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Antigenic structure of the E2 glycoprotein from transmissible gastroenteritis coronavirus

Isabel Correa, Gustavo Jiménez, Carlos Suñé, María J. Bullido
and Luis Enjuanes

*Centro de Biología Molecular (CSIC-UAM), Facultad de Ciencias, Universidad Autónoma, Canto Blanco,
28049 Madrid, Spain*

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Summary

The antigenic structure of transmissible gastroenteritis (TGE) virus E2 glycoprotein has been defined at three levels: antigenic sites, antigenic subsites and epitopes. Four antigenic sites (A, B, C and D) were defined by competitive radioimmunoassay (RIA) using monoclonal antibodies (MAbs) selected from 9 fusions. About 20% (197) of the hybridomas specific for TGE virus produced neutralizing MAbs specific for site A, which was one of the antigenically dominant determinants. Site A was differentiated in three antigenic subsites: a, b and c, by characterization of 11 MAb resistant (*mar*) mutants, that were defined by 8, 3, and 3 MAbs, respectively. These subsites were further subdivided in epitopes. A total of 11 epitopes were defined in E2 glycoprotein, eight of which were critical for virus neutralization. Neutralizing MAbs were obtained only when native virus was used to immunize mice, although to produce hybridomas mice immunizations were made with antigen in the native, denatured, or mixtures of native and denatured form. All neutralizing MAbs reacted to conformational epitopes. The antigenic structure of the E2-glycoprotein has been defined with murine MAbs, but the antigenic sites were relevant in the swine, the natural host of the virus, because porcine sera reacted against these sites. MAbs specific for TGE virus site C reacted to non-immune porcine sera. This reactivity was not directed against porcine immunoglobulins. These results indicated that TGE virus contains epitope(s) also present in some non-immunoglobulin component of porcine serum.

TGE virus; Antigenic structure

Correspondence to: L. Enjuanes, Centro de Biología Molecular (CSIC-UAM), Facultad de Ciencias, Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain.

Introduction

Transmissible gastroenteritis virus of swine is a member of the *Coronaviridae* family that causes a disease of the gastrointestinal tract, producing transitory enteritis in adult animals and 80 to 100% mortality in animals less than 10 days old (Bohl, 1975; Siddell et al., 1983; Sturman and Holmes, 1983).

The virus particle is spherical with a diameter of 100 nm, contains a lipid envelope and a positive single-stranded RNA of approximately 20 kb (Hu et al., 1984; Kapke and Brian, 1986), that is infectious (Brian et al., 1980). The virus particle is composed of two glycoproteins (E2 and E1) and one nucleoprotein (N). The E2 glycoprotein induces neutralizing antibodies and forms the characteristic club-shaped projections of the particle (Garwes et al., 1978-79; Jiménez et al., 1986).

Effective protection of swine against TGE virus infection is only induced by virulent strains of the virus (Moxley and Olson, 1986). The development of an efficient anti-TGE vaccine (synthetic or genetically engineered) will require knowledge of: (i) the immunogenicity, antigenicity and variability of the virus; (ii) the mechanism(s) of virus neutralization; (iii) the conditions for induction of mammary and gut immunity; and (iv) the cloning and expression of genes coding for E2-glycoprotein or other proteins that may be relevant in the induction of protection.

In a previous publication (Jiménez et al., 1986) we have described some properties of six epitopes critical in TGE virus neutralization. In this article we describe the antigenic structure of the E2 glycoprotein at three levels by defining antigenic sites, antigenic subsites and epitopes, based on competitive radioimmunoassay (RIA) and characterization of monoclonal antibody resistant (*mar*) mutants, and present information on their nature. To accomplish the antigenic characterization of TGE virus, a large collection (1,015) of TGE virus positive hybridomas were used in an attempt to obtain representative data and to characterize MAbs able to recognize sequential determinants in the different antigenic sites. Interestingly, the dominant site on TGE virus neutralization was subdivided in three antigenic subsites. In addition, the crossreactivity between one antigenic site from TGE virus and a component of porcine sera has been described.

Materials and Methods

Cells

The epithelial swine testicle cell line ST developed by McClurkin and Norman (1966) was obtained from Dr. Kemeny, National Animal Disease Center, Ames, Iowa. Cells were grown as monolayers in growth medium consisting of Dulbecco's modified Eagle (DME) medium (GIBCO Europe) and 10% newborn calf serum (Flow Laboratories, Inc.).

Animals

BALB/c mice, originally obtained from R.A. Fox, Frederick Cancer Research Center, Frederick, MD., were used for immunization and as a source of thymocytes and peritoneal macrophages.

Viruses

The Purdue strain of TGE virus (PUR 54) was plaque purified and grown in ST cells as described previously (Jiménez et al., 1986). Virulent Miller strain passed in swine (Wesley and Woods, 1976), was kindly provided by R. Wesley (National Animal Disease Center, Ames, Iowa).

