > 0.05$).

Total immunoglobulin level in serum and milk

The mean quantities of immunoglobulin classes in serum and milk on the day and 10 days after challenge of piglets are presented in Table 2. No difference was seen between the two samples at the times in the levels of IgG, IgM or IgA in serum or milk. The individual variation of the level of milk immunoglobulins was very high. A correlation existed between the level of each class of immunoglobulin in the milk and serum (Spearman test, $P < 0.05$, results not shown). The only parameter which correlated with protection was the increase in the serum IgM titre.

TGE antibody classes activity and protection

The antibody class responses to TGE virus of 10 immune sows are shown in Fig. 1 (serum) and in Fig. 2 (milk wheys).

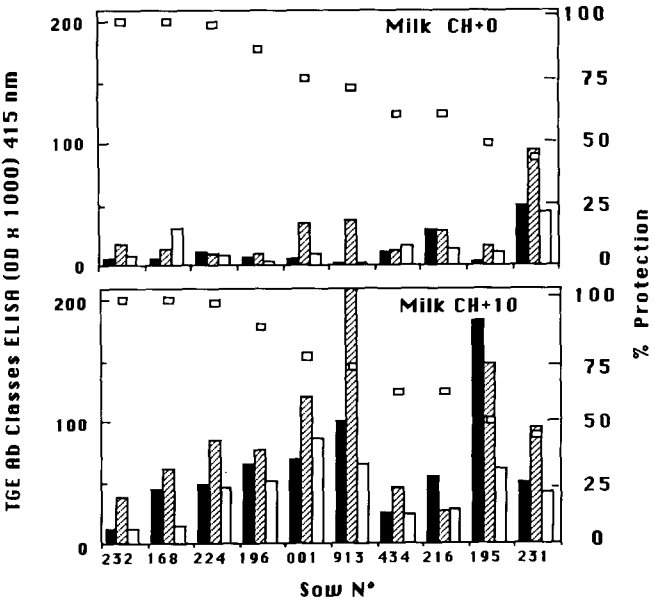

Fig. 2. TGE antibody class response in milk (optical density, ELISA technique) and passive protection rate of 10 litters, on the day or 10 days after virulent challenge exposure of the piglets. Closed bars, IgG antibodies; hatched bars, IgA antibodies; open bars, IgM antibodies; open rectangles, protection rate (% surviving piglets).

On the day of challenge, no direct correlation was seen between the passive protection rate and the level of any class of TGE antibody in serum or milk. Statistical analysis (Table 3), showed no other associations except a linkage between the level of the IgG, IgA and IgM antibodies in the serum. No correlation was seen with protection.

Ten days after challenge, neutralizing activity (Table 1) and TGE antibody classes detected by ELISA were greatly increased in the serum (Fig. 1) and milk (Fig. 2) of the sows (except sow no. 232). The inverse relationship between protection rate and the antibody titre at this time was confirmed by statistical analysis (Table 3). Further analysis of statistical correlation between the level of antibody activity in serum and milk is given in Table 4. No relationship was seen between the different antibody titres in samples taken on the day and 10 days after challenge, neither in milk nor in serum. However, on the day of challenge, a linkage was seen between the serum neutralizing antibody level and the milk IgG TGE antibodies. Ten days after challenge, the pattern of the relationship between the activities of the antibody class in serum and milk, was compatible with an anamnestic response in the serum of sows and of diffusion into the milk (Table 4).

TABLE 3

Relationship between passive protection rate and titre of specific anti-TGE antibody classes (IgG, IgA, IgM) in serum and milk, on the day and 10 days after challenge

		Serum[a]				Milk[b]			
		IgG	IgA	IgM	Neut. Ab	IgG	IgA	IgM	Neut. Ab
Ch[c]	IgA	P				—			
	IgM	—	PP			—	—		
	Neut. Ab	—	—	—		—	—	—	
	Protection	—	—	—	—	—	—	—	—
Ch+10[d]	IgA	PP				P			
	IgM	P	PP			PP	P		
	Neut. Ab	—	-	P		—	—	—	
	Protection	N	NN	NN	—	—	—	—	N

Non-parametric Spearman rank analysis.
[a]In serum: class IgG, IgA and IgM anti-TGE antibodies determined by ELISA technique, Neutralizing antibodies (Neut. Ab) and litter protection (Protection)
P = Spearman's correlation coefficient $rs > 0.74$ (ddl = 8), positive correlation $P < 0.05$.
PP = $rs > 0.88$, positive correlation $P < 0.01$.
N = $rs > -0.74$, negative correlation $P < 0.05$.
NN = $rs > -0.88$, negative correlation $P < 0.01$.
— = No correlation.
[b]In milk, same key as for serum.
[c]Samples collected on the day of challenge exposure of piglets.
[c]Samples collected 10 days after challenge exposure of piglets.

