Antibody Response in Pigs Inoculated with Transmissible Gastroenteritis Virus and Cross Reactions Among Ten Isolates

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ABSTRACT

Groups of two or three day old pigs were inoculated intravenously with cell culture grown transmissible gastroenteritis virus. A single or a multiple dosage schedule was used. The magnitude of immune response was measured in terms of serum neutralization indices. A single dose of relatively attenuated virus caused mild clinical signs of transmissible gastroenteritis infection in the pigs and induced a low level of antibody in the serum by the seventh day after inoculation. Repeated injections of virus at seven day intervals stimulated little increase in antibody titers. However, high serum antibody titers were obtained for all pigs if the time interval between injections was extended to 15 days. Sera obtained early after exposure to live transmissible gastroenteritis virus contained mainly IgM antibody whereas sera obtained later after exposure contained mainly IgG antibody. Ten plaque purified isolates of transmissible gastroenteritis virus, comprising eight American isolates, one Japanese isolate and one British isolate were indistinguishable by means of reciprocal plaque reduction neutralization tests.

RÉSUMÉ

Cette expérience visait à injecter, une ou plusieurs fois, par la voie intra-veineuse, différentes souches du virus de la gastro-entérite transmissible, propagées en cultures tissulaires, à des groupes de porcelets âgés de deux et trois jours. On mesura l'ampleur de la réaction immunologique en termes d'indices de séro-neutralisation. Une seule injection de virus relativement atténué provoqua l'apparition de signes cliniques mitigés de gastro-entérite transmissible et ne révéla qu'une faible production d'anticorps sériques, le septième jour après l'inoculation. La répétition d'injections de virus, à intervalles de sept jours, stimula une production un peu plus élevée d'anticorps. Tous les porcelets développèrent cependant plus d'anticorps, lorsqu'on espaçait de 15 jours les injections de virus. Le sérum prélevé tôt après l'infection contenait surtout des immunoglobulines IgM; ultérieurement, il contenait surtout des immunoglobulines IgG. Dix souches du virus de la gastro-entérite transmissible, purifiées par la méthode des plages et comprenant huit souches américaines, une souche japonaise et une souche britannique, s'avérèrent indiscernables lors d'épreuves de neutralisation croisées, basées sur la réduction du nombre de plages.

INTRODUCTION

The development of a plaque assay for transmissible gastroenteritis (TGE) virus of swine in cell culture has made possible the study of the immunological response of swine to natural viral infection or after vaccination by quantitative virus neutralization tests. The plaque reduction neutralization test may also serve as a basis for serological diagnostic procedures (11,12,15) and for demonstration of antigenic relationships among various TGE virus isolates. Using neutralization tests in cell culture, Bohl and Kumagai (2) compared the American Purdue and Station strains with the Japanese Shizuoka virus. Cartwright (4) tested the British FS-216/64

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Submitted May 23, 1975.

isolate with the Purdue strain. Harada *et al* (8) studied the serological relationships of three Japanese isolates and the New York-I virus. These workers were unable to detect any antigenic differences among their strains tested.

The objectives in this study were: (a) to study primary and secondary immune responses of pigs after inoculation with cell culture grown virus, (b) to determine appropriate intervals for reinoculation with virus as indicated by the magnitude of the secondary response and (c) to investigate whether antigenic differences exist among ten laboratory maintained isolates of TGE virus. These differences can be determined by the plaque reduction neutralization test with homologous and heterologous antisera.

MATERIALS AND METHODS

Viruses

TGE viruses isolated from pigs in eight states and in Great Britain and in Japan were used (Table I). These viruses have been collected over a period of years from sources shown and stored at –70°C in a mechanical freezer. Unfortunately the passage history of these viruses could not be determined accurately. Each isolate was purified by three single plaque passages in swine testes (ST) cells. The agar overlay technique was used (2). Viruses used for inoculation of pigs and for neutralization tests consisted of cell free virus suspensions from infected cultures and were stored at –70°C in a mechanical freezer.

Cell Cultures

A continuous line of ST cells was propagated in 250 ml plastic bottles by techniques similar to those described by McClurkin (12). The growth medium was Earle's basal medium containing Eagle's additives, 0.5% lactalbumin hydrolysate and 10% porcine serum obtained from young (under six months of age) specific pathogen-free swine. The serum was inactivated at 56°C for 30 minutes before use. The medium used for maintenance of cell cultures was serum free. All media contained 50 μg of gentamicin sulfate/ml.