Virus titration, neutralization and purification

TGE virus plaques were isolated and the virus was titrated on ST cells as previously described (Jiménez et al., 1986). A plaque reduction assay was performed by incubating TGE virus in 50 μ l of phosphate-buffered saline (PBS) with 2% foetal calf serum in the presence of 50 μ l of hybridoma supernatant at 37°C for 60 min. Dilutions (10-fold) of the virus-MAb mixture in DME medium containing 2% foetal calf serum and 40 μ g of DEAE-dextran per ml (Pharmacia) were carried out. Portions containing 50 μ l of each dilution were applied to cells. After 1 h of virus adsorption, the inoculum was replaced with medium containing 2% foetal calf serum, 40 μ g of DEAE-dextran per ml and 0.1% agarose, and the cells were incubated at 37°C for 2 to 3 days in a humidified CO₂ incubator. Cells were fixed with 10% formaldehyde and stained with 0.1% crystal violet, and the plaques were counted. The neutralization index was determined by dividing the number of PFU of virus per milliliter mixed with normal serum by the number of PFU of virus per milliliter in the presence of mouse antiserum.

To purify TGE virus, ST cells were grown in roller bottles and incubated with virus at a multiplicity of infection of 10 PFU per cell. Virus from clarified culture supernatants was concentrated by centrifugation for 1 h at 34 000 rpm and 4°C in a Sorvall A-641 rotor through a 31% (w/w) sucrose cushion in 0.01 M Tris hydrochloride-1 mM disodium EDTA-1 M NaCl (pH 7.4) supplemented with 0.2% Tween 20. The sediment was diluted on the above buffer supplemented with 0.05% Tween 20 and sedimented through a 30 to 42% (w/w) sucrose gradient by centrifuging for 2 h at 25 000 rpm and 4°C in a Sorvall AH-627 rotor. Fractions containing the virus were pooled, diluted with one volume of the above buffer, and sedimented in an AH-627 rotor for 2 h at 25 000 rpm and 4°C.

RIA and competitive RIA

For radioimmunoassay, TGE virus protein [0.25 μ g per well in 50 μ l of PBS] was adsorbed to polyvinyl disposable "U" plates (Dynatech Laboratories, Inc.) by overnight incubation at 37°C. The subsequent steps of the assay were performed as

described by Sanz et al. 1985, using a second antiserum (mouse immunoglobulins specific rabbit antiserum) to amplify the reaction and ^{125}I -labelled protein A to develop the assay.

For competitive RIA, MAbs were purified by high pressure liquid chromatography (HPLC) from ascitic fluids (Deschamps et al., 1985), ^{125}I -labelled (Greenwod et al., 1963), and the inhibition of their binding to purified virus by unlabelled MAbs was studied in a single step competitive RIA. Briefly, 150 μl of 5% bovine serum albumin in PBS per well were added to virus coated plates as indicated above, and the plates incubated for 2 h at 37°C. Then five-fold dilutions of each unlabelled MAb in PBS containing 0.1% BSA were mixed with a ^{125}I -labelled MAb (5×10^5 cpm per well, specific activity 10^7 cpm per μg of immunoglobulin), and incubated for 2 h at 37°C in the antigen-coated wells. Plates were washed six times with the buffer described above supplemented with 0.05% Tween 20, the wells were cut and the radioactivity was determined in a 1275 Minigamma counter (Wallac, LKB).

Hybridoma production, selection and characterization

To obtain hybridomas secreting TGE virus-specific MAbs, cells from the mouse myeloma cell line X63/Ag 8653 (Kearny et al., 1979) were fused with spleen cells from BALB/c mice immunized with purified native, denatured, or both native and

TABLE 1
INDUCTION OF TGE VIRUS NEUTRALIZING MAbs.

Fusion number	Immunization		Serum titre		Hybridoma	
	Antigen ^a	Duration ^b	RIA ^c	Neutralization index ^d	Positive by RIA ^e	Neutralizing ^f
1	N	43	ND	ND	200	15
2	D _d	40	ND	ND	2	0
3	D _d	44	ND	<1	69	0
4	N	44	ND	<1	18	0
5	N	51	$10^{3.5}$	$>10^{3.5}$	5	0
6	D _d + N	51	10^4	$>10^{3.5}$	48	1
7	N	131	ND	$>10^4$	96	3
8	D _{d+t} + N	521	$10^{5.5}$	$10^{4.2}$	438	152
9	D _{d+t} + N	461	$10^{5.5}$	$10^{3.5}$	139	26

^a The antigen used in the different fusions was native (N) or denatured by treatment (3 min in boiling water) with 1% Nonidet P-40, 1% SDS and 2.5% 2-ME alone (D_d) or followed by incubation (15 min at 4°C) in the presence of 5% trichloroacetic acid (D_{d+t}).

^b Expressed in days.

^c Serum titre was determined by RIA using purified TGE virus as antigen. ND, not determined.

^d The neutralization index was determined by dividing the number of PFU of virus per milliliter mixed with normal serum by the number of PFU of virus per milliliter in the presence of mouse antiserum. ND, Not determined.

^e Number of hybridomas selected for their high titre in the first evaluation performed after fusion.

^f Number of hybridomas, among the ones selected by RIA, neutralizing TGE virus infectivity more than 100-fold.

denatured virus, as summarized in Table 1. Denatured virus was obtained by incubation with 1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, and 2.5% 2-mercaptoethanol (2-ME), for 3 min in boiling water. When indicated (see Table 1) a second incubation (15 min at 4 °C) in the presence of 5% trichloroacetic acid was performed. The characterization of the hybridomas from fusions 1 to 6 has been described previously (Jiménez et al., 1986). The characterization of the hybridomas from fusions 7 to 9 is described below.