TABLE 4

Relationship between specific TGE antibody classes in serum and those in milk, on the day and 10 days after challenge exposure

		Serum[a]		Ch[c]		Milk[b]		Ch+10[d]	
		IgG	IgA	IgM	Neut. Ab	IgG	IgA	IgM	Neut. Ab
Milk[b]	IgG	—	—	—	P	—	—	—	—
Ch[c]	IgA	P	—	—	—	—	—	—	—
	IgM	—	—	—	—	—	—	—	—
	Neut. Ab	—	—	—	—	—	—	—	—
Serum[a]	IgG	—	—	—	—	P	P	P	—
Ch+10[d]	IgA	—	—	—	—	P	P	—	—
	IgM	—	—	—	—	—	—	—	—
	Neut. Ab	—	—	—	—	P	P	P	—

Non-parametric Spearman rank analysis.
Same footnotes as for Table 3.

DISCUSSION

The efficacy of the vaccinal N strain was estimated against standardized challenge of 1000 LD_{50} of Gep-II virulent virus (Aynaud et al., 1985). According to our previous experiments, we estimated that in this age range (4 to 9 days old) the use of only one seronegative susceptible control was sufficient to show the validity of the challenge. Unfortunately, this field virulent strain does not grow in tissue culture, so it is not possible to analyse the neutralizing antibodies against this virus. However, we have analysed the neutralizing activity of many monoclonal (Delmas et al., 1986) and polyclonal antibodies against many different strains of the virus (personal data), and we have never found a fundamental difference between the neutralizing activities. For convenience we choose the Purdue-115 virus to analyse our samples.

The most interesting result of the preliminary report about the immune response of sows vaccinated with N strain of TGE (Shiraï et al., 1988) was the absence of correlation between the passive protection rate of the litter and the neutralizing antibody titre in serum or milk of sows on the day of challenge. In the present work, using eight other sows, we confirmed the absence of linkage between protection and neutralizing antibody level on the day of challenge. We also demonstrated a statistically significant inverse relationship between the neutralizing antibody titre in milk 10 days after challenge and protection of the litters (Table 3). Ten days after challenge, it is clear that an inverse relationship exists between the protection and the titre of all the antibody classes in serum and milk (Fig. 1 and 2, Table 3). This secondary response was modulated by the quantity of virus excreted by the piglets and the immune status of the digestive tract. Absence of, or a low secondary immune response of sows 10 days after challenge could be a good a posteriori marker of lactogenic passive protection.

In a previous report (Shiraï et al., 1988), we analysed the antibody classes which were responsible for the neutralizing activities in serum and milk 10 days after challenge by chromatographic separation. The low titres in the sample collected on the day of virulent challenge obliged us to search for a more sensitive technique. We used therefore an appropriate ELISA (Bernard et al., 1986) to allow us to study the antibody class distribution on the day of challenge. The pattern of the IgG, IgA and IgM TGE antibody classes did not show any relationship with protection. With high virulent TGE virus, antibody of the IgA class was the main immunoglobulin involved in the passive protection of the suckling piglets (Bohl and Saif, 1975; Saif and Bohl, 1979; Saif and Bohl, 1981). However, the protection mediated by IgA is also dependent on the oral route of vaccination with virulent virus since intramuscular injection does not elicit IgA synthesis (Abou-Youssef and Ristic, 1975; Kodama et al., 1981; Woods, 1984). With cell culture-attenuated TGE virus neutralizing antibody, but not IgA antibody, was detectable in the serum of sows which had been

either orally or intramuscularly inoculated (Kodama et al., 1980; Woods, 1984). With the attenuated N strain it seems that IgA antibody activity is not sufficient to explain the protection of the litters. However, purified IgG TGE antibody gives broadly the same passive protection as IgM and IgA to artificially-fed piglets (Stone et al., 1977) and bovine hyperimmune colostrum whey, rich in IgG1, considerably reduced the mortality of passively-fed piglets following TGE challenge (Stepanek et al., 1982). It is very difficult to compare an in vitro antibody detection test with the in vivo protection rate due to attendant complex multiparametric physiological phenomena.

Characteristics of the N strain such as resistance to acidity and to digestive enzymes (Aynaud et al., 1985) and its high content of structural antigen (Nguyen et al., 1987) is sufficient to explain the in vitro immunogenicity but not to explain the in vivo protection induced by this virus. According to Bohl (Bohl et al., 1972a,b; Bohl and Saif, 1975) the routes of immunization of the sow, the virulence of the virus, the time of immunization and the processing of the viral epitope would be parameters that affect the pattern of the immune response. The mastery of successful oral vaccination will be achieved only with a greater understanding of all these parameters together with many others.

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