Virus Assay

For virus titrations, 60 x 15 mm plastic culture dishes with confluent ST cell sheets were inoculated with 0.2 ml of serial tenfold dilutions of virus in PBS, three dishes per dilution. After an adsorption time of 90 minutes at room temperature an overlay medium containing equal volumes of double strength maintenance medium and 1.2% agarose in distilled water with 50 μg of gentamicin sulfate/ml was added without rinsing. Cultures were incubated for 40 hours at 37°C in an atmosphere of 5% CO_2 and 2 ml of 10% formalin was added to 90 minutes at room temperature, an overlay was discarded and the monolayers were stained with 1% crystal violet to facilitate counting of plaques. The virus titer was expressed as plaque forming units (PFU) per ml of the undiluted viral suspension.

Plaque Reduction and Neutralization Test

All antisera were heated at 56°C for 30 minutes before testing. Neutralizing antibodies to TGE virus in sera were assayed by a modification of a plaque reduction test described by Karabatsos *et al* (9). After preliminary experiments, tenfold virus dilutions were mixed with equal volume of a 10^{-2} dilution of immune pig sera. Preinoculation serum was included for control with each test. The serum-virus mixtures were incubated at 37°C for 60 minutes and ST cell cultures were inoculated with 0.2 ml of the mixture. After an adsorption time of 90 minutes, overlay medium was added and the plates were incubated at 37°C in an atmosphere of 5% CO_2. Plaques were counted after 40 hours incubation. The neutralization index (NI) of each immune serum was calculated as the $\log_{10}$ difference between the titer of virus in the presence of nonimmune pig serum and the titer in the presence of the immune serum. All ten TGE virus isolates were tested. Homologous single dose and triple dose antisera and heterologous antisera were used. Antigenic similarity was considered to exist if the difference between homologous and heterologous titers was less than 1.5 $\log_{10}$.

Pigs

Hysterectomy-derived, colostrum-deprived pigs two to three days of age were used. They were kept in isolation units and fed

TABLE I. Designation and Source of Various Isolates of Transmissible Gastroenteritis Virus Used in Serological Comparisons

Virus Isolate	Isolation Area	Obtained from	Reference
California	California	McClurkin	Konishi and Bankowski (11)
FS-216/64	Great Britain	Bohl	Cartwright *et al* (4)
Hormel	Minnesota	McClurkin	Young *et al* (18)
Illinois	Illinois	Ristic	Ristic *et al* (14)
Iowa	Iowa	McClurkin	McClurkin (12)
Kansas	Kansas	McClurkin	None
Miller	Ohio	Tamoglia	Bohl and Kumagai (2)
New York-II	New York	McClurkin	Sheffy (15)
Purdue-37	Indiana	McClurkin	Haelterman and Pensaert (7)
Shizuoka-25	Japan	Bohl	Harada *et al* (8)

a modified cow's milk diet (1). Preexposure sera were obtained and pigs were inoculated intravenously (i.v.) with infectious cell culture grown virus and were bled for serum at various times from the anterior vena cava.

Immunization Procedures

The following immunization schedules were used to produce antisera against the various TGE virus isolates:

(a) Four groups of three pigs each were inoculated i.v. with the 50th cell culture passage of Miller isolate at weekly intervals. In the first group, a single injection of 1 ml of cell culture fluid containing $10^{7.5}$ PFU of virus was inoculated and the pigs were bled for serum at seven, 14, 21, 28 and 35 days postinoculation (DPI). In the second, third and fourth groups of pigs one, two, or three additional injections of virus (1 ml each) were given at weekly intervals respectively.

(b) Antisera were prepared against the ten virus isolates by inoculating two pigs i.v. with each isolate either once or three times, with 15 day intervals between inoculations. Pigs that were given a single dose of virus were given 1 ml of cell culture fluid containing approximately $10^{6.2}$ to $10^{7.4}$ PFU/ml of virus (Table III) and were bled for serum 15 days later. Pigs that were given three injections received increasing doses of virus in 1 ml, 3 ml and 5 ml of infectious cell culture fluid. Blood for serum was collected from these pigs 15 days after the third inoculation.

(c) Anti-ST cells control serum was prepared by injecting pigs i.v. with 5 ml of noninfected cell culture fluid three times at 15 day intervals. The pigs were exsanguinated 15 days after the third inoculation. All sera were stored at -20°C until used.

Mercaptoethanol Treatment

The serum samples were treated with 0.1 M 2-mercaptoethanol (final concentration) for 18 hours at room temperature and subsequently dialysed against 0.1 M iodoacetamide in PBS and then against PBS alone (13).