Selection of monoclonal antibody resistant (mar) mutants

The selection of antigenic variants resistant to the neutralization by specific MAbs (*mar* mutants) with thirteen MAbs was performed by incubating about 10^9 PFU of TGE virus (Purdue 54 strain) in 0.1 ml of PBS with 2% foetal calf serum, with one volume of each hybridoma supernatant. Then ST cell monolayers growing in petri dishes were inoculated with 0.2 ml portions of 10-fold dilutions of the virus-MAb mixtures and incubated for 1 h at 37 °C, before adding an agar overlay containing 0.2 volumes of the corresponding hybridoma supernatant per dish (diameter 35 mm; Costar). At 2 days after infection, virus from plaques were collected, and the selection procedure was carried out two more times. The *mar* mutants isolated were neutralized less than $10^{0.5}$ -fold by the homologous MAbs, whereas these neutralized the original virus more than $10^{4.8}$ times. The frequency of the *mar* mutants was defined as the inverse of the number of PFU of virus neutralized by a MAb per each PFU resistant to the neutralization by this MAb.

Immunodot assay

The assay was performed in a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories) using nitrocellulose membranes (Trans-Blot transfer membranes, cat. No. 162-0115, Bio-Rad Laboratories). Basically, the procedure of Hawkes et al. (1982) was followed. Briefly, native or denatured purified TGE virus (0.5 µg per well) was bound to nitrocellulose paper prewashed for 30 min with PBS buffer. Virus was denatured by incubation in boiling water for 10 min with 0.5% SDS in PBS. After denaturation the virus was diluted 10-fold in PBS and bound to nitrocellulose paper by incubating 10 µl samples for 1 h at room temperature. After filtration by vacuum, 250 µl of blocking solution (3% BSA in PBS) per well were added, incubated for 0.5 h and filtered. Then, one hundred-fold dilution of the mouse serum in PBS containing 0.1% BSA or undiluted hybridoma supernatant was added, and the incubation continued overnight at 4 °C. The wells were washed with PBS supplemented with 0.05% Tween 20 using vacuum, the nitrocellulose membrane was removed from the microfiltration apparatus and incubated 0.5 h at room temperature in the presence of 5% (w/v) nonfat dry milk (Molico, Nestlé) in PBS. Then, a 10^3 -fold dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulins (heavy and light chains) (Cappel Laboratories) in PBS supplemented with 1% BSA was added and incubated for 1 h. The filters were washed, color development solution (3 mg of 4-chloro-1-naphthol per ml of methanol and five vol of

0.02% H₂O₂ in PBS) (Hawkes et al., 1982) was added and the incubation continued until optimum staining was obtained.

Immunoblotting analysis

The binding of polyclonal or MAbs to viral proteins transferred to nitrocellulose paper was performed by the method of Towbin et al. (1979) with minor modifications. Briefly, the proteins were separated by SDS-PAGE (Laemmli, 1970) in the absence or in the presence of 2-ME. The gel was equilibrated with 20% methanol in 25 mM Tris base-192 mM glycine (pH 8.3) and then the proteins were electrophoretically transferred to a nitrocellulose membrane filter. After the transfer, the nitrocellulose membrane was washed in 500 mM NaCl-20 mM Tris hydrochloride (pH 7.5), then the incubation was continued in washing buffer (see above) containing 5% nonfat dry milk, to saturate remaining protein binding sites. The membrane was then incubated in hybridoma supernatant or in a 1 : 40 dilution of the antiserum in the washing buffer (see above) containing 1% nonfat dry milk, overnight at 4 °C. Bands specifically recognized were developed by the 4-chloro-1-naphthol technique (Hawkes et al., 1982), after incubation with peroxidase-labelled rabbit antiserum specific for mouse immunoglobulins.

Results

Selection of TGE virus specific MAbs

A total of 1015 hybridomas TGE virus positive, derived from independent cell to cell fusion events (as the cells were cloned immediately after hybridoma formation) were obtained in 9 different fusions. Hybridoma growth was observed in 70% of the wells, suggesting that statistically there was only one hybridoma per well. Table 1 summarizes the antigen administered in the immunization of the BALB/c mice used in the fusions, the duration of the immunization, the titres of sera from mice providing the immune cells and the number of hybridomas positive by RIA and producing neutralizing MAbs. The characteristics of the MAbs in the first 6 fusions have been described previously (Jiménez et al., 1986). In these fusions, 342 MAbs were positive by RIA for TGE virus. Based on their high titre in the RIA, we selected 48 MAbs (secreted by hybridomas which were cloned at least three times). Sixteen of these neutralized virus infectivity. All neutralizing MAbs were specific for the E2 glycoprotein and recognized epitopes sensitive to denaturation with SDS plus 2-ME. In order to induce E2 specific neutralizing MAbs which would be able to recognize antigenic determinants representative of the major immunodominant sites and resistant to denaturation, fusions 7, 8 and 9 were performed using cells from mice immunized with native virus alone (fusion 7) or with native and denatured virus (fusions 8 and 9). From the last three fusions 673 MAbs were initially selected, based on their high titre in the RIA. When tested in the neutralization assay, 181 MAbs (27%) were positive, i.e., neutralized virus infectivity more than 100-fold.

TABLE 2
REACTIVITY OF MAbS IN IMMUNODOT AND IMMUNOBLOTTING ASSAYS.

Treatment ^a	Serum ^b		MAbs ^c					
	TGEV specific	Control	Neutralizing		Non-neutralizing			
			1G.A7	1A.F10	1D.B12	6A.A6	1D.G3	8D.H8
Immunodot								
Nil (native virus)	+	-	+	+	+	+	+	+
SDS 0.1%	+	-	-	-	±	+	-	±
SDS 0.5%	+	-	-	-	±	+	-	±
SDS 0.5% + 2% 2ME	+	-	-	-	±	+	-	±
Immunoblotting								
SDS 0.1%	+	-	+	+	+	+	+	+
SDS 2.5%	+	-	+	+	-	+	+	ND
SDS 2.5% + 5% 2ME	+	-	-	-	-	-	+	ND

^a In the immunodot assay the virus was denatured by incubation in boiling water for 10 min in the presence of the indicated reagent. SDS, sodium dodecyl sulfate; 2ME, 2-mercaptoethanol. In the immunoblotting the concentrations of SDS and 2ME refer to the maximum reagent concentration with which the virus was treated during the immunoblotting.

^b +, Reactive; -, non-reactive.

^c The antigenic sites were defined by competitive RIA. +, Positive reactivity; ±, weak reactivity; -, no reactivity. ND, Not determined.

Twenty of these neutralizing MAbs were selected by hybridomas cloned more than three times.

To study whether the MAbs secreted in fusions 7, 8 and 9 were able to recognize sequential determinants, their reactivity to native and denatured TGE virus was analyzed in an immunodot assay (Table 2). Although all MAbs recognized native virus, none of the 181 neutralizing MAbs reacted to denatured virus. The reactivity of MAbs to TGE virus in the immunodot and the immunoblotting assays analyzed after treatment with different SDS concentrations and with 2-ME indicated that the pattern of reactivities was different in the two systems (Table 2). Only 6 MAbs, two neutralizing (1G.A7 and 1A.F10) and four non-neutralizing (1D.B12, 6A.A6, 1D.G3 and 8D.H8) reacted in the immunoblotting after treatment with 0.1% SDS, whereas only 3 MAbs showed strong (6A.A6) or weak (1D.B12 and 8D.H8) reactivity in the immunodot after similar treatment.

Antigenic sites of E2 glycoprotein determined by competitive RIA

Initially we studied the interference of unlabelled MAbs, purified by HPLC, in the binding of 21 ¹²⁵I-labelled E2-specific MAbs to TGE virus, by one-step competitive RIA. The study was performed with each possible pair of MAbs in both directions. Binding inhibition curves, as those shown in Fig. 1, were obtained. All MAbs inhibited the binding of the homologous ¹²⁵I-labelled ones by more than 90%. Different degrees of competition, that are illustrated for representative MAbs, were obtained: (i) only one MAb blocked the binding completely (Fig. 1,A) or partially