RESULTS

Increasing Plaque Count with Time of Incubation

Plaques usually appeared from 24 to 72 hours after virus infection, depending on the dose of virus inoculated. The number and size of the plaques increased after inoculation up to the sixth day. Changes in plaque counts were not observed after six days although cell cultures could be maintained in a viable state for at least ten days. Plaques were counted routinely for virus assay after 40 hours of incubation.

The Effect of Time Interval Between Injections on Antibody Response

The combined data concerning the effect of 2-mercaptoethanol on the neutralizing antibody and the level of antibody that developed in four groups of pigs inoculated at weekly intervals with one to four doses of Miller isolate of virus are presented in Table II. A single dose of virus induced a low level of antibody by seven DPI. The NI in the sera increased from 0.4 $\log_{10}$ to 1.7 $\log_{10}$ by 14 DPI and persisted at this level up to 35 days. Multiple inoculations of the pigs given seven days apart, generally did

TABLE II. Effect of 2-Mercaptoethanol on Neutralizing Antibody and Level of Antibody in Pigs Inoculated Intravenously at Weekly Intervals with the Miller Isolate of Transmissible Gastroenteritis Virus

No. Pigs in Group	No. of Inoculation[b]	Log₁₀ Neutralization Index[a] of Antiserum Time Postinoculation											
		0 Day		7 Days		14 Days		21 Days		28 Days		35 Days	
		Serum	2-ME[c]	Serum	2-ME	Serum	2-ME	Serum	2-ME	Serum	2-ME	Serum	2-ME
3	1	0	0	0.4	0.1	1.7	1.3	1.6	1.6	1.7	NT[d]	1.8	1.7
3	2	0	0	0.4	0.1	1.8	1.3	1.8	1.7	1.8	1.8	2.1	1.9
3	3	0	0	NT	NT	1.8	1.3	1.6	1.5	1.6	1.6	2.2	2.0
3	4	0	0	NT	NT	1.8	1.3	NT	NT	1.8	1.7	2.2	2.1

[a]Log_{10} difference between the plaque forming units of virus in the presence of nonimmune pig serum and in the presence of test antiserum. Mean value of two titrations
[b]Virus was given on days 0, 7, 14 and 21
[c]2-ME = 2-mercaptoethanol
[d]NT = not tested

not result in an increased antibody titer, although pigs that were given multiple injections had higher antibody titers at 35 days than those that received a single dose of virus.

As shown in Table II, treatment of sera with 2-mercaptoethanol reduced antibody activity in early sera collected at seven to 14 days after exposure but not in late sera collected 21 to 35 days after exposure. These results indicate that IgM antibody was predominantly present in early sera and IgG antibody was predominantly present in late sera.

Cross Neutralization

Ten plaque purified isolates of TGE virus comprising eight American isolates, one Japanese isolate and one British isolate were compared by reciprocal cross neutralization tests in which antisera produced by one or three inoculations of TGE virus into pigs were used. The results are presented in Table III. As compared with the homologous serum, there were less than 1.5 log_{10} differences in the NI among the ten TGE isolates when either single or triple dose antisera were used. In the single dose sera, the NI were in the range of 0.9 log_{10} to 2.6 log_{10} by 15 DPI. In the triple dose sera, the NI were in the range of 2.6 log_{10} to 5.0 log_{10} by 45 DPI. The amount of antibody apparently increased as the time interval between inoculations of virus was extended from seven to 15 days. Because the ten isolates were indistinguishable by reciprocal neutralization tests we concluded that they were antigenically closely related and, by this test, are of a single serotype.

DISCUSSION

This study was undertaken primarily to evaluate procedures that could be used to produce specific antisera to TGE virus and to compare ten cell culture grown isolates serologically. Antisera from pigs inoculated i.v. with TGE virus by different immunization schedules were examined by plaque reduction tests for neutralizing antibody. As might be expected, antibody titers ob-

TABLE III. Reciprocal Cross Neutralization Plaque Reduction Tests Among 10 TGE Virus Isolates with Pig Serum Produced by One or Three Intravenous Inoculations of Virus with 15 Day Intervals