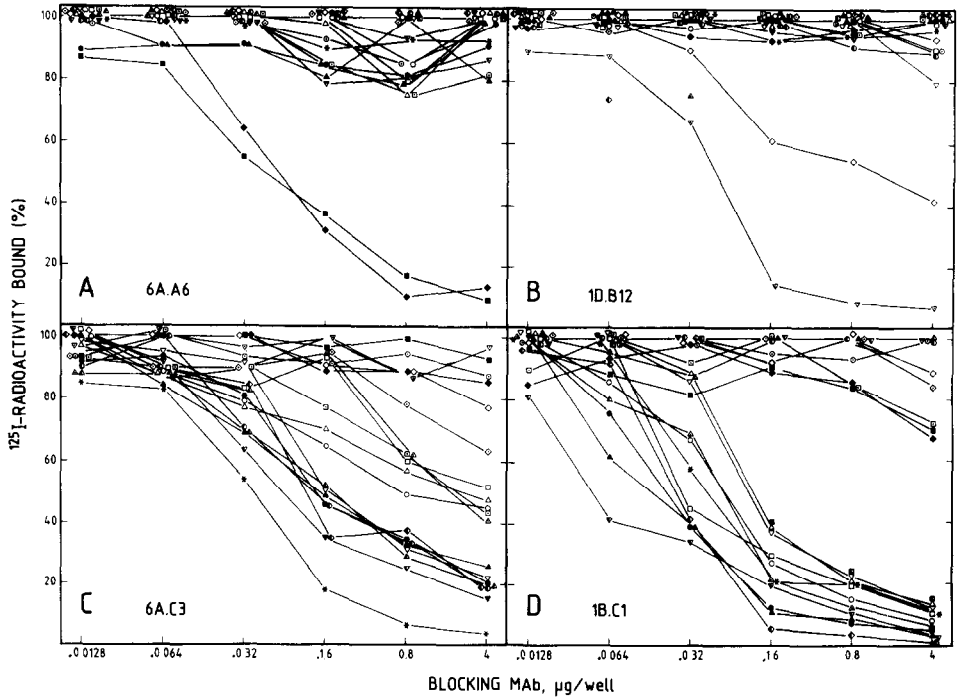


Fig. 1. Inhibition of MAb binding to TGE virus by E2 protein-specific MAbs in a competitive RIA. The binding of the MAb, indicated in each section of this graph (A, MAb 6A.A6; B, MAb 1D.B12; C, MAb 6A.C3; and D, MAb 1B.C1), was inhibited as described in Materials and Methods by the other MAbs: 1D.B3 (\blacklozenge), 1E.H8 (\blacktriangle), 1H.C2 (∇), 1B.H6 (\bullet), 1A.F10 (\square), 1B.B5 (\blacktriangle), 1D.E8 (\diamond), 1B.B11 (\blacksquare), 1D.E7 (\square), 1G.A7 (\triangle), 1E.F9 (Δ), 6A.C3 ($*$), 1H.D2 (∇), 1B.C1 (\bullet), 1G.A6 (\circ), 1D.B12 (∇), 1B.H11 (\diamond), 1D.G3 (\circ), 5B.H1 (\blacklozenge), 6A.A6 (\blacksquare).

(Figure 1,B); (ii) different MAbs inhibited the binding to a different extent (Figure 1,C); and, (iii) the competing MAbs could be classified into high and low inhibitors of the labelled one (Figure 1,D). The results of bidirectional competitive RIA were processed and all MAbs that inhibited the binding more than 70% were assigned to the same antigenic site. Figure 2 shows a summary of these results. Four antigenic sites (named A, B, C and D) could be differentiated. These sites were defined by 16, 2, 2, and 1 MAb, respectively. Interestingly, all neutralizing MAbs mapped at site A. Conversely, all MAbs against site A were neutralizing. We extended this study to the 181 neutralizing MAbs selected in fusions 7, 8 and 9. The inhibition of the binding of ^{125}I -labelled MAbs representative of each site (6A.C3 and 1D.B3, site A; 1B.H11, site B; 5B.H1, site C; 1D.G3, site D), by supernatants from each hybridoma was studied. Figure 3 shows the result of typical competitive RIAs. All 181 neutralizing MAbs from the last three fusions inhibited the binding of the MAbs representative of site A more than 70%, but not the binding of those representing sites B, C or D indicating that they reacted with site A epitopes. Two MAbs, 8B.E3 and 9F.C11, were selected from fusions 8 and 9, respectively, as representative for

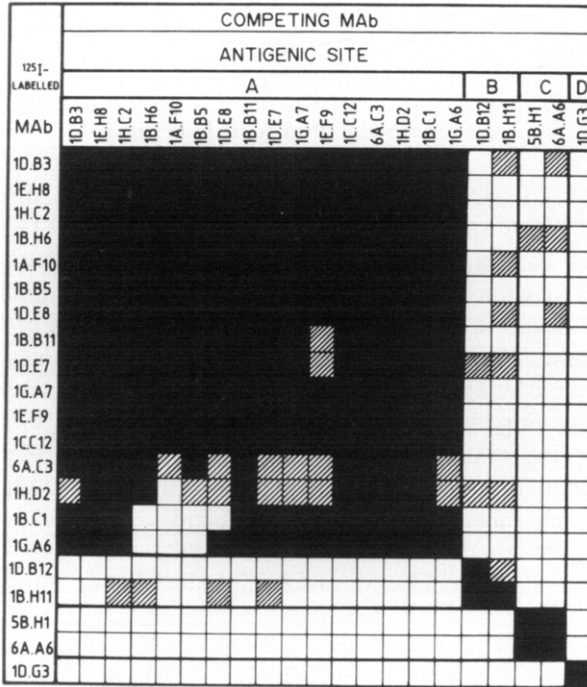


Fig. 2. Antigenic map of E2 glycoprotein from TGE virus. Results from competitions similar to the ones shown in Fig. 1 were represented according to the criteria: competition > 70% (■), between 30 and 70% (▨), < 30% (□).

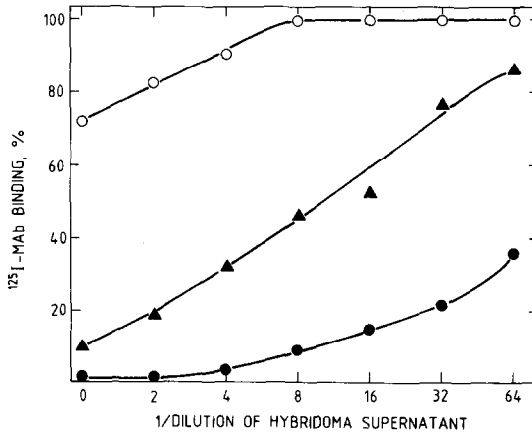


Fig. 3. Competitive RIA of the binding of ^{125}I -labelled MAbs by hybridoma supernatants. The binding of MAb 1C.C12 representative of antigenic site A was inhibited by dilutions of supernatants from cloned hybridomas specific for site A: (1C.C12 (●) and 1E.F9 (▲)), but not for site D: (1D.G3 (○)).

site A. In addition, MAbs 8F.B3 and 8D.H8 were selected as representative of sites B and D, respectively.

Antigenic subsites in E2 glycoprotein defined with mar mutants

A collection of 11 *mar* mutants (Fig. 4) was obtained with isolation frequencies ranging from $10^{-4.8}$ to $10^{-7.0}$. With two MAbs (6A.C3 and 1B.B11) it was not possible to isolate *mar* mutants, as they neutralized all virus used (10^9 PFU per assay), indicating that the frequency of their putative *mar* mutants was lower than 10^{-9} . The characterization of the 11 mutants, with a collection of E2 glycoprotein-specific MAbs, showed lack of neutralization of the *mar* mutants by the MAbs used in their selection and by MAbs different from the one used in the isolation of each *mar* (Fig. 4), suggesting that these MAbs recognized epitopes located in the same antigenic subsite, and that this antigenic area was altered in these *mar* mutants. These results permitted a grouping of the MAbs in three sets of 8, 3, and 3 MAbs, which defined three subsites designated a, b, and c, respectively. Subsite a, in addition to being the one defined by the largest number of MAbs, was modified in most *mar* mutants (8 out of 11). MAb 6A.C3 reduced *mar* mutant 1B.B5 plaque

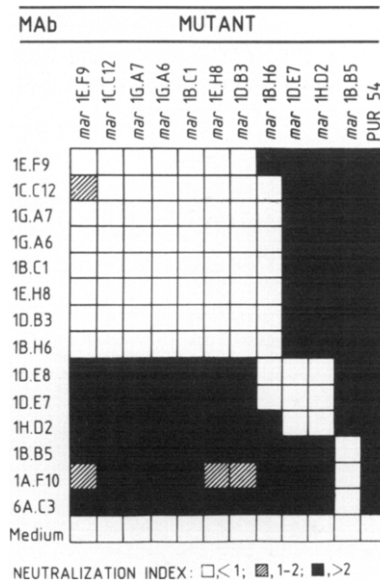


Fig. 4. Typification of *mar* mutants by neutralization with a collection of MAbs. The indicated *mar* mutants were tested for their sensitivity in a plaque reduction assay with a panel of MAbs. The neutralization index was determined by dividing the number of PFU of virus per milliliter mixed with normal medium by the number of PFU of virus per milliliter in the presence of a MAb and was expressed as the \log_{10} of this ratio. White, hatched, and black squares represent a neutralization index of <1, between 1-2, or >2, respectively. MAb 6A.C3 reduced the *mar* mutant 1B.B5 plaque size.

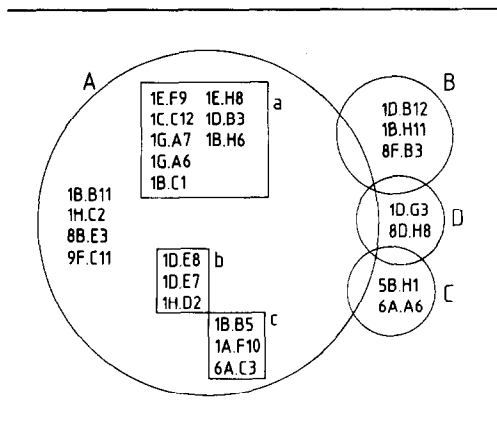


Fig. 5. Antigenic map of TGE virus E2 glycoprotein. Circles A, B, C and D represent the four antigenic sites of E2 glycoprotein. The MAbs defining each site are indicated in the corresponding circle. Site A was subdivided in three antigenic subsites (a, b and c) based on the typification of the *mar* mutants by neutralization with a panel of MAbs. The MAbs defining each antigenic subsite are shown inside a square. MAbs 1B.B11, 1H.C2, 8B.E3 and 9F.C11 have not been located in the antigenic subsites.

TABLE 3

EPITOPES OF TGE VIRUS E2 GLYCOPROTEIN.

MAb	Neutralizing activity ^a	Antigenic site ^b	Critical subsite ^c	Sensitivity to ^d			Epitope presence in Toyama virus ^e	<i>mar</i> mutant frequency ^f
				NP-40	SDS	SDS + 2ME		
1B.C1	+	A	a	+ *	+	+	+	1×10^{-7}
1G.A6	+	A	a	-	+	+	+	$1 \times 10^{-6.2}$ *
1G.A7	+	A	a	-	- *	+	+	$1 \times 10^{-5.6}$
1E.F9	+	A	a	-	+	+	± *	1×10^{-6}
1D.E8	+	A	b *	-	+	+	ND	ND
6A.C3	+	A	c *	-	+	+	+	$< 1 \times 10^{-9}$ *
1A.F10	+	A	c	-	- *	+	+	ND
1H.C2	+	A	ND	-	+	+	- *	ND
1D.B12	-	B *	NA	-	+	+	ND	ND
6A.A6	-	C *	NA	-	-	+	ND	ND
1D.G3	-	D *	NA	-	-	-	ND	ND

^a Determined in a plaque reduction assay. +, Neutralizing; -, not neutralizing.

^b Determined by competitive RIA. *, relevant differential characteristic.

^c Determined by typification of *mar* mutants. ND, not determined; NA, not applicable; *, relevant differential characteristic.

^d Determined by studying the reactivity of the MAb in the immunoblotting and immunoabsorption assays. NP-40, 1% Nonidet P-40; SDS, 2.5% sodium dodecyl sulfate; 2ME, 2% 2-mercaptoethanol; +, sensitive; -, not sensitive; *, relevant differential characteristic.