Virus Isolate	Initial Virus Titer[a] (log_{10} PFU/ml)	Log_{10} Neutralization Index[b] of Antiserum to Isolate									
		California	FS-216/64	Hormel	Illinois	Iowa	Kansas	Miller	New York II	Purdue-37	Shizuoka-25
California	6.2	1.2 **3.6**	1.1 2.4	1.9 3.2	1.4 3.0	1.6 3.0	1.4 2.9	1.1 3.0	1.1 2.7	0.9 2.3	1.3 2.5
FS-216/64	6.7	1.6 4.5	1.3 **5.0**	2.0 4.9	1.9 4.2	2.2 4.9	2.4 4.6	2.0 4.8	1.5 4.7	1.6 4.8	1.5 4.6
Hormel	7.0	1.4 3.1	1.7 3.3	2.5 **4.2**	2.5 3.8	2.4 4.2	1.7 3.1	2.0 3.3	1.4 3.8	1.2 3.8	1.4 3.4
Illinois	7.0	2.0 3.7	2.6 4.4	2.0 3.8	2.6 **4.7**	2.2 4.6	2.4 3.5	1.9 3.7	2.0 4.0	2.2 4.1	2.2 4.1
Iowa	6.9	1.6 3.7	1.7 4.0	2.2 3.7	2.0 3.6	1.7 **4.0**	1.3 3.8	1.3 3.8	2.2 3.7	2.2 3.8	1.6 3.9
Kansas	6.2	1.6 3.0	0.9 2.3	2.0 2.7	1.8 3.5	1.7 3.4	1.6 **3.4**	2.1 3.6	1.3 3.1	0.9 3.3	2.2 3.3
Miller	7.4	1.0 4.7	1.6 3.8	1.6 4.7	1.7 3.7	2.0 4.1	1.7 3.6	1.5 **4.7**	1.7 4.5	1.8 3.8	2.0 3.9
New York II	7.0	1.8 4.0	1.7 3.0	1.4 4.1	1.2 4.4	2.0 4.4	1.3 3.1	1.7 4.4	1.8 **4.4**	2.0 3.8	1.4 3.4
Purdue-37	6.4	2.0 3.8	1.4 4.1	2.0 3.8	2.2 3.5	2.2 3.8	2.2 2.4	1.9 2.5	2.0 3.7	2.2 **3.8**	1.0 3.2
Shizuoka-25	7.2	1.3 4.1	1.5 4.0	2.0 4.4	1.9 4.1	1.6 4.7	1.7 3.8	2.5 3.0	1.4 4.0	1.3 4.0	1.5 **4.7**

[a]Mean value of three or more titrations
[b]Log_{10} difference between the plaque forming units of virus in the presence of nonimmune pig serum and in the presence of test antiserum. The upper figures were obtained with single dose serum, the lower figures with triple dose serum

tained after seven days were lower than those after 15 days. The titers, however, had little increase after multiple administration of virus at seven day intervals. This finding of an immune response was seemingly paradoxical because multiple doses of antigen would be expected to induce higher levels of antibodies. Previously, Stone *et al* (16) reported that pigs that were vaccinated with attenuated African swine fever virus failed to show an anamnestic response after challenge with virulent virus. They attributed this finding to the persistence of virus and intermittent viremia after inoculating pigs with modified live virus. Burrows *et al* (3), working with foot-and-mouth disease virus, reported little or no secondary response in cattle, sheep and swine after a second injection of virus if the time interval between two inoculations was less than 14 days. The cause of this finding, however, has not been explained. In the present study we report similar observations with TGE virus. Because pigs did have increases in antibody when the injections were given at 15 day intervals, we suggest that TGE virus induced immunological response of swine is similar to immunization of horses (10) or mice (6) with nonreplicating antigens. In these latter studies it was demonstrated that the height of the secondary antibody response increased as the time interval between antigen injections was lengthened. The proper timing of a second injection of live virus in immunization of swine appears to be important and should be considered when planning a vaccination schedule against TGE in the field. In human viral infections, induced interferon (17) may also be considered to suppress secondary antibody formation. However, no study on sensitivity of TGE virus replication to inhibition by interferon has yet been reported.

The sequential appearance of early IgM and late IgG antibodies in the immune response to TGE viral antigen agrees with the results of other studies obtained with a number of viruses (5, 16).

Although the ten isolates had varying degrees of pathogenicity for newborn pigs when given i.v. they were indistinguishable by means of reciprocal plaque reduction neutralization tests. The variations in pathogenicity of the different strains of TGE virus that have been passaged in the laboratory many times are probably due to the degree of attenuation by cell culture passage.

ACKNOWLEDGMENTS

The author wishes to thank Dr. S. S. Stone of this laboratory for his valuable suggestions, discussions and review of the manuscript and Mr. L. V. Wiltsey for technical assistance.

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