^e Determined by the ability of the MAb to neutralize the TOY 56 strain of TGE virus. +, Presence; -, absence; ±, partially modified; ND, not determined; *, relevant differential characteristic.

^f Determined by *mar* mutant isolation. ND, not determined; *, relevant differential characteristic.

size, suggesting that the epitope recognized by MAb 6A.C3 was only partially modified in that *mar* mutant. Since all MABs used to define the antigenic subsites were located in site A, the antigenic structure of the E2 protein, based on the results of competitive RIA and on the characterization of *mar* mutants, could be represented diagrammatically, as shown in Fig. 5.

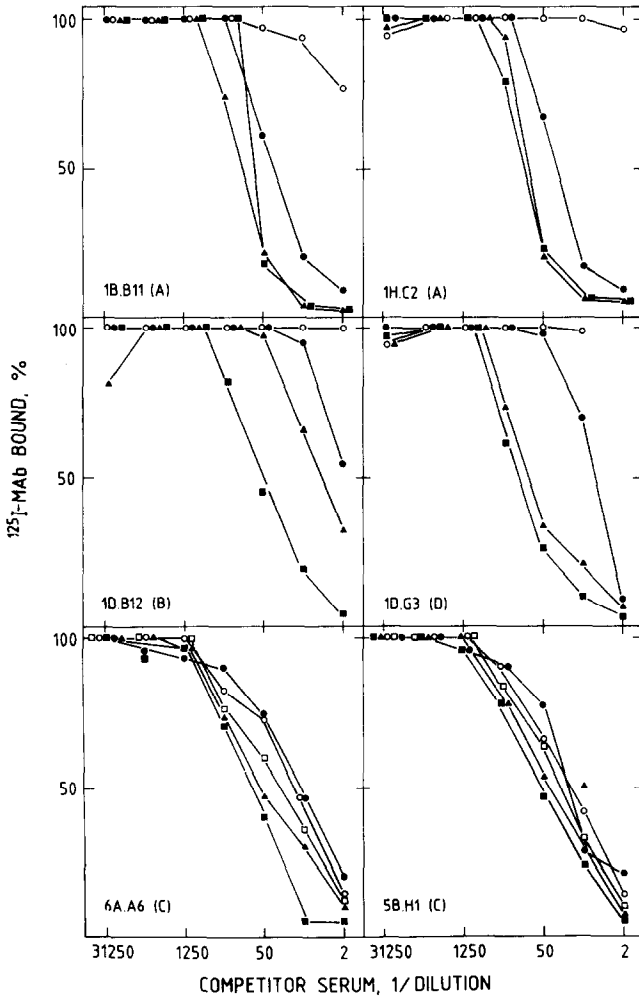


Fig. 6. Inhibition of E2 glycoprotein specific MABs binding to TGE virus by specific polyclonal swine antisera. The binding of the ¹²⁵I-labelled MAB indicated in each graph (letters in parenthesis indicate the antigenic site of which the MAB was representative) was inhibited by immune sera (▲, ■, ●) or sera from nonimmune animals (□, ○). Swine sera were from animals immunized with the strain PUR 54 of TGE virus inactivated with UV light (▲, ■), or from animals first infected with the virulent strain of Miller virus and then immunized with inactivated virus (●) (kindly provided by R. Wesley).

Epitopes defined in the E2 glycoprotein

The data obtained by competitive RIA, as well as by *mar* mutant characterization, resistance to detergents and 2-ME, determination of their presence in natural isolates (Jiménez et al., 1986), and on the *mar* mutant isolation frequency, led us to define a minimum of 11 epitopes in E2 glycoprotein, of which 8 were critical in the neutralization of TGE virus (Table 3). Next, we studied the relevance of the antigenic sites so defined, in TGE virus infections of swine, its natural host.

Epitope recognition by swine serum

To study whether the different E2 glycoprotein antigenic sites defined by murine MABs were also recognized by swine antisera, the inhibition of the binding of E2 glycoprotein-specific MABs to TGE virus by specific polyclonal swine antisera from infected and immune animals was determined. These studies were performed with six MABs, representative of the four antigenic sites of E2 protein: 1B.B11 and 1H.C2 (with high and low relative avidity, respectively, data not shown), site A; 1D.B12, site B; 6A.A6 and 5B.H1, site C; and 1D.G3, site D. The results (Fig. 6) indicate that the three swine antisera tested inhibited the binding of MABs representative of the four antigenic sites.

An unexpected result was that control antisera also inhibited the binding of the two MABs (6A.A6 and 5B.H1) specific for antigenic site C, but not the binding of MABs specific for the other antigenic sites. To determine whether the inhibition of site C-specific MABs binding was due to an occupation of the binding sites by normal swine serum, mediated by its reactivity with TGE virus, or by a direct reactivity of the labelled MABs with the serum, the competitive RIAs were performed following two protocols. In the first one, both the swine serum and the labelled MABs were incubated simultaneously in virus-coated wells. In the second one, the serum was incubated first, the plates washed to remove unbound serum, and then the labelled MAB was added. The inhibition in the binding of the ¹²⁵I-labelled MABs was observed only in the first case, suggesting that the blocking was due to a reactivity of MABs with sera from non-immune animals. To study if the reactivity of the MABs was directed against the immunoglobulins present in the sera, we analyzed the inhibition of the binding of this MAB to the virus in the RIA by purified porcine immunoglobulins. The results (not shown) indicated that the porcine immunoglobulins did not inhibit the binding, suggesting that the reactivity was directed against other component(s) of porcine sera.

Discussion

The antigenic structure of E2 glycoprotein of TGE virus has been determined using 1015 TGE virus positive hybridomas selected from independent cell-to-cell fusion events. Based on this data, and on the percentage of wells with hybridoma growth (70%), we considered for statistical conclusions, that we are dealing with

monoclonal reagents. Nevertheless, 68 hybridomas (48 from the first 6 fusions, and 20 from fusions 7, 8, and 9) were cloned at least three times. MAbs representative of the antigenically dominant sites were obtained. We introduced the characterization of *mar* mutants to subdivide the critical antigenic site in smaller antigenic areas that were defined as antigenic subsites, which most probably represented domains of the virus surface physically differentiable.

Four antigenic sites (A, B, C and D) were defined by competitive RIA. All (197) neutralizing MAbs were specific for site A. These MAbs were secreted by a large proportion (around 20%) of the 1,015 positive hybridomas analyzed (as in our cloning conditions each well contained, statistically, around one hybridoma per well). This result suggests that the critical site A is one of the antigenically dominants, in agreement with our previous results (Enjuanes et al., 1987). Delmas et al. (1986) also have identified an immunodominant site in TGE virus neutralization. We studied the neutralization of TGE virus (PUR 54 strain) by a MAb induced with the Miller strain of this virus (provided by R. Woods and R. Wesley). This MAb, which neutralized reversibly the PUR 54 strain of TGE virus (probably because of its low avidity) did not inhibit the binding of MAbs representative of antigenic sites A, B, C or D (I. Correa, R. Woods, R. Wesley, and L. Enjuanes, unpublished results). These data indicated that either TGE virus has minor critical epitopes distinct from site A, or that this MAb reacted also to the critical site A, but due to its low avidity did not inhibit the binding of site A representative MAbs.

All epitopes in site A are probably critical in virus neutralization, as all MAbs reacting to this site were neutralizing. This area must be conformation-dependent since: (i) none of the 197 neutralizing MAbs recognized the viral proteins denatured with SDS plus 2-ME (Table 2), and (ii) neither the virus nor the E2 glycoprotein denatured by different procedures induced neutralizing polyclonal nor monoclonal antibodies (Table 1 and Jiménez et al. (1986)). Although we cannot discard the possible existence of MAbs able to recognize primary structure, we were unable to isolate any MAb of this type. The selection of this MAb type is unlikely to be due to the lack of neutralization by the polyvalent antisera induced by denatured virus.

Interestingly, MAbs representative of sites A, B, C and D reacted to E2 glycoprotein partially denatured with 0.1% SDS, which might be useful for the physical differentiation of peptides representative of all antigenic sites (Table 2 and Fig. 1).

The antigenic sites of E2 glycoprotein were defined by competitive RIA (Fig. 1). The competitions were performed in both directions and, in most cases, the interference in the binding between a pair of MAbs was reciprocal: either they inhibited each other or no blocking of the binding was observed. Only a few exceptions were detected (Fig. 2) that could be explained by differences in the avidity of the MAbs. An alternative explanation would be that, in those cases, the inhibition in the binding by one MAb was due to a change in the conformation of the epitope recognized by the other MAb and that this conformational change was not reciprocal.

The characterization of 11 *mar* mutants made it possible to differentiate site A of E2 glycoprotein into three antigenic subsites (a, b and c). These subsites represented

areas in the virus surface that could be independently modified. This form of differentiation of antigenic domains critical in virus neutralization could be the basis for the selection of different peptides to be used in synthetic or subunit vaccines. In other viral systems, the alterations detected in the surface of *mar* mutants, in relation with the wild type virus, have been shown to be due to a single amino acid change, which was the consequence of a change in a single nucleotide, whenever the sequences of the *mar* mutants were determined (Seif et al., 1985; Vandepol et al., 1986; Van-Wyke Coelingh et al., 1987; Yewdell et al., 1986). It will be possible to correlate these functional subsites in the virus surface to small fragments of the TGE virus genome by determining their nucleic acid sequence.

The antigenic structure of the E2-glycoprotein of TGE virus was further subdivided into epitopes by using MAbs with different properties. A total of 11 epitopes were defined in the E2 glycoprotein. All 8 critical epitopes were located in site A and, reciprocally, all epitopes defined on site A were critical, indicating that this antigenic site of the virus was "untouchable" by antibodies without inactivating the virus. The number of epitopes (11) defined on the E2 glycoprotein with a relative molecular weight of 200 000 is in the order of the numbers defined in well characterized viruses (one epitope for each fragment with a relative molecular weight of 12 000) (Jackson et al., 1982; Niman and Elder, 1982; Stone and Nowinski, 1980).

The epitopes defined with murine MAbs in the E2 glycoprotein were relevant for the natural host, as porcine sera from infected or immunized swine reacted to the four antigenic sites defined by competitive RIA with the MAbs. The MAbs reacting to antigenic site C also reacted to an undetermined non-immunoglobulin component of swine sera. Thus TGE virus infection could induce an autoimmune response, that could produce an immunocomplex disease in chronically infected animals (Oldstone, 1984).